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Review article

Chemical Engineering of RNase Resistant and Catalytically Active Hammerhead Ribozymes

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1. Introduction

In the last few years, both the development of efficient methods for the chemical synthesis of oligonucleotides and of new concepts for gene inactivation opened promising new therapeutic perspectives as illustrated by the antisense approach.[1] The potential targets of these new strategies include cellular genes with undesired phenotypes or foreign genes introduced by pathogens such as viruses. If antisense technologies could attract so much interest it is because, in theory, antisense oligonucleotides can be designed simply on the basis of Watson–Crick base pairing with their RNA target.[2] Concomitantly to the early developments of antisense research it was discovered that in a biological context a large number of naturally occurring RNA motifs, termed ribozymes, were capable of catalytic self-cleavage (not to mention the trans-acting RNase P). Of particular interest are the small endonucleolytic motifs found in plant pathogens which are considered to replicate by a rolling circle mechanism.[3] These natural ribozymes can be adapted to cleave in trans any specific targeted RNA in a sequence specific manner. Among these, the hammerhead ribozyme[4] is the shortest and the most studied since it can be readily synthesized by chemical[5] and biochemical methods.[6] Unfortunately, the progress of oligonucleotide-based therapies is currently faced with many obstacles. Among the major hurdles, slowing down the development of exogenous administration of oligonucleotides, are the poor biological stability and the inefficient uptake into the target cell.[7] The instability issue can be resolved by oligonucleotide synthetic chemistry using nucleoside analogues which have the capacity to confer nuclease resistance to artificial nucleic acid type constructions.[8] However, in the case of ribozymes, care must be taken in choosing the correct chemical modifications so that they do not hinder catalytic cleavage activity and (or) hybridization.

This review will summarize our present knowledge on the structure and cleavage mechanism of hammerhead ribozymes with emphasis on the chemical modifications which have given structural and functional informations and were useful in view of designing active and stable ribozymes. Finally the current in vivo applications of the modified ribozymes will be evoked.

2. Characteristics of Hammerhead Ribozymes

2.1. Hammerhead ribozyme secondary structure

In the last decade it was found that the replication of several plant pathogens involved an internal cleavage of

the multimeric form of their RNA genome. This reaction was shown to occur auto-catalytically at well defined sequences manifesting a common conserved nucleotide arrangement. Following this remarkable discovery, it was realized that RNA cleavage reactions could be reproduced in vitro in trans using a suitable combination of two (or three) oligoribonucleotides.[4]

Thus, bimolecular cleaving reactions are generally observed using the hammerhead ribozyme motif when two oligoribonucleotides can form the secondary structure[4b] shown in Figure 1. A divalent metal ion (Mg^{2+}, Mn^{2+}...) is absolutely required for the reaction that generates two products which are terminated with 2′,3′-cyclic phosphate and 5′-hydroxyl groups, respectively (Fig. 2). In the bipartite hammerhead ribozyme construction, the enzyme strand (R) associates with the substrate (S) via base pairing forming helices I and III. It can be adapted to cleave any RNA sequence on the 3′ side of a NUX triplet, N being any nucleotide and X being different of G. The catalytic domain is made of helix II and of two short stretches of apparently unpaired residues which join helices I and III to helix II.[10] The importance of the latter residues has been revealed by mutational analysis showing that, except for position 7, when they were changed the catalytic activity was either greatly diminished or lost. Similarly, the importance of helix II for the activity has been examined revealing that the conserved base pair G10.1-C11.1 can neither be changed or inverted without causing a dramatic decrease of the k_{cat} with little variation of the K_m.[11] However the sequence of helix II together with that of its corresponding loop can be modified. It is assumed that this helix does not play a critical role in the catalysis provided it contains at least three base pairs, including G10.1-C11.1. However, helix II would be important for the stabilization of the transition state. At the cleavage site, residues C or A are those for which the higher cleavage rates are observed. This rate is decreased twentyfold in the case of U compared to C while G is not tolerated, presumably because it forms a base pair with C3.[10a,12] Finally, the presence of U16.1, which makes a base pair with A15.1, is necessary while the replacement of G16.2-C15.2 by A16.2-U15.2 or U16.2-A15.2 provokes a moderate activity decrease, whereas the base pair C16.2-G15.2 is not tolerated.[10a,13]

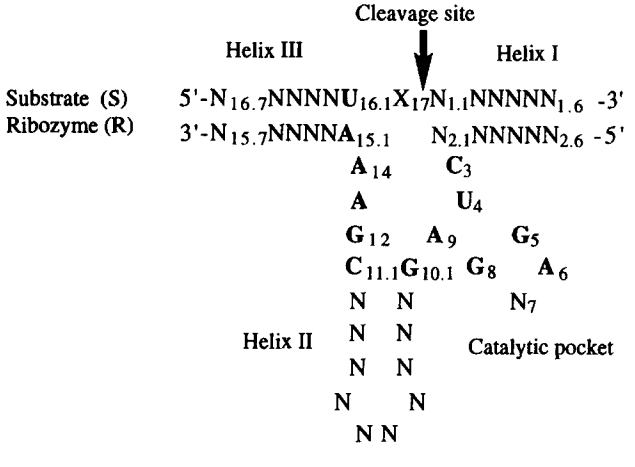


Figure 1. Hammerhead ribozyme domain with numbering according to ref 9. Bold letters indicate conserved residues of the central core and the arrow the cleavage site.

