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Review article

A critical survey of the structure-function of the antisense oligo/RNA heteroduplex as substrate for RNase H

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Dedicated to the 70th Birthday of Professor J.J. Fox (Sloan Kettering Memorial Cancer Center, NY, USA)

Abstract

The aim of this review is to draw a correlation between the structure of the DNA/RNA hybrid and its properties as a substrate for the RNase H, as well as to point the crucial structural requirements for the modified AONs to preserve their RNase H potency. The review is divided into the following parts: (1) mechanistic considerations, (2) target RNA folding–AON folding–RNase H assistance in AON/RNA hybrid formation, (3) carbohydrate modifications, (4) backbone modifications, (5) base modifications, (6) conjugated AONs, (7) importance of the tethered chromophore in AON for the AON/RNA hybrid interactions with the RNase H. The structural changes in the AON/RNA hybrid duplexes brought by different modifications of the sugar, backbone or base in the antisense strand, and the effect of these changes on the RNase H recognition of the modified substrates have been addressed. Only those AON modifications and the corresponding AON/RNA hybrids, which have been structurally characterized by spectroscopic means and functionally analyzed by their ability to elicit RNase H potency in comparison with the native counterpart have been presented here. © 2001 Published by Elsevier Science B.V.

Keywords: Structure; DNA/RNA hybrid; AONs; RNase H

RNase H is an endogenous enzyme, which hydrolyses the RNA strand in an RNA/DNA hybrid in a catalytic manner [26,78]. It produces short oligonucleotides with

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5′-phosphate and 3′-hydroxy groups as final products [14]. Bivalent cations as Mg^{2+} and Mn^{2+} are found to be necessary cofactors for enzymatic activity [5,26,77]. The enzyme is widely present in various organisms [14], including retroviruses, as a domain of the reverse transcriptase [91]. The RNase H1 from *Escherichia coli* is the most characterized enzyme in this family [38,39,94]. Even though the physiological functions of *E. coli* RNase H1 have not been understood clearly, it has been suggested to be involved in DNA replication and repair [8]. This enzyme was found to be required for the initiation of Col E1 DNA replication in vitro [16,33]. It was suggested that after RNA has served as a primer, it is eliminated from product by RNase H. This enzyme is also involved in the chromosomal DNA replication [30,31,41,64].

RNase H-promoted cleavage of the viral mRNA via formation of the duplexes with complementary oligo-DNAs (antisense strand) is one of the strategies to treat viral infections [13,57]. Recent isolation of the human RNase H1 and RNase H2 highlights the importance of the development of the antisense drugs utilizing this mechanism of action [11,20,92,93].

The aim of this review is to draw a correlation between the structure of the DNA/RNA hybrid and its properties as a substrate for the RNase H, as well as to point the crucial structural requirements for the modified AONs to preserve their RNase H potency. The review is divided into the following parts: (1) mechanistic considerations, (2) target RNA folding–AON folding–RNase H assistance in AON/RNA hybrid formation, (4) backbone modifications, (5) base modifications, (6) conjugated AONs, (7) importance of the tethered chromophore in AON for the AON/RNA hybrid interactions with the RNase H. We will concentrate on the structural changes in the AON/RNA hybrid duplexes brought by different modifications of the sugar, backbone or base in the antisense strand, and the effect of these changes on the RNase H recognition of the modified substrates. Only those AON modifications and the corresponding AON–RNA hybrids have been presented here, which have been structurally (by spectroscopic means) and functionally (by RNase H potency) correlated to the native counterpart.

1. The mechanistic considerations of AON/RNA hybrid interactions with the RNase H

The service-end of the RNA cleavage reaction is the 2′-OH of the RNA strand in the antisense (AON)/RNA hybrid [34,37,83,84]. The stereochemical location of the 2′-OH group of the RNA strand is in the minor groove of the AON/RNA hybrid; hence, the site for the RNase H-promoted catalytic cleavage activity is highly sensitive on how the conformation of the minor groove changes upon introduction of any 2′-modification (F, NH_2) in the ribo configuration [83] or backbone modification (phosphorothioate with either Rp or Sp) [34] in the RNA strand. It turned out that the RNA strand in the heteroduplex with any substitution of 2′-OH was not substrate to RNase H (For the effect of Rp or Sp thioates, see the chapter on backbone modification). This work also proved the importance of the 2′-OH group in the minor groove for the RNase H catalysis. Presumably an outer sphere complex of Mg^{2+} ion with water molecules binds to the 2′-OH of the RNA strand [84], and positions itself for accepting the departing

conjugate base, 3′-oxyanion, liberated during the phosphate hydrolysis. Such a scenario might explain the importance of steric availability of the 2′-OH of the RNA strand, as well as stereoelectronic accommodation of Mg^{2+} ion complexed with water in the minor groove for the catalytic activity of the RNase H. Much attention is given to the fact how the minor groove width in the hybrid changes, upon introduction of any modification, compared to the DNA/DNA and RNA/RNA duplexes. An important issue is how flexible the minor groove should be to accommodate the conformational adjustment required by the 2′-OH for assisting in the RNase H-promoted hydrolysis.

The conformational rigidity imposed on the antisense strand locked in the O4′-*endo* conformation [18,48] may not be adequate for the optimal RNase H cleavage activity because of at least three reasons: (i) interference with the spines of hydration in the minor groove (often by introduction of fatty substituent); (ii) interference with the flexibility required in the minor groove for the approach of RNase H/Mg^{2+}-hydrate for interaction and binding; (iii) interference with the flexibility of the sugar-phosphate backbone (i.e. imposing high activation-energy barrier) required for the pseudoroation of the trigonal bipyramidal phosphorane to be formed upon the OH^- attack to the potentially scissile phosphate.