2.2 Kinetics of the hammerhead enzymatic reaction

In bipartite hammerhead systems a single ribozyme can cleave as many as one hundred substrate molecules (i.e., it behaves as a true enzyme). Thus the catalytic RNA binds its RNA target, catalyzes cleavage and then the reaction products dissociate allowing the ribozyme to enter in a new cycle. A minimal kinetic scheme has been proposed for this process (Fig. 3).[14] It includes substrate binding to R with the formation of the R.S complex (k_1), cleavage (k_2) to generate a new complex R.P1.P2 which releases the reaction products P1 (k_3, k_6) and P2 (k_4, k_5). The scheme accounts for the reverse reactions: products binding to R (k_{-3} to k_{-6}), catalytic ligation of products (k_{-2}) and finally substrate dissociation (k_{-1}).[14,15] The rate constants for each step have been determined under various conditions and more particularly with ribozymes differing by the length and stability of stems I and III. The bimolecular binding steps (k_1, k_{-3} to k_{-6}) occur with rate constants (10^7–10^8 M^{-1} min^{-1}) as observed with small complementary oligonucleotides. In some cases however, a high substrate concentration is necessary to allow formation of R.S (resulting in a high K_m value) due to the propensity of oligonucleotides to adopt internally folded conformations.[16] The dissociation rate constants (k_{-1}, k_3 to k_6) are, to a first approximation, found in agreement with the respective stabilities of the involved stems.[14] The cleavage rate constants termed k_{cat}, when determined under catalytic conditions (multiple turnover), and k_2 for single turnover reactions (ribozyme in large excess) are generally in agreement for systems with short arms I and III (within a factor of 2).[17] Typically, k_2 values range between 1 and 20 min^{-1} at pH 8 (37 °C, 20 mM Mg^{2+}), which constitutes a 10^8-fold rate enhancement over the estimated rate of cleavage of RNA at neutral pH in the absence of a catalyst. However, the much less specific RNase A enzyme, cleaves RNA with a four orders of magnitude higher

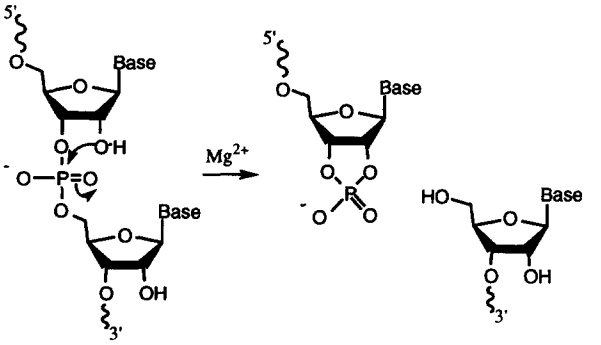


Figure 2. Hammerhead ribozyme-mediated cleavage reaction.

rate.[14] A comprehensive study of the ligation reaction has shown that the equilibrium constant largely favours the cleavage reaction ($k_2/k_{-2} \approx 130$). The favourable free energy of the cleavage reaction benefits from an entropic contribution overcoming the unfavourable enthalpy change.[15]

It is now accepted that five to seven base pairs on each side of the cleavage site (stems I and III) suffice to achieve optimal cleavage activity. Extending the length (and stability) of both stems results in a burst kinetic under catalytic conditions, the amount of free ribozyme R being limited by product dissociation from R.P1.P2. Interestingly, it also results in a lower k_2 value as determined under single turnover conditions.[18] To explain this finding it was suggested that R.S might undergo a conformational change leading to the true catalytic complex.[19] The two helices behave anisotropically with respect to the cleavage rate constant. Introduction of a mismatch into one of the four innermost positions of helix III results in a low k_2, whereas it is only the case for the first base pair of helix I (positions 1.1 and 2.1), with those in the vicinal positions showing little effect.[20] Unexpectedly, however, k_2 is very sensitive to the length of helix I reaching its optimal value at four to six base pairs and then decreasing abruptly.[18,19]

When using long substrates (or ribozymes) susceptible to adopt internally folded structures that hampers the R.S association step, RNA binding proteins or facilitator oligonucleotides may favour the overall cleavage reaction. A typical case is that of the retroviral nucleocapsid (NC) protein which pertains to a group of nonspecific RNA binding proteins. The latter are known for their ability to speed association and dissociation of oligonucleotides by an as yet unknown mechanism. Interestingly, these proteins contribute to enhance the ribozyme cleavage activity.[21,22] On the other hand, facilitators are oligonucleotides hybridizing the 5′ end (or the 3′ end) of long substrates thus leading to an extension of stem III (or stem I) in the R.S complex. They improve the cleavage activity at subsaturating substrate concentrations and with the examples analysed so far, facilitators were revealed more efficient when bound at the target 5′ end[23] than at its 3′ end.[24] A recent detailed analysis of a 5′ end facilitator mechanism of action indicated that it does not affect the cleavage step (k_2), but rather enhances the association rate constant k_1 (sixfold) presumably by destabilizing alternative substrate folded forms. In addition, it decreases the dissociation rate constant k_{-1} (initially higher than k_2) leading to a eightfold lower K_m value.[17]

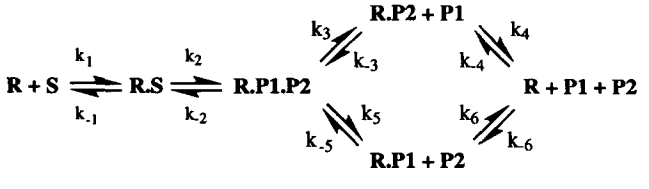


Figure 3. Minimum kinetic scheme of the hammerhead ribozyme cleavage reaction.[14]