This is particularly demonstrated in case of LNA and bc-ANA hybrid with RNA [56]. This comparison also has shed light on the kind of conformational flexibility required for the AON/RNA hybrid to become a substrate for RNase H. It is not clear what is the minimal structural change required to coax the AON strand to adopt the O4′-*endo* conformation, yet retaining sufficient flexibility in the AON/RNA hybrid to accommodate the RNase H activity!

2′F–ANA and ANA strand of the hybrid duplexes with native RNA are known to adopt the O4′-*endo* sugar conformation [15,56,62] with 2′-F or 2′-OH groups pointed into the major groove of the AON/RNA duplex in such a way that they do not interfere with the catalytic cleavage of the RNA strand by RNase H/Mg^{2+} hydrate complex in the minor groove, which was recently supported by molecular dynamics calculations [86]. This means that in these AON/RNA hybrids, the outer sphere Mg^{2+} hydrate and RNase H can approach the 2′-OH (and the scissile phosphate) without any stereochemical or stereoelectronic hindrance. Thus, the F–ANA/RNA and ANA/RNA duplexes are the only examples of the 2′-*O*-modified AON/RNA complexes that are recognized and cleaved by the RNase H [15,62] because they adopt the most energetically favorable O4′-*endo* sugar conformation, giving minimal energy penalty for any of the structural requirements, such as the flexibility of the minor groove and the sugar-phosphate backbone and hydration required for RNase H interaction and function. The criteria of a best antisense strand for modulating the target RNA strand into a “cleaving conformation” are not only based on the fact that it is locked and preorganized into a DNA-type strand upon the hybrid duplex formation. This is evident from a recent work [56] on (3.3.0) bicyclo-ANA (bc-ANA), which was found to be locked in the desired O4′-*endo* conformation, but no RNase H cleavage. Thus, a $d(T)_{14}/r(A)_{14}$ duplex containing thirteen bc-ANA residues failed to activate the RNase H. On the basis of the molecular modeling, one can suggest that the additional atoms of the (3.3.0)bicyclo moiety introduced between the 2′-*O* and 3′-*C* of the arabinose are protruding into the major groove of the duplex, which probably should not have interfered with the cleavage.

Clearly, this would have been the case *if only the conformational, as well as hydration, requirement around the 2′-OH and the scissile phosphate could have been fulfilled*. It is likely that the (3.3.0)bicyclo moiety has frozen the conformation of the scissile sugar-phosphate backbone, it has also perhaps purged water out from the proximity of the 2′-OH and the vicinal scissile phosphate groups, thereby has made the approach of RNase H/Mg^{2+} hydrate in the minor groove energetically unfavorable. Clearly, an optimal hydration around the 2′-OH and the scissile phosphate is vital because it allows the approach of the hydrated complex of Mg^{2+} to deliver activated (more acidic) water molecules to donate a proton to scavenge the conjugate base, the 3′-oxyanion. It may be noted that histidine 124 of RNase H is located in the major groove to produce OH^- ion required for the bimolecular phosphate hydrolysis (clearly accessibility of the active water around histidine 124 is also advantageous), whereas the liberating 3′-oxyanion needs a proton to drive the cleavage reaction to completion. In this process, any lipophilic (i.e. oily) function interfering with the hydration process in and around the vicinity of the minor groove will produce deleterious effect (water poisoning) [47,48–50].

The NMR and CD data suggested that both the enzyme and the substrate undergo some structural changes upon binding [63]. The direction and extent of these changes cannot be assessed because no crystal or NMR structure of the enzyme-substrate complex is available to date. However, such dynamic adjustment of the local geometry at the cleavage sites of the hybrid substrate is perhaps necessary to allow binding to the outer sphere Mg^{2+} hydrate–RNaseH complex in the minor groove. This means that the activation-energy barrier for the local geometrical change of the RNA strand (particularly, at the scissile internucleotidyl site) mediated by either AON and/or RNase H should not be very high. Therefore, one should assess the nature of the geometrical constraints one imposes upon constraining the AON strand into a fixed geometry.

Introduction of conformationally constrained nucleotides can make the AON strand indeed locked to mimic the DNA- or RNA-type conformation [28], but there is an energy penalty for it. The very high activation-energy barrier for complete loss of flexibility of the AON strand and, consequently, of the RNA strand in the AON/RNA hybrid makes the minor groove irresponsive to the structural adjustments required for recognition and interaction by RNase H. Compared to these AONs with locked N-type conformation, 2′F–ANA shows a thermodynamic preference for the O4′-*endo* conformation; nevertheless, it can be steered to a different sugar pseudorotamer and, consequently, can steer the intermediary trigonal bipyrimidal phosphorane pseudorotation to a conformational hyperspace required for the RNase H cleavage chemistry operating at the scissile sugar-phosphate of the RNA strand.

The width of the minor groove of the duplex is thought to be responsible for the substrate recognition by the RNase H [18,72]. From the crystallographic data (Table 1), it has emerged that the minor groove width in DNA/RNA duplex is rather large, indeed very close to the one in the A-type RNA/RNA duplex compared to DNA/DNA duplex, which is relatively much narrower. The width of the minor groove of DNA/RNA hybrids can vary from 8.9 to 10.5 Å compared to the one of the DNA/DNA duplexes (varying from 3 to 7.4 Å), and RNA/RNA (varying from 9.3 to 10.8 Å) counterparts. The comparison of the entries shown in Table 1 suggests that the width of the minor groove of the hybrid duplex is sequence-dependent. The variation of the minor groove

Table 1
Minor groove width as a function of sequence and type of the nucleic acid duplex from X-ray studies