2.3. Three-dimensional structure of the hammerhead ribozyme

Recently, two crystallographic structures have been reported for bipartite hammerhead ribozyme domains.[25] Although the crystals of both domains were obtained in different conditions, with different design and substrate analogues, the reported structures exhibit an identical tertiary fold. In one case[25a] the wild substrate was replaced by a DNA oligonucleotide forming helices I and III, giving rise to a DNA–RNA complex. In the other one[25b] a RNA substrate forming helices I and II (thus containing helix III) incorporating a 2′-OMe cytidine at the cleavage position was used. Overall, the ribozyme domain was shown to adopt an Y form with stems I and II in close juxtaposition and stems II and III almost collinear (Fig. 4). All three stems are present as A-form helices, stem II being extended by three non canonical base-base interactions involving A9-G12, G8-A13 and U7-A14. Thus stems II and III form a long continuous helix interrupted by the connection of stem I which is mediated by a four nucleotide 'catalytic loop' (C3-U4-G5-A6). This loop adopts a conformation similar to the uridine turn found in the $tRNA^{Phe}$ anticodon loop. The central core of the ribozyme is stabilized by a network of weak interactions including hydrogen bonds between bases and ribose 2′-OH groups.

Various approaches, such as hydrodynamic measurements[26], migration in native gels[27] and evaluation of distances between individual residues by energy transfer[28] have been used to explore the folding of the

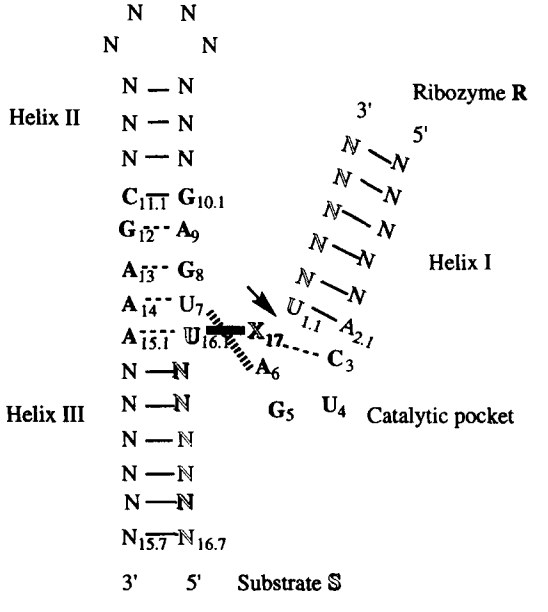


Figure 4. Planar projection of the crystal structure of the hammerhead ribozyme domain.[25] (Arrow indicates the cleavage site.)

hammerhead domain in solution. All these methods led to the conclusion that, in the presence of Mg^{2+}, the ribozyme adopts a Y form with stems I and II side by side. By hydrodynamic measurements the angle formed between the axes of stems II and I was found larger (110°) than observed in the crystal structure (60°). Interestingly, at low ionic strength (5 mM Na^+) the domain adopts a more opened conformation.[27]

A more direct insight into the hammerhead ribozyme domain conformation in solution, was obtained by application of photoaffinity labelling using site-specific introduction of thio-modified residues within substrate analogues. The presence of 4-thio-2′-deoxyuridine in place of uridine in the substrate analogues induces a minimal structural perturbation since they still form active complexes with the ribozyme. Irradiation of the complexes led to the formation of cross-links between residues able to come into contact.[29] These cross-links were identified, yielding a number of representative tertiary interactions which were used as constraints to build a plausible structure by molecular modelling.[30] The derived solution model is consistent with the X-ray structure.[25] In particular the pair U16.1-A15.1, in the vicinity of the cleavage site, is weakened and C17 lies in a pocket made up the C3-U7 sequence. The central core is stabilized by a network of hydrogen bonds and stacking interactions between conserved residues which are distinct from the ones observed in the crystal.[25] The overall picture derived from the photo-cross-linking data indicates a high conformational flexibility of the central region together with the presence of alternative conformers, even under cleavage conditions.[29a]

Localisation of the phosphate groups involved in the binding of divalent cations and important for the cleavage activity was first deduced from 'rescue' experiments where addition of thiophilic Mn^{2+} (or Cd^{2+}) restored cleavage activity of substrate analogues having a R_p phosphorothioate linkage at the cleavage site (see 2.4). In addition to the metal bound at the cleavage site, these experiments have identified three phosphates, in the extension of helix II containing GA and AU mismatched base pairs, which are away from the cleavage site and probably strictly required for maintaining an active structure.[31] Initial crystallographic analysis did localize unambiguously one such metal site[25] and suggested the presence of several other sites, two of which being coordinated with adjacent purine N7 positions and chelated with the amino groups of C3 and C17. Recently, Scott et al. were able to analyze the crystal structure of a full RNA hammerhead construct either in the absence or after addition of Mg^{2+}.[32] This catalytically active structure exhibited the previously observed folding. Moreover, several Mg^{2+} binding sites, common to this structure and to the previous inactive ones, were detected. Interestingly, an intermediate could be captured at pH 8.5 by freeze-trapping. This intermediate underwent a conformational change restricted to C17 and residues A6 and G5 of the catalytic loop together with the positioning of an additional Mg^{2+} at the pro-*R* oxygen of the scissile phosphate.