X-ray			
Type of the duplex	Sequence	Minor groove width[a] (Å)	NDB[b] index
RNA/RNA (A-type)	5′-r(UAGCCCC)-3′, 5′-r(GGGGCUA)-3′	9.5 –9.9	AR0010
	5′-r(pGGGGGGGGGGG)-3′, 5′-r(pCCCCCCCCCCC)-3′	9.8–10.1	AR0020
	5′-r(CGUACG)-3′	10.5–10.8	ARF0108
	5′-r(CCCCGGGG)-3′	9.9–10	ARH064
	5′-r(UUAUAUAUAUAUAA)-3′	9.8–10.7	ARN035
	5′-r(CCCCGGGG)-3′	9.6–10	ARH074
	5′-r(UAGCGGUGC)-3′, 5′-r(GCACCGCUAC)-3′	9.3–9.9	AR0021
	5′-r(GCACCGUUGGUAGCGGUGC)-3′, 5′-r(GCACCGCUACCAACGGUGC)-3′	9.3–10.2	AR0021
	5′-r(UAGCUCC)-3′, 5′-r(GGGGCUA)-3′	9.7–10	AR0013
DNA/DNA (B-type)	5′-d(ACCGACGTCGGT)-3′	6.6–7.2	BD0001
	5′-d(CGCGAATTCGCG)-3′	3.7–4.7	BD0004
	5′-d(CCGCCGGCGG)-3′	7–7.1	BD0015
	5′-d(GCGAATTCGCG)-3′	3–3.9	BD0018
	5′-d(CCGCTAGCGG)-3′	6.6–6.7	BD0028
	5′-d(CGCGAATTCGCG)-3′	3.1–4.4	BD0029
	5′-d(CGATCGATCG)-3′	4.6–6.5	BDJ025
	5′-d(CGCATATATGCG)-3′	3.5–4.8	BDL007
	5′-d(ACCGGCGCCACA)-3′	7–7.4	BDL018
	5′-d(CGCTCTAGAGCG)-3′	6.2–7	BDL070
	5′-d(ACCGACGTCGGT)-3′	7.1–7.4	BD0002
	5′-d(ACCGGTACCGGT)-3′	6.7–7.2	BD0003
	5′-d(CGCGAATTCGCG)-3′	3.4–4.2	BD0005
	5′-d(CATGGCCATG)-3′	5.5–6.7	BDJ051
	5′-d(CTCTCGAGAG)-3′	5.4–6.4	BDJ060
	5′-d(CCACTAGTGG)-3′	3.6–4.9	BDJ061
DNA/RNA (A-B-type)	5′-r(GAAGAGAAGC)-3′, 5′-d(GCTTCTCTTC)-3′	9–10.3	AH0001
	5′-d(CTCTTCTTC)-3′, 5′-r(GAAGAAGAG)-3′	9.5–10.4	AH0005
	5′-d(CTCCTCTTC)-3′, 5′-r(GAAGAGAGAG)-3′	8.9–10.2	AH0010
	5′-d(TTCTTC + pTTC)-3′, 5′-r(GAAGAAGAA)-3′	9.3–10	DR0003
	5′-r(UUCGGGCGCC)-3′, 5′-d(GGCGCCCGAA)-3′	9.7–10.5	UHJ055

[a] Based on P–P distances minus 5.8 Å.
[b] The Nucleic Acid Database Project Rutgers, The State University of New Jersey: http://ndbserver.rutgers.edu/NDB/NDBATLAS/index.html.

width found in DNA/RNA hybrid duplex is not statistically different than the average minor groove width found in the A-type of RNA/RNA duplex, unlike many reports in

the literature [18,56,72]. There is a high degree of similarity in the X-ray (taken from the Nucleic Acids Data Bank) and NMR data regarding the spread of the minor groove width in the RNA/RNA and DNA/RNA duplexes (Table 2). Taken together, all these data show that the minor groove width is important; however, it not the ultimate requirement for the substrate recognition. This was also demonstrated [61] on the 2′-*O*-methylated chimer DNA/RNA hybrid duplex containing an RNA/DNA segment in the center. NMR study revealed that all DNA residues in this duplex, except one, had typical C3′-*endo* conformation (A-type RNA) and the duplex had a wide minor groove (11.5–12.5 Å) similar to those of the RNA/RNA hybrids. Nevertheless, this duplex is recognized by the RNase H and cleaves at the specific position [32,60].

We suggest that not only the width, but the content of the minor groove, have crucial effect on the recognition and cleavage of the AON/RNA hybrids by the RNase H. The absence of 2′-OH function (as in DNA/DNA structures) or the presence of any additional substituents (2′F–DNA/DNA) or an additional presence of a 2′-OH group as in an RNA/RNA duplex in the minor groove might result in alteration of the interactions with the outersphere Mg^{2+}–water complex (in RNase H). Similarly, for the catalytic cleavage, the loss of flexibility for the conformational adjustment of the ternary complex (not possible with the extremely rigid antisense strand as with the constrained nucleotide units) might result in RNase H resistance. Alterations in the hydration

Table 2
Minor groove width as a function of sequence and type of the nucleic acid duplex from NMR studies

NMR			
Type of the duplex	Sequence	Minor groove width[a] (Å)	PDB[b] number or reference
RNA/RNA (A-type)	5′-r(CGACUCAGG)-3′, 5′-r(CCUGCGUCG)-3′; (CU mismatch)	10.8–10.9	1C4L
	5′-r(CGCGCG)-3′	11.1	1PBM
	5′-R(GGCGAGCC)-3′; (GA mismatch)	8–8.4	1YFV
DNA/RNA (A-B-type)	5′-d(GCGTCAGG)-3′, 5′-r(CCUGACGC)-3′	8.7–9.3	8DRH
	5′-d(GPsCPsGPsTPsCPsAPsGPsG)-3′, 5′-r(CCUGACGC)-3′	8.7–9.5	8PSH
	5′-d(GTCACATG)-3′, 5′-r(CAUGUGAC)-3′	8.5–9.5	Ref. [18]
	5′-d(TCAATC)-3′-Pzn, 5′-r(GAUUGAA)-3′	8.6–9.5	Ref. [47]
DNA/DNA (B-type)	5′-d(GGCAGGTGGTG)-3′, 5′-d(CACCACCTGCC)-3′	6.4–7.5	1AFZ
	5′-d(GCCGTTAACGGC)-3′	3.3–4.9	132D
	5′-d(CGGACAAGAAG)-3′, 5′-d(CTTCTTGTCCG)-3′	6.1–7.2	1AGH

[a] Based on P–P distances minus 5.8 Å.