2.4. Reaction mechanism: role of Mg^{2+} ions

As other RNA enzymes, hammerhead ribozymes are metalloenzymes functioning in the presence of divalent cations.[33] Even when other counterions such as spermine or Na^+ are added to stabilize the folded structure, divalent metal ions (Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Cd^{2+}) are still required. Thus, these cations promote both ribozyme folding and catalysis.[34] The dependence of the cleavage rate upon Mg^{2+} concentration is suggestive of the presence of a catalytic Mg^{2+} which binds with moderate affinity, the apparent dissociation constant being close to 10 mM.[35] Analysis of cation binding, using a fluorescent reporter group placed at position 16.1 next to C17, revealed a strong binding site (K_D = 1 mM) and a weak one (K_D = 100 mM), thus implicating 'structural' Mg^{2+}.[36] However, our present understanding of the cleavage mechanism derives mainly from the inspection of the pH profile rate data obtained in the presence of different metal cations.[37] While a direct interaction of Mg^{2+} with the attacking 2′-OH group has been proposed,[38] the pH profile data strongly suggested that a metal hydroxide was responsible for the proton abstraction of this group.

Analysis of the configuration of the reaction products from substrates containing a S_p (or R_p) phosphorothioate linkage at the cleavage site, showed inversion of configuration at the phosphorus centre indicating an in-line attack of the scissile phosphorus oxygen bond by the adjacent deprotonated 2′-hydroxyl group.[39] However, a reduced activity was observed in the presence of Mg^{2+} when the pro-*R* (but not the pro-*S*) phosphate oxygen was replaced by a sulfur at the cleavage site. The activity was partially rescued after addition of thiophilic Mn^{2+} ions.[40] It was thus proposed that a divalent metal ion was directly coordinated to the pro-*R* oxygen being able to fulfil, as a metal hydroxide, the deprotonation of the 2′-OH nucleophile (Fig. 5).[37b] Although questioned on the basis of a quantification of the 'thio-effect',[41] this view was recently reinforced, if not definitively established, by the recent crystallographic analysis of a hammerhead trapped intermediate (see 2.3).[32]

The cleavage of the P-O(5′) bond, which represents the second step of the cleavage reaction, appears to be the rate-limiting step, at least in non-enzymatic systems.[42] Since no neat kinetic isotope effect (kD_2O/kH_2O) was

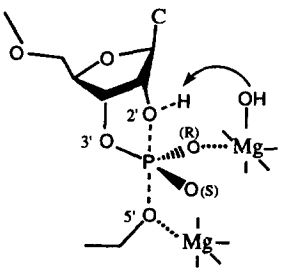


Figure 5. Possible catalytic role of Mg^{2+} ions in the hammerhead mediated cleavage reaction.[38]

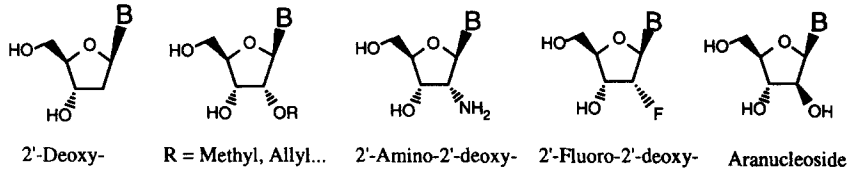
observed in hammerhead ribozyme catalyzed cleavage it was concluded that: (1) deprotonation of the 2′-hydroxyl group was not the rate-limiting step, (2) cleavage of the P-O(5′) bond was assisted by a catalyst distinct from a proton. Accordingly, another catalytic Mg^{2+} was suggested to act as a Lewis acid coordinating the 5′-leaving oxygen.[38] However, substitution of this 5′ oxygen by sulfur did not accelerate the cleavage rate as expected when thiophilic Mn^{2+} ions were used in place of Mg^{2+}.[43] These apparent contradictions were raised arguing that, in the case of the thio-deoxy-substrate analogue used by Kuimelis,[43] the rate limiting step was the attack of phosphorus by the deprotonated 2′-hydroxyl whereas in the natural substrate it was the Mg^{2+} assisted departure of the 5′ hydroxyl group.[44]

3. Chemical Engineering of Hammerhead Ribozymes: RNase Resistance and Catalytic Activity

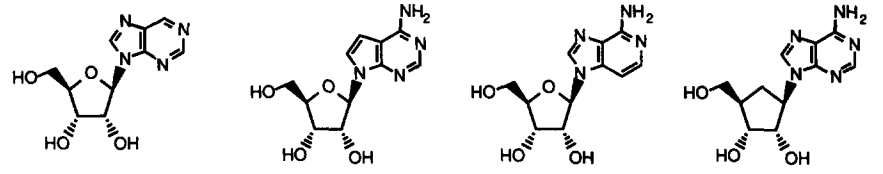
As mentioned above, because of its unstability in biological fluids, unmodified RNA cannot be used for therapeutic applications. In the hammerhead series one way to overcome this serious obstacle consists of introducing judicious chemical modifications in the ribozymes thereby increasing their stability with respect to both exo- and endonucleases while retaining catalytic activity. Because of their relatively small size hammerhead ribozymes are well suitable to test a large body of chemical modifications which can be performed by taking advantage of the recent progresses in the synthesis of oligoribonucleotides.[5]

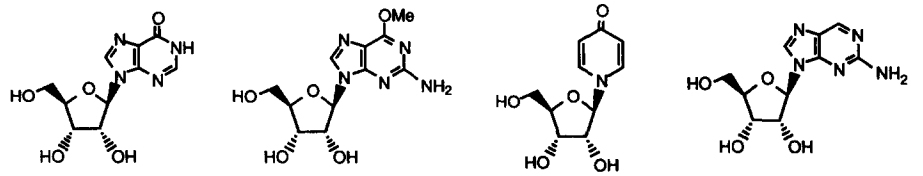
Protection against exonucleases can be achieved using standard protocols leading to the synthesis of ribozymes containing several phosphorothioate linkages at their 3′ ends.[45] Resistance to 3′-exonucleases activity, which in general predominate in biological fluids, can be conferred by modification of the ribozyme 3′ end. It has been accomplished by introducing an abasic residue, an inverted (3′–3′) linked dT[45], or even a diol (butane diol, cyclohexane diol) unrelated to nucleosides.[46a] Another procedure consists of extending the ribozyme 3′ end by a stable mini-hairpin.[46b]