[b] Berman HM, Westbrook J, Feng Z, Gilliland G, Bhat TN, Weissig H, Shindyalov IN, Bourne PE. The protein data bank. Nucleic Acids Res. 2000;28:235-42. (PDB; http://www.rcsb.org/pdb/).

pattern, as well as steric hindrance of the 2′-OH of the RNA, could also contribute to this loss. This can also be seen from the last example [61], where no significant differences in the structure of the DNA/RNA and 2′OMe–RNA/RNA parts of the duplex were observed. However, only the DNA/RNA part was susceptible for cleavage. The methyl groups of the 2′OMe–RNA/RNA part are directed towards the minor groove and result in formation of the hydrophobic layer affecting the hydration pattern in the minor groove [2,61,66]. Thus, we conclude the following on the minor groove in AON/RNA duplexes: (1) The AON/RNA duplex is preferred to have a helical structure with the minor groove wide enough to accommodate the chemistry of the RNase H cleavage site (closer to the A-type rather then B-type). (2) AON should possess enough flexibility to support the conformational change required for the realization of the RNase H cleavage. (3) Any modification in the minor groove or its proximity should be avoided to preserve availability of the 2′-OH of the RNA and the associated hydration pattern.

The CD spectroscopy is a very convenient way to determine the melting transitions [17] and the global helical structure of the nucleic acids complexes [82]. The special features of the CD spectra (the CD band intensities, wavelength of the CD bands maxima and minima as well as the crossover points) allow fast and easy classification of any nucleic acids complexes into the above-mentioned structural classes. In agreement with X-ray data, CD suggests that the conformation of DNA/RNA hybrid duplexes is intermediate between A (RNA/RNA) and B (DNA/DNA) geometry [43]. Shifts in the absorption bands or changes in the relative magnitudes of Cotton effect reflect the change brought into the structure of duplex upon the duplex modification (nucleoside or backbone modifications in the AON strand) and allow us to qualitatively estimate the alterations of the minor groove (using X-ray or NMR structures as the reference points), which is apparently so important for the recognition. Although it is a very valuable method to gather the helical information of the complexes, the sensitivity of CD to the minor or local structural distortions in AON/RNA hybrid has proven to be inefficient and insensitive for the prediction of the RNase H recognition, interaction and cleavage [67].

Recently, the effect of single modification, with N-type constrained sugar moiety in thymidine nucleotide block, on the conformational characteristics of the AON/RNA duplex has been examined by CD and RNaseH degradation studies [67]. Four 15 mer AONs holding a single 1-(1′,3′-*O*-anhydro-β-*D*-psicofuranosyl)thymine block [locked in the N-form (3′-*endo*)] systematically incorporated at various sites of an AON sequence were hybridized to the target RNA. CD failed to detect any structural perturbances of the modified hybrids compared to the native counterpart; however, the RNase H cleavage pattern clearly showed the local conformational changes in the hybrid substrate spanning a total of five nucleotides towards the 5′-end of the AON (3′-end of RNA). This was evident from the fact that the 5 nt region of the RNA strand in the hybrid duplex became completely inactive to the catalytic cleavage reaction by RNase H, although it remained suitable for the enzyme binding, thereby showing how a conformationally constrained nucleotide enforces the neighboring nucleotides to compromise their conformations, and adopts the conformation of the constrained nucleotide. Thus, CD cannot detect, but RNase H can sense, this local change of the conformational character of the hybrid, and

chooses not to act for up to a 5 nt stretch in the RNA strand of the hybrid duplex owing to adoption of a A-RNA/A-RNA-type conformation. This work, therefore, constitutes an example of how microscopic local conformational character is transmitted [1,81] from one nucleotide residue to the others in the neighborhood. It also provides a new example of how the local conformation of a substrate can be monitored exploiting the stereochemical sensitivity of an enzyme to map the local microscopic conformational change invisible to the spectroscopic technique.

Recently, structural studies [6] have been carried out by NMR and CD spectroscopy on hybrid duplex formed by AON containing one locked nucleic acid, LNA, and RNA to examine if there have been any conformational changes in the microenvironment around the modification site. It was found that 3′-nucleotide flanking the modification site adopts a clear N-type conformation. Notable difference was also observed in CD, and it was concluded that overall A-type conformation exists in the AON strand of the duplex stretching only one nucleotide both at 3′- and 5′-ends of the LNA nucleotide modified site, which means that this part of the duplex takes up a more A-RNA/A-RNA-type helix. However, no report of the RNase H cleavage pattern of the above single LNA-modified AON has been available to assess how far the local structural deformation has taken place compared to what has been observed by NMR. Clearly, the cleavage pattern of the RNase H degradation of these duplexes should provide an answer about the extent and direction of the structural changes from the "enzyme point of view", and only those changes are of importance for the further development of the antisense technology.