Such modifications increase the ribozyme stability without any detectable effect on the activity.[47] On the other hand, overcoming the ribozyme degradation by the endonucleases is more problematic. It is not possible to envisage an uniformed substitution strategy such as the replacement of all ribonucleotides by 2′-modified-2′-deoxynucleotides[49] or all the phosphodiester functions by phosphorothioates.[50] Also to discuss the present status of the effects of the chemical modifications (Fig. 6) which have been performed in the hammerhead series two important regions of the system will be distinguished: the non catalytic region which is made of helices I, II, and III and the catalytic region which includes the non canonical base pairs and the catalytic pocket. Most of this modification work have been accomplished in view of structure-function studies, the results of which are extremely important for designing artificial ribozymes. Unfortunately, little information on their potential effects regarding ribo-



Nucleosides modified at the 2′-ribose position





Selected modified ribonucleosides

Figure 6.

zyme stability in biological fluids has been reported, however, whenever possible we shall comment on this point.

3.1. Modifications of helices I, II and III

In principle, except for the A15.1-U16.1 and G10.1-C11.1 conserved base pairs, the three helices I, II, and III as well as stem II of the hammerhead can accomodate many types of modified ribonucleotides. The preliminary works directed at the construction of RNase resistant ribozymes proposed to synthesize chimeric DNA/RNA ribozymes. Indeed, the simple modification consisting of removing the 2′-hydroxyl of a number of residues was very appealing since this functional group participate in RNA digestion by RNases.[49–52] It was found that a ribozyme having the arms of helices I and III as well as the stem-loop II made entirely of DNA, exhibited a higher K_m value as a consequence of the lower stability of hybrid helices while k_{cat} or k_2 remained either unaffected or enhanced.[50b,51–54] Particularly striking is the behaviour of small DNA-armed ribozymes, the k_{cat} of which increased, with Mg^{2+} concentration to reach 100 min^{-1} (at 800 mM Mg^{2+}, pH 8, 37 °C). In contrast, the k_{cat} of the corresponding all-RNA system remained constant (18 min^{-1}) between 30 and 800 mM Mg^{2+}.[55]

However the DNA part of such modified ribozymes could be sensitive to DNases. Thus, a more appropriate solution of this problem was proposed making use of oligonucleotides incorporating 2′-*O*-alkylribonucleotides known to be fully resistant by most endonucleases.[56] Accordingly, ribozymes containing 2′-*O*-methyl- or 2′-*O*-allylribonucleosides at helices I and III sites as well as within stem/loop II have been synthesized. Another good reason to incorporate such 2′-*O*-alkylribonucleosides in ribozymes can be ascribed to their known preference to adopt the same 3′-*endo* conformation as their parent ribonucleosides, allowing formation of standard type A helices. Moreover it is of interest to note that 2′-*O*-alkylribonucleotides are hydrogen bond acceptors although they are no more hydrogen donors. Finally, substitution of the residues involved in substrate base pairing and in loop II by 2′-*O*-methyl- or 2′-*O*-allylribonucleosides resulted in molecules which retained the efficiency of cleavage of their parent all RNA ribozyme.[45,57] Moreover, with regard to their stability in various sera, the modified ribozymes exhibited a marked resistance compared to their unmodified analogues.[45,57]

In an interesting line of research, work has been accomplished to determine how much helix II of a ribozyme could be reduced in size and modified without losing the activity. As a result of this study, shorter ribozymes, which were termed minizymes, were found to have a cutting activity. In one such active minizyme, helix II was replaced by a stretch of four thymidines while the arms forming helices I and III were replaced by DNA.[58] Other closely related minizymes having linkers different to T_4 proved to be less efficient.[59] Another effort was undertaken to explore the importance of helix II. Thus, various tetraloops have been probed showing little effect suggesting that the thermodynamic stability of the loop does not seem to contribute to the catalyst efficiency. However, this study established the importance of having at least two base pairs in helix II to observe efficient cleavage.[11] As a consequence, a number of reports have proposed modified ribozymes having a short helix II loop which was replaced by different types of linkers, some of them being non-nucleosidic. Thus the simple replacement of the four nucleotides of loop II by hexaethylene glycol, or other related motifs, appeared to have very little effect, under stoichiometric conditions, in comparison with the native ribozyme. However, shortening the linker to less than nine atoms was detrimental for the activity.[60] In view of observing a maximum activity with minizymes, which were DNA-armed in helices I and III, it was suggested to increase the length of the ethylene glycol linker in order to reach 25 atoms.[61] Instead of using a long flexible linker in place of loop II, abasic sites have been incorporated with the expectation that they might somehow maintain its atom backbone. The data which were gathered after this modification together with the shortening of helix II indicated that it was compatible with the catalytic activity.[62] As a complement to this work, 2′-aminoacyl-2′-deoxyuridines, the acyl group being an aminoacid residue (alanine, phenylalanine, lysine or asparagine), were incorporated in the loop of helix II. Here again the ribozyme activity was retained.[48] Another clever modification of loop II, which might simplify the chemical synthesis of hammerhead ribozymes, resulted of a reductive alkylation reaction between a 5′-aminohexyl-3′-half ribozyme and the product of periodate cleavage at the 3′ end of the 5′-ribozyme half. The ribozyme which had a morpholino-link in loop II proved to be active.[63]

3.2. Modifications within the catalytic core

In most cases the catalytic core cannot be modified without provoking deleterious effects. Unfortunately, in spite of the remarkable advances in the structural studies of the hammerhead domain (see section 2.3), it is not yet possible to fully rationalize these effects in term of tertiary interactions[64] and thus utilize this wealth of structural informations to optimize the design of stable catalytically active ribozymes. Below we briefly review, at the level of each residue of the catalytic core, the modifications which are (or are not) compatible with activity and indicate, whenever possible, those which could improve RNase resistance. Since these modifications were in general characterized under many different reaction conditions, care must be exercised in the comparison of the results. A modification will be considered satisfactory if the ratio k_{mod}/k_{wt}, is close or over 0.5 (in steady state or single turnover conditions) for experiments which in general show little effect on the K_m.