2. Target RNA folding–AON folding–RNase H assistance in AON/RNA hybrid formation

Cleavage by the RNase H is a three-component process, which requires the presence of the all three participants: the RNA target, the AON and the RNase H itself with Mg^{2+}. Experimental dissection of this complex process into separate simplified steps [i.e. (i) binding of the AON to the target RNA and then (ii) the study of RNase H cleavage reaction] might result in misleading conclusions. This becomes clear from some recent reports, showing that the RNase H is the active participant of the DNA/RNA duplex formation [44]. In this work, 15 nt target sequence in the middle of a 79 nt RNA transcript extensively involved into the secondary structure formation was examined by RNase H in the presence of complementary AONs, which were in the form of single-stranded, duplex or hairpin structures. Detailed thermodynamic investigation and gel mobility shift assay showed no hybrid duplex formation with some of the structured AONs (especially with the hairpin structure) and the 79 nt target-folded RNA. Interestingly, RNase H assay revealed up to 50% cleavage of the target RNA promoted by these structured AONs. These experiments also showed that, in many cases, the target was digested to the comparable extent in the presence of the single-stranded or the structured AONs. It was suggested that RNase H can promote formation and cleavage of a AON/RNA duplex, even if the equilibrium reaction of the RNA and DNA alone does not favor the hybrid formation. This implies that formation of the three-component

complex—DNA/RNA/RNase H—might be favorable, while in the absence of the enzyme, the formation of the two-component complex—DNA/RNA hybrid—was thermodynamically prohibited.

The role of the RNase H in unwinding the RNA target for the successful DNA/RNA/RNase H complex formation and subsequent cleavage was demonstrated in our latest studies [96], where various native and modified single-stranded AONs (with no apparent tertiary structure) were tested on their ability to promote the RNase H-mediated cleavage of the RNA targets having different degree of the folded structures, evident by different free-energy of stabilizations, which were characterized by UV and CD spectroscopy. The result showed that the extent of hydrolysis of the target RNA in the AON/RNA hybrid was not significantly affected by different degree (i.e. relative free-energy difference) of stabilizations of any of the RNA self-folding motifs. It was concluded from this work that RNase H cleavage itself is a slower process than the rates of the folding/unfolding of the target RNA; hence, the latter process, i.e. the state of the folding/unfolding equilibrium of the target RNA, has no significant effect on RNase H-promoted cleavage of the target RNA. The slower cleavage rate of RNase H cleavage requires that the actual substrate (i.e. AON/RNA hybrid) concentration need not be very high; only a very small active population of the hybrid is formed independent of the free-energy of stabilizations of various folded states of the target RNA, and that is sufficient to drive the cleavage reaction.

In another report [46] investigating the ability of the RNase H to hydrolyze structured substrates, the well-characterized 47 nt RNA hairpin structure was targeted with AONs at the different parts of the loop region, and the rates of hydrolysis were compared with those for the non-aggregated short RNA target. The complex of the single-stranded RNA at the loop region and AON exhibited fewer cleavage sites, and each site was cleaving slower than in the unstructured substrates consisting of short 13 nt RNA and AON. On the basis of molecular modeling studies, this result was explained as a steric hindrance of the enzyme by the structured substrate at the loop region. The strategy to target AON to the loop part of the RNA has an intrinsic structural disadvantage in that all single-stranded loop RNA preferentially adopts 2′-*endo* conformation (S-type), which is a "more stretched-out conformation" than what is achieved with a 3′-*endo* sugar (N-type)-containing nucleotide. This is perhaps the reason why the rate of RNase H cleavage of the hybrid in the loop part was found to be slower than the hybrid formed with relatively unfolded single-stranded RNA region.

The question remains which part of the RNA—the single-stranded (loop) part or the duplex part—should be targeted to provide the best availability for the AONs to form the hybrid duplex, followed by RNase H assisted cleavage. To answer these questions, various methods have been developed and they were tested in vitro and in vivo, which have been reviewed recently [75,76]. Among these, the methods based upon mapping of the transcripts with RNase H and oligonucleotide arrays are found to be promising. In few reports, AONs were targeted to the complex mRNA and accessible sites were identified by RT-PCR assays [55,74]. Many of those reports failed to establish a clear correlation between the features of the local RNA structure in the target and activity of the AONs to elicit RNase H response. Recent work [29] has failed to show the correlation between the secondary structures of RNA predicted by computational

methods [87] and the RNase H response on the resulting RNA/AON hybrids. It has been suggested [29] that current level of sophistication of RNA structure prediction has very limited utility for the selection of AON sequences. Very recently [65], a computational semiemperical method was suggested to evaluate the accessibility of the RNA target based on structural predictions by *m*-fold programme. This method is based on the assumption that the most accessible sites are those regions in the target having unpaired bases (i.e. in loops, bulges, junctions or free ends). This assumption shadows the utility of this method because the role of RNase H assistance in the hybrid formation is neglected. Although a better understanding of the mechanism of the RNase H/substrate interactions is necessary, a practical solution for the problem of finding the optimal antisense sequence for the target RNA has been already suggested through assay based on combinatorial AONs arrays [55]. An array of AONs of various lengths complementary to different regions of rabbit β-globin mRNA was fabricated on the surface of the glass chip. Antisense activity of these AONs was measured by RNase H assay and by in vitro translation. The results helped to find the optimal sequences for AONs and the concentration required to inhibit translation by 50%. It is noteworthy that in this work also, no correlation was found between predicted secondary structure of the target RNA and heteroduplex yield observed with the most effective AONs.