3.2.1. Modifications of the non-canonical base pairs. Hammerhead ribozyme X-ray structural analysis has unexpectedly revealed a particular region of the domain in which three non-canonical base pairs were observed, namely U7-A14, G8-A13 and A9-G12. Besides base-pairing, the tertiary interactions between these six residues manifested an array of hydrogen bonds implicating the donor and acceptor sites of their ribonucleosides and phosphates. Such an intricate hydrogen bond network is certainly crucial for the activity since there are many studies which demonstrate the importance of the integrity of the residues involved in this structure.

In the X-ray structure[25] as well as in the modelled structure,[30] base pairs G8-A13 and A9-G12 form reversed-Hoogsteen hydrogen bonds. The sugar pucker of G8 is shifted towards C2′ endo with its 2′-hydroxyl forming hydrogen bonds with N^7 of G10.1 and G12 exocyclic amino groups. These observations correlate well with the decrease of activity noticed after either the replacement of G8 by 2′-deoxyguanosine,[35,65a,b] as well as araguanosine[66] or the substitution of its 2′-hydroxyl by fluoro or amino groups.[65b] Similarly the importance of the 2-amino group of G8 was underlined by the loss of activity after inosine substitution.[65c,67] For A13, the complementary base of G8, its replacement by a deoxyadenosine resulted in a moderate decrease of activity.[68] However, the introduction at this position of a 2′-fluoro-2′-deoxyadenosine[68] or an isoguanosine is well tolerated, a result which is in agreement with the observed G8-A13 base pairing.[69]

A number of modifications at positions A9 and G12 have been tested. Thus, A9 could be replaced by 2′-deoxyadenosine,[65a,68] 2′-*O*-allyl-[57] and 2′-fluoro-2′-deoxyadenosine[68] without damage. However, other functional group modifications led to an unacceptable decrease of activity. It is surprising that the replacement of A9 by 7-deazaadenosine (tubercidin)[65a] or N^9-purine riboside (nebularine)[65a,67] produced a relatively active ribozyme since, according to the three dimensional structure data, these modifications should deeply weaken the hydrogen bond network involving A9. It is also noteworthy that the N^3 position of A9 does not play a critical role in the chemical cleavage step since its replacement by a 3-deazaadenosine residue gave rise to a ribozyme which exhibited the same behaviour as the native system.[70] Interestingly, for the base pair A9-G12, introduction of deoxynucleosides is well tolerated. This is more particularly the case for G12 substitution where an increase of activity can be observed.[65c] It could indicate that the hydrogen bond between the oxygen of its 2′-OH and a hydrogen of the 6-amino group of A9 does not play an important role for the activity. However, all the other types of modification such as the replacement by inosine[65c,67] 2-aminopurine riboside[65c] and O^6-methyl-guanosine[71] had dramatic effects. In contrast the introduction at this position of a 7-deazaguanosine is well tolerated.[72] It would be interesting to know if this substitution confers RNase stability.

In this context one has to consider the modifications at the N7-A14 base pair site. Nucleotide N7 is most commonly a U, although natural hammerheads containing A and C at this position have been encountered.[3] It was observed, that U7 could be efficiently photo-cross-linked to a 6-thiodeoxyinosine[73] or a 4-thiodeoxyuridine[29a] introduced in position 16.1 of a substrate analogue indicating that it is close to the cleavage site. In the crystal structure, the uracil ring forms hydrogen bonds involving its N^3H with O^6 of G8 and its O^2 with the exocyclic amino group of A14. These bonds are not required for activity since C, A and G are well tolerated at position 7.[10a]

Interestingly, substitution of this position by nucleoside analogues favouring 3′-*endo* sugar puckering was found to enhance the rate of the cleavage step. Thus replacement of uracil by a 4-pyridone residue increased k_2 by 12-fold.[74] On the other hand introduction of an abasic site at this position resulted in a 50-fold lower activity[60,75] indicating that base stacking between N7 and U16.1 is important for maintaining the active structure. These observations suggest that N7 might function as a hinge between the extended stem II and the catalytic loop. Moreover, some degree of conformational freedom at this position could allow an enhanced catalytic activity. Another important point, with regard to U7, deals with its susceptibility in the presence of RNase. This will be discussed in section 3.2.3 together with that of the other pyrimidine nucleosides of the catalytic pocket.