3. Carbohydrate modifications

As for other modifications, much attention has been devoted to explore various carbohydrate modifications in order to produce AONs with enhanced binding affinity to the target RNA. The 2′-modifications with an electronegative substituent can confer an RNA-like conformation to the AON, which accounts for their higher binding affinity [51]. In this perspective, if we look at the recent advances, it is easy to note that the loss of RNase H activity of various sugar modified AONs is due to the conformational drive of these modifications, which steer the AON strand to adopt an RNA-type helix in the AON/RNA hybrid duplex. The 2′F–RNA/RNA [40] hybrid duplexes and duplexes formed by the 1′,5′-anhydrohexitol nucleic acids (HNA) with RNA [27] could not activate RNase H irrespective of their higher thermodynamic stability compared to the native counterpart (ΔT_m = 1.2–2.3°C/modification for 2′F–RNA and 1.4–2.5°C/modification for HNA). This can be well understood from the RNA/RNA-type helical conformation observed in the CD spectra of these hybrids. Among various 2′-*O*-alkyl modification of the sugar, 2′-*O*-(methoxyethyl) and 2′-*O*-(aminopropyl) showed [51,53,58] high affinity to target RNA (1–2°C increase in binding affinity per modification). Even though the AONs bearing these modifications showed higher binding affinity compared to the unmodified AONs, they failed to activate RNase H [45,51]. Here also, the 3′-*endo* conformation of the sugar moiety in such modified nucleosides has resulted in the formation of A-type helixes typical of RNA/RNA duplexes [80].

Among the nucleic acids containing one or more conformationally constrained nucleotides, only a few of them have been tested for their ability to elicit RNase H: Introduction of the *N*-methanacarbothymidine block in which the sugar is fixed in the N-form into an oligo chain caused increase in binding affinity to the target RNA

($\Delta T_m = 1.3$°C/modification), but resulted in the complete loss of RNase H activity [52]. Recently reported [90] Locked Nucleic Acid (LNA), where a methylene bridge connects 2′-*O* and 4′-*C* locking the sugar in the 3′-*endo* conformation (A-type RNA/RNA duplex) as revealed by the CD spectrum of LNA/RNA duplex [42], had an unprecedented binding affinity to the target RNA ($\Delta T_m = +46$°C), but elicited no RNase H potency. The α-anomeric oligodeoxyribonucleotides [22], even though exhibited high affinity to the RNA target ($\Delta T_m = +26$°C), failed to show RNase H-promoted cleavage. Here, the loss of enzymatic activity was attributed to the perturbation of the enzyme-substrate complex as a result of the change in polarity of the duplex in conjunction with the parallel binding of α-DNA to the complementary RNA.

Typically, all sugar modified AONs eliciting RNase H activity have global A–B-type helical conformation similar to that of native DNA/RNA heteroduplex, and most of them have lower thermodynamic stability compared with the native. For example, arabino nucleic acids (ANA) have been recently [15,62] tested for their ability to activate RNase H. Both the sequences tested had lower thermodynamic stability in comparison with the natural DNA/RNA hybrid duplex. CD spectra of these duplexes showed close resemblance to the native DNA/RNA duplexes. Although no quantitative data is available, the duplexes formed by ANA and complementary RNA were found to be poorer substrates for RNase H-assisted cleavage compared to the native counterpart. However, when Mn^{2+} was used instead of Mg^{2+} in the reaction medium, nearly complete degradation of the target RNA was observed [62]. The 2′F–ANA has also been explored for RNase H potency [15]. Their hybrids with RNA showed higher T_m than the native DNA/RNA hybrid duplex ($\Delta T_m = +5$°C) and also exhibited global helical conformation similar to native DNA/RNA hybrids as revealed by CD spectroscopy. RNase H-promoted cleavage of these hybrids were found to be similar to that observed for native DNA/RNA and DNA-thioate/RNA hybrids. The other supporting factor concerning the RNase H-eliciting power of ANA and 2′F–ANA is that in their hybrids with RNA, the 2′-OH and 2′-F groups are projected into the major groove of the duplex where it should not interfere with the binding and catalytic processes of RNase H. These exemplify the fact that the stereochemical availability of Mg^{2+} hydrate near the scissile phosphate to stabilize the 3′-oxyanion and availability of 2′-OH group of the RNA strand in the minor groove is more important than binding to or interaction with any 2′ substituent of the AON strand.

Recently, cyclohexenyl nucleosides have been incorporated to AONs (CeNA), and found to have stabilizing effect with the target RNA [89]. The CD spectra of CeNA/RNA hybrid showed close resemblance to the native counterpart. Incorporation of one, two, or three cyclohexenyl-A nucleosides in the DNA strand increases duplex stability with +1.1, +1.6, and 5.2°C. The stabilization effect as expected also depends on the site of introduction. However, when tested for RNase H activity, they were found to be a relatively poorer substrate for the enzyme in comparison with the native.

AONs having one or more 4′-*C*- and 5′-*C*-substituted thymidine residues showed $\Delta T_m = -1.7°$ to +0.7°C per modification, but when tested for RNase H activity some of them showed same extent of cleavage as of native while the others were poorer substrates compared to the native [88]. Recently, Kanazaki et al. [36] studied the RNase H-eliciting power of AONs containing 4′-α-*C*-amininoalkylthymidines. These AONs

showed lower binding affinity to the target RNA compared to the native counterpart ($\Delta T_m = -2.5$ to -2.2°C) and exhibited very small difference in the CD spectra compared with the non-modified duplex. RNase H-promoted hydrolysis rates for the hybrid duplexes formed by 4′-α-*C*-amininoalkylthymidine-containing AONs were found to be similar to that of unmodified counterpart, but no quantitative data is available for comparison [36]. Also, it was reported that hydrolysis rates were decreased when five residues of 4′-α-*C*-amininoalkylthymidine have been introduced into the AON.