A number of modifications have been introduced at A14. Except for nebularine (N^9-purine ribonucleoside)[67] which is well tolerated, these substitutions provoke a decrease of activity. This decrease is moderate for 2′-deoxy-, 2′-fluoro-2′-deoxyadenosine,[68] 3-deazaadenosine[70] and aristeromycin (4′-carbaadenosine) replacements. In the latter case an increased RNase resistance was observed at the modified position whereas it was suggested, to explain the damaging effect, that the presence of aristeromycin at position 14 might have reinforced hydrogen bonding with U7 impairing the flexibility at the catalytic pocket level.[76]

3.2.2 Modifications at positions G10.1, C11.1 and A15.1. Residues G10.1.and C11.1 form a Watson–Crick base pair that must be retained in order to maintain the catalytic activity. Indeed, when this base pair was replaced by a C10.1-G11.1 base pair a 300-fold decrease of the initial cleavage rate was observed.[10a,11] However, modification of the 2′-functional group of C11.1 can be envisaged. In particular a 2′-fluoro-2′-deoxycytidine could be introduced at this position without affecting the activity.[77] More interestingly, a base pair d(G10.1-C11.1) made of 2′-deoxynucleosides was tolerated.[50b] In view of this result a base pair incorporating either 2′-*O*-methyl- or 2′-*O*-allyl-nucleoside derivatives was found compatible with a reasonable activity.[45,57] This observation is important because such residues offer a strong RNase resistance.

In general, substitution of A15.1 led to a one to two order of magnitude loss of activity. This was observed after adenosine replacement by 2′-deoxyadenosine, 2′-deoxy-2′-fluoroadenosine or 3-deazaadenosine.[68,70] Similarly, the introduction of 2′-*O*-alkyl nucleoside cannot be recommended at this position.[45,57] In the case of tubercidin (7-deazaadenosine) there is a disagreement between two reports. In the earlier one[78] a ten-times decrease was observed while a slight increase was noticed in the second one.[79] For this position, the best results were apparently obtained after substitution of A15.1 by aristeromycin which provoked a twofold decrease both of the k_{cat} and the K_m so that the ratio k_{cat}/K_m remained the same for the considered ribozyme.[76]

3.2.3. Modifications within the catalytic pocket. Another very critical sequence of the hammerhead ribozyme includes the four residues C3, U4, G5, and A6 of the so-called catalytic pocket. This short oligonucleotide stretch exhibits a conformation identical to that of the U turn found in the anticodon loop of $tRNA^{Phe}$.[25a] Since photo-cross-linking data have revealed the high conformational flexibility of this sequence, it might be suggested that cooperatively these residues must play an important role in the elaboration the active ribozyme conformation.[29] Indeed, with a few exceptions (C3 can be replaced by 2-pyrimidinone ribonucleoside with moderate activity decrease albeit in relatively high Mg^{2+} concentration),[80] none of these can be modified without a dramatic loss of the activity. However, the introduction of nebularine unit in place of A6 can be tolerated.[67,78] The presence of aristeromycin, which is a close adenosine analogue, in place of A6 induced a 30-fold decrease of the catalytic activity.[76]

Accordingly, for the design of active but nuclease resistant ribozymes one must be very careful at changing any residue of this short sequence whose importance is certainly due to its role in clustering the Mg^{2+} ions necessary for the catalytic step.[32] Studies on the stabilities of ribozymes in either foetal bovine or human serum have shown that they give the same pattern of degradation and that these serum RNases have a RNase A character, demonstrating a significant preference for pyrimidine nucleoside sites.[50a] Accordingly, to design stable and active ribozymes, it was found reasonable to limit the modifications at the exposed pyrimidine positions of the 5′-CUGA sequence of the hammerhead. Inspection of the literature indicates that a promising approach can be recommended. It proposes to concomitantly undergo these modifications at the U4 and U7 positions since U7 has been found to be particularly susceptible to endoribonucleases. In the case of a highly modified DNA/RNA chimeric ribozyme, thiophosphates were introduced on the 5′ side of C3, U4, G5, and G8, which is vicinal to U7. This modified ribozyme had a k_{cat} comparable to that of the wild ribozyme but with a 200-fold increased K_m.[50a] Similarly, the effects of the introduction of 2′-amino-2′-deoxyuridine and 2′-fluoro-2′-deoxyuridine at positions 4 and 7 were compared.[77,81] In regard to the activity, a real beneficial effect was found when these two positions were occupied by 2′-amino nucleoside derivatives, the cleavage capacity being very close to that of the native ribozyme whereas the lifetime of this ribozyme in foetal calf serum was greatly increased.[77] However, when the U4 and U7 positions were substituted by 2′-acetamido-2′-deoxyuridine, the corresponding ribozyme was unable to cleave its substrate. It is believed that the 2′-acetamido modification is too bulky to maintain an active structure. Surprisingly, the ribozymes which incorporated a number of 2′-acetamido-2′-deoxynucleosides did not reveal an enhanced stability compared to the unmodified ribozyme when incubated in the presence of foetal calf serum.[46a]

The above results illustrate how difficult it is to modify the catalytic core of the ribozyme without dramatically perturbing the cleavage capacity. Thus, the design of nuclease resistant, but enzymatically active, ribozymes should take advantage of the freedom of allowing the introduction of a large body of modifications in helices I, II and III while modification of the catalytic core should be kept minimum. To date, two research groups have elaborated synthetic ribozymes in which all the residues, except for five residual positions G5, A6, G8, G12, and A15.1, were modified. In terms of nuclease resistance and activity these constructions represent the most satisfactory compromise. In one case, except for the five critical positions, the modified ribozyme was mostly made of 2′-*O*-allyl ribonucleotides. Surprisingly, this ribozyme, which was found to have a reduced activity in vitro, demonstrated efficacy in mice.[82] Similarly, modified ribozymes containing the more accessible 2′-*O*-methyl ribonucleotides and five crucial purines in the central core have been synthesized. These molecules manifested a remarkable serum stability together with a relatively high catalytic activity compared with their all-RNA parent ribozymes.[45] In this work a systematic study has been undertaken to determine the best choice to modify positions U4 and U7. After trying many combinations, it was found, in agreement with another study, that the introduction of a 2′-amino-2′-deoxyuridine at positions 4 and 7 represents the best compromise.