4. Backbone modifications

Among various backbone modifications, only phosphorothioates [10,79] and boranophosphates [69] were shown to support RNase H hydrolysis. The CD experiments on the hybrid duplexes formed by the stereo-enriched and random phosphorothioate oligodeoxynucleotides with target RNA showed [95] that substitution of the PO backbone with PS did not alter the global conformation of the duplex, and that the stereochemistry of PS-oligo backbone did not have any major influence on the helical structure. The RNase H digestion study revealed that all these duplexes are the substrates for the RNase H. It was also shown that the thio-AONs enriched with the Rp PS-linkages provided faster RNA target degradation by the enzyme compared to the Sp-enriched oligos. This observation is in agreement with previous work [83,84], where the PO linkage at the cleavage site was changed into a PS linkage. Two diastereomers generated by this modification were separated and tested on their ability to activate the RNase H. In the case of the duplex containing a Sp-phosphorothioate, where the Sp-sulfur atom is accessible to the enzyme through the minor groove, lower extent of hydrolysis was monitored compared to the Rp-phosphorothioate-containing duplex, and the cleavage site was shifted. This result supports the importance of the interactions between the enzyme and the minor groove of the substrate duplex, and shows that the RNase H is also sensitive towards the modifications in the junction of the minor and major grooves, responsible for the substrate recognition.

Boranophosphate oligothymidines (11 mer borano-AON, where one of the nonbridging oxygens is replaced with borane) were reported to support RNase H hydrolysis of poly(rA) with efficiency higher than nonmodified thymidine oligos regardless of their poor affinity towards the target RNA [69]. The borano modification produces minimal changes in the CD spectrum of the thymidine dimer compared to the native counterpart and both diastereomers adopt B-type conformation (the same as unmodified d(TpT) dimer). Unfortunately, there is no CD or any other structural data available on the hybrid duplexes of such borano-AONs with RNA, which makes it impossible to assess the structural background for the recognition of these duplexes as the substrates by the RNase H vis-à-vis natural counterpart.

All the other backbone modifications examined for the RNase H potency failed to recruit the enzyme to cleave the target RNA. Hybrid duplexes formed by methylphosphonates [21,54] phosphoro-*N*-morpholidates, phosphoro-*N*-butylamidates [3] methylenemethylimines [59] and N3′ → P5′ phosphoramidates [24] were found to be resistant to the RNase H. As it was demonstrated by NMR and CD spectroscopy, 3′-aminonucleo-

side 2′-deoxyfuranose in N3′ → P5′ phosphoramidates adopts the N-type conformation and the phosphoramidate duplexes adopt a A-type RNA with C3′-*endo* sugar puckering [24]. NMR and modeling studies showed that introduction of the methylenemethylimine internucleotide linkage also drives the sugar conformation to a C3′-*endo* pucker, thus preorganizing AON into the A-geometry [59]. No structural data is available for the DNA/RNA heteroduplexes formed by the AONs containing the methylphosphonate, phosphoro-*N*-morpholidate or phosphoro-*N*-butylamidate internucleotide linkages. A common feature of all these modifications is the lack of the negative charge as the result of the replacement of the natural phosphodiester backbone. Studies on the crystal structure of the RNase H [38,39,94] and NMR studies on the solution structure of the DNA/RNA hybrids [18] suggested extensive interactions between the enzyme and the DNA strand of the hybrid. According to the models proposed in these studies, side chains of Asn16 and Asn45 are located in close proximity to the DNA sugar-phosphate backbone, and can be engaged in direct hydrogen bonds with the DNA phosphate groups. A part of the DNA backbone interacts with Gln76, Gln80, Trp81, and Arg138 is assumed to interact with the phosphate group two nucleotides away from His124 in the same backbone. The proposed specific interactions with the DNA backbone take place exactly across the minor groove of the hybrid duplex, which, as it was shown above, was proven to be responsible for the substrate recognition by the RNase H. Also, the kinetic analysis [35] of RNase H with different DNA/RNA substrates suggested that the DNA residues complementary to the RNA residues located six or seven bases upstream from the cleavage site are crucial for the enzyme binding. This demonstrates the critical importance of complementary DNA residues and explains why most of the backbone modifications (which cannot provide the necessary hydrogen bonding to the enzyme) failed to support RNase H-mediated cleavage of RNA.

5. Base modifications

Unfortunately, the studies employing DNA/RNA heteroduplexes formed by the base-modified AONs, as substrates for RNase H, did not provide any information on the structural changes brought by these modifications. However, valuable information has been obtained regarding the sensitivity of the enzyme towards the changes in the minor or the major groove of the hybrid duplex.

5-phenyl-2′-deoxyuridine was introduced [83] instead of thymidine opposite to the RNA cleavage site, where the phenyl group resides in the major groove, and blocks interactions in this groove by the steric hindrance. In another example, guanosine moiety was substituted with 2-*N*-methylguanosine, affecting the binding in the minor groove. Both modifications did not significantly destabilize the duplex. The results of the RNase H cleavage experiments showed that the K_m value for the duplex containing 2-*N*-methyl-2′-deoxyguanosine (modification is projected into the minor groove) was greatly increased compared to the base-unmodified duplex, while a bulky phenyl group (projected into the major groove) had little effect on the enzyme binding. These results again indicate that the active site of the *E. coli* RNase HI interacts with its substrate duplex in the minor groove.

The AONs containing 5-propyne, -butyne or -dimethylthiazole moieties in 2′-deoxyuridine and 2′-deoxycytidine nucleosides [25], as well as the recently reported 9-(aminoethoxy)phenoxazine analogue of cytosine (G-clamp), showed [19] enhanced binding affinity towards RNA, but did not improve the ability to recruit RNase H as compared with nonmodified AONs. Especially valuable is the case with the G-clamp, where the significant gain in the thermodynamic stability ($\Delta T_m = 18°C$) as the result of the single modification in the 10-bp-long duplex did not provide a faster RNase H hydrolysis rate; in fact, the extent of hydrolysis of the modified duplex was slightly lower compared to the native counterpart. As it was shown on the duplexes containing aminopropyl modifications in sugars [45], the presence of the positive charge in the antisense sequence can affect the binding of the enzyme to the substrate because of electrostatic repulsion between the protonated amino group and the basic protrusion of the enzyme, which is extensively involved in the binding to the substrate. In the case of the G-clamp, such a repulsion can be provided by the positively charged 9-(aminoethoxy)phenoxazine moiety.