4. In Vivo Applications of RNase Resistant Hammerhead Ribozymes

In the past few years, it was experimentally demonstrated that hammerhead ribozymes could be used for the inactivation of genes in oocytes and for the inhibition of viral replication in infected cells after microinjection[83] or liposome mediated transfection.[84] In principle, for viral infections provoked by a retrovirus, ribozymes can be tailor made to cleave specifically the viral RNA transcripts without touching cellular transcripts. Unfortunately, this is easier said than done and at present there is very little information about the stability and activity of modified ribozymes in an intracellular environment. Recently, an antistromelysin

nuclease-resistant hammerhead ribozyme was designed[85] that contained mostly 2′-*O*-methyl-nucleotides together with 2′-amino-2′-deoxyuridines at positions U4 and U7, four phosphorothioate linkages at the 5′ end and a 3′-3′ inverted thymidine at the 3′ end. The ribozyme was directly administered into the intra-articular space of the knee joint of New Zealand rabbits. After 24 h, 80–90% of the modified ribozyme remained intact in this synovial tissue. As a result of a delivery study an average 60% reduction of the level of the target protein, stromelysin, was observed.[85] Another important investigation which was performed in a biological context used a nuclease resistant ribozyme. In this ribozyme, pyrimidine nucleosides were replaced by their 2′-fluoro analogues, except for U4 and U7 which were substituted by 2′-amino-2′-deoxyuridines while several thiophosphates replaced phosphodiester bonds at the 3′- and 5′-end. The investigation of the cleavage of HTLV-1 *tax* RNA in nuclei suspension showed that, compared to the rate of cleavage of isolated *tax* RNA, cleavage was enhanced 30-fold in the nuclear compartment.[22] This increased efficiency was ascribed to a greater accessibility, in the presence of the nuclear proteins, of *tax* RNA to ribozyme mediated cleavage. In a recent ex vivo study a chimeric RNA/DNA ribozyme with phosphorothioate at its 3′ end, was delivered into porcine aortic vascular smooth muscle cells by transfection with cationic lipids.[86] This ribozyme, which was designed to cleave a sequence within leucocyte 12-lipoxygenase (12-LO) mRNA, caused a dose-dependent decrease of 12-LO mRNA at levels where neither antisense (or sense) oligonucleotides would be effective. Interestingly, as a control an inactive modified ribozyme, containing a single nucleotide substitution in the catalytic core, showed less inhibition than the intact ribozyme. This indicates that, in this particular case, the modified ribozyme is a more potent inhibitor of mRNA translation than an antisense oligonucleotide. In the case of DNA armed ribozymes such controls are important, because interpretation of the cleavage results might be biased by an RNase H activity involving helices I and III heteroduplexes.[87]

5. Concluding Remarks

The major prospective applications of hammerhead ribozymes in the field of the control of gene expression by means of cleavage of such gene products, as those associated with cancer and retroviral infection (AIDS), are beyond the scope of this review. Its main objective was to present the large body of information which is now available in the domain of hammerhead ribozymes in a perspective which might be useful in the field of medicinal chemistry. We have emphasized the current difficulties to clarify the role of the functional groups of the important residues of hammerhead ribozymes. Generally it is impossible to give a reasonable explanation of the effects of modifications in the context of both the kinetic scheme and the now well known ground state conformation. In many cases functional groups that were identified as important for catalysis were not found to be involved in specific tertiary interactions which correspond to the ground state situation of the complex. Indeed, modifications can affect the ribozyme structure disrupting either base-base stacking and/or hydrogen bonding with dramatic consequences on either the correct folding and/or the binding of divalent metal ions and more particularly those required as co factors. Moreover, modifications can disturb the transition state geometry affecting one of the steps in the catalytic pathway: 2′-OH deprotonation, alignment of 2′-O^- and P-O(5′) or rupture of the P-O(5′) bond.

Despite the limitations of our knowledge concerning hammerhead ribozymes, active and stable ribozyme analogues were synthesized incorporating a great number of well tolerated 2′-modifications provided they were restricted to well defined regions. The role of stem-loop II has been thoroughly studied; as a result it can be shortened and considerably modified making easier the synthesis of artificial ribozymes. Finally, one might ask whether ribozymes exercise a true cleaving activity or simply act by an antisense-like process. The question can be answered by utilising inactive ribozymes such as those obtained by mutations in the central core. However, with antisense oligonucleotides which in several cases exhibit an unexpected non-antisense biological activity, it cannot be ascertained that the biological effects of in vitro active ribozymes correspond to a cleavage activity if the reaction products cannot be characterized. In conclusion, hammerhead ribozymes are fascinating molecules which might inspire chemists to design an even better catalyst for RNA cleavage.

In a recent paper[88] the influence of metal ions on hammerhead activity was critically reexamined. It was concluded that a model involving two divalent cations directly coordinated to the attacking 2′ oxygen and to the 5′ leaving oxygen, respectively, and one of them being chelated to the pro-*R* oxygen, fitted the data more easily than the previously proposed one-metal-hydroxide ion model.

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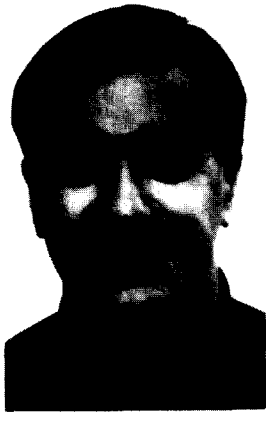

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