AONs, containing 5-(*N*-aminohexyl)carbamoyl-2′-deoxyuridine residues [85], were shown to form more stable AON/RNA hybrid duplexes compared to the native counterpart and CD confirmed that the helical structure of the duplex was only slightly affected by this modification. However, the rates of the RNase H hydrolysis were found to be lower, which was attributed to the presence of the amino groups of aminohexyl-linkers interfering with the phosphate backbone of the duplex.

Duplexes formed by the 22 mer AONs, where thymine bases were replaced by 6-azathymine or 5-bromouracils [73], were recognized by the *E. coli* RNase HI. Similar cleavage results were found when these duplexes were treated with mammalian RNase H (HeLa cells extract). No cleavage pattern or extent of cleavage is available in these works. N^2-imidazolylpropylguanine and N^2-imidazolylpropyl-2-aminoadenine modifications were found [70] to have a very moderate stabilizing effect on the DNA/RNA duplexes, and their ability to activate the RNase H was suggested but never shown.

6. Conjugated AONs

Conjugation of various chromophores and hydrophobic moieties to AONs has emerged as a promising area in the antisense research in view of their potential ability to form stable AON/RNA duplexes with preserved helical structural parameters [49,68,96] of the native counterpart, their utility for cellular delivery [12] and stability towards various exonucleases [7,71].

However, only few of these conjugated AONs have been investigated on their ability to activate RNase H. Acridine-conjugated AONs targeted to β-globin mRNA were tested for RNase H potency in wheat germ extract, which has the RNase H activity, and it was found that the acridine-linked 11 mer AON was more potent inhibitor of β-globin synthesis than the unmodified counterpart [9]. Cholesterol residue was conjugated to the 3′- or 5′-end of the AON and targeted to 27 bp fragment of *Ha-ras* oncogene mRNA, and tested for their RNase H-eliciting power [23]. Results showed that at low concentration, the conjugated AONs were able to promote higher extent of target RNA hydrolysis

by RNase H compared to the nonconjugated counterpart. At higher AON concentrations, the cleavage was inhibited, which was suggested to be the result of either hydrophobic interaction of the cholesterol moiety with the active site of the enzyme or the self-association of the cholesterol conjugates leading to the formation of higher-order complexes, which decreases the available concentration of free AON. The formation of such complexes has been revealed by gel mobility shift experiments. Unfortunately, no information on the above conjugates is available to evaluate the structural background for the improvement of the RNase H activity.

It was shown [68] that tethering of chromophores to the 5′-end of the AONs increases the affinity of the AONs to the RNA target and, yet, the heteroduplexes were found to retain global structures very similar to the corresponding native AON/RNA duplexes. The RNA component in these 5′-modified AON/RNA heteroduplexes were found to be improved substrates of the RNase H hydrolysis compared to the native counterpart. Recently, we have also investigated the effect of different chromophores and polyaromatic metal complexes conjugated to the 3′- and 5′-terminals of the AONs on the recognition of the tethered AON/RNA duplexes by the RNase H [96]. This study showed that the nature of the tether can dictate not only the rate of the AON/RNA duplexes hydrolysis, but also the cleavage pattern of the RNA component. For all the modifications, the 3′-end conjugation resulted in the maximal RNase H potency, increasing the extent of RNA hydrolysis for some 3′-modified AONs to 91% compared with 40% for the unmodified counterpart. The 3′-conjugation also dramatically increased the stability of the AONs towards the 3′-exonucleases (snake venom phosphodiesterase), which should extend the life time of such AONs in the cell media. Similar results were obtained with conjugated phosphorothioate AONs.

7. The importance of the 3′-tethered chromophore in AON for the AON/RNA hybrid interactions with the RNase H

Recent work from our laboratory [4] has shown that AONs with various 3′-modifications [with tethered dipyridophenazine (Dppz) and phenazine (Pzn)] form AON/RNA duplexes with very different thermodynamic stabilities. To understand if the RNase H recognition and cleavage properties of Dppz–AON/RNA and Pzn–AON/RNA hybrid duplexes are dictated by their relative stabilities or/and on the nature of 3′-tethered chromophores, we have examined their RNase H cleavage potency under a condition using a large excess of AONs relative to the target RNA in order to bypass the influence of the relative differences in the thermodynamic stabilities. We found that, under such a saturation condition, the extent of the target RNA hydrolysis, at $t_{99.9\%}$, are indeed different for different AON/RNA duplexes. This means that the catalytic activity of RNase H towards the RNA component in different AON/RNA hybrid duplexes is indeed dictated by the nature of the tethered aromatics at the saturation condition, not by the thermodynamic stability of duplexes. The change of competing K_m and k_{cat} for each hybrid duplex substrate by the enzyme is due to either different recognition and interaction owing to the uniqueness of the 3′-tethered aromatic chromophore or (and) a slight conformational change of the helix of the hybrid substrates. Since the CD spectra

of all three AON/RNA hybrids (i.e. Dppz–AON, Pzn–AON and the antive–AON) were found to be identical (meaning that their global helical conformation have remained to be the same) despite the fact that the chemical nature of the 3′-terminal has been altered in all three cases, one can rule out the second possibility. This confirms that the change of the aromatic nature of the 3′-tethered chromophore (Dppz vs. Pzn) changes the substrate recognition and catalysis by RNase H, when an excess of AON is used as is the case in an actual pharmacological condition of AON administration. Furthermore, the preferred recognition and cleavage of the tethered AONs compared to native counterpart, Dppz–AON > Pzn–AON > Native, show the importance of the nature of 3′-chromophore substituent. This means that we should be able to steer the AON/RNA hybrid recognition and cleavage by RNase H more effectively in the future, depending upon how well we can engineer the properties of the tethered chromophore in the AON strand.

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