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PNAzymes That Are Artificial RNA Restriction Enzymes

Merita Murtola,* Malgorzata Wenska, and Roger Strömberg*

Department of Biosciences and Nutrition, Karolinska Institutet, Novum, S-14183, Huddinge, Sweden

Received February 1, 2010; E-mail: Roger.Stromberg@ki.se; Merita.Murtola@ki.se

Abstract: DNA-cleaving restriction enzymes are well-known tools in biomedical and biotechnological research. There are, however, no corresponding enzymes known for RNA cleavage. There has been an ongoing development of artificial ribonucleases, including some attempts at sequence selectivity. However, so far these systems have displayed modest rates of cleavage, and in most cases, the cleaver has been used in excess or in stoichiometric amounts. In the current work, we present PNA-based systems (PNAzymes) that carry a Cu(II)-2,9-dimethylphenanthroline group and that act as site and sequence specific RNases. The general basis for the systems is that the target is cleaved at a nonbase paired region (RNA bulge) which is formed in the substrate upon binding of the PNAzyme. With this copper based system, cleavage takes place at virtually only one site and with a half-life of down to 30 min under stoichiometric conditions. Efficient turnover of RNA-substrate is shown with a 100-fold excess of substrate, thus, demonstrating true enzyme behavior. In addition, alteration of the sequence in the RNA bulge or a mismatch in the base-pairing region leads to substantial decreases in rate showing both kinetic resolution and binding discrimination in the substrate selectivity. The selectivity is further demonstrated by the substrates, with two potential cleavage sites differing in only one base, are cleaved only at the site that either does not have a mismatch or is kinetically preferred. We suggest that these systems can serve as a basis for construction of RNA restriction enzymes for *in vitro* manipulations.

Introduction

Restriction enzymes recognize DNA sequences very specifically. These enzymes are outstanding biological catalysts that also are invaluable tools in biotechnology.[1] On the other hand, RNA manipulation/processing have not been much employed and efficient sequence-selective scissions of RNA molecules are also hard to accomplish. More advanced biotechnology would be possible if sequence-selective RNA manipulation can become available. Ribozymes are an alternative for site-selective RNA cleavage, but these often chemically modified enzymes are costly and cumbersome to make or are susceptible to degradation by natural ribonucleases.[2,3] Their construction also set some inherent limitations to which sequence that can be targeted and accessed.[4] Development of completely artificial custom-tailored enzymes that are capable of performing RNA cleavage of a chosen sequence with high levels of efficiency and selectivity is still quite a challenge. Artificial RNA cleaving enzymes could be used as restriction enzymes in molecular biology research, in gene silencing as catalytic antisense oligonucleotides, to map the structure of nucleic acids, or as accurate genotyping of single nucleotide polymorphisms[5] and insertion/deletion polymorphisms.[6]

Quite some effort has been directed toward finding systems capable of catalyzing RNA chain cleavage. Artificial systems using activators and metal aquo ions free in solution as well as complexed in metal chelates and metal ion-independent cleaving agents have been developed.[7−10] However, most systems developed are slow and either not shown to be catalytic (i.e., not tested for turnover) or give limited sequence selectivity. Oligonucleotide based systems[5,11−22] where the artificial cleaver (e.g., a metal chelate) is guided to the target RNA should presumably give reasonable target selection since they are based on an initial recognition by Watson−Crick base pairing. Some oligonucleotide based systems have even displayed turnover[11,14,18,21] but typically with limited cleavage site specificity, thus, creating multiple fragments.

A couple of oligonucleotide based systems have been reported to give reasonable site selectivity in RNA cleavage, and most notable are a dinuclear Zn(II) complex[23] and the acridine/Lu(III) based systems[5,6,24] developed in Komiyamas laboratory and the guanidine system from Goebels group.[25] However, in these

(1) Roberts, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102* (17), 5905–5908.
(2) Takagi, Y.; Ikeda, Y.; K., T. *Top. Curr. Chem.* **2004**, *232*, 213–251.
(3) Zaug, A. J.; Been, M. D.; Cech, T. R. *Nature* **1986**, *324* (6096), 429–433.
(4) ChristiansenJ.K., J. K.; Lobedanz, S.; Arar, K.; Wengel, J.; Vester, B. *Bioorg. Med. Chem.* **2007**, *15*, 6135–6143.
(5) Kuzuya, A.; Mizoguchi, R.; Sasayama, T.; Zhou, J. M.; Komiyama, M. *J. Am. Chem. Soc.* **2004**, *126* (5), 1430–1436.
(6) Sasayama, T.; Kato, M.; Aburatani, H.; Kuzuya, A.; Komiyama, M. *J. Am. Soc. Mass Spectrom.* **2006**, *17* (1), 3–8.
(7) Trawick, B. N.; Daniher, A. T.; Bashkin, J. K. *Chem. Rev.* **1998**, *98* (3), 939–960.
(8) Häner, R. *Chimia* **2001**, *55* (12), 1035–1037.
(9) Niittymäki, T.; Lönnberg, H. *Org. Biomol. Chem.* **2006**, *4* (1), 15–25.
(10) Kuzuya, A.; Komiyama, M. *Curr. Org. Chem.* **2007**, *11*, 1450–1459.
(11) Magda, D.; Wright, M.; Crofts, S.; Lin, M.; Sessler, J. L. *J. Am. Chem. Soc.* **1997**, *119* (29), 6947–6948.
(12) Hall, J.; Huesken, D.; Pieles, U.; Moser, H. E.; Haener, R. *Chem. Biol.* **1994**, *1* (3), 185–190. Hall, J.; Huesken, D.; Haener, R. *Nucleic Acids Res.* **1996**, *24* (18), 3522–3526. Huesken, D.; Goodall, G.; Blommers, M. J. J.; Jahnke, W.; Hall, J.; Haener, R.; Moser, H. E. *Biochemistry* **1996**, *35* (51), 16591–16600.
(13) Putnam, W. C.; Bashkin, J. K. *Chem. Commun.* **2000**, 767–768.

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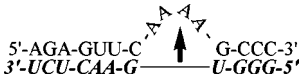

Figure 1. Schematic representation of the concept of forming a bulge in the target RNA to which an attached artifical cleaver is directed. The RNA target is the upper structure and the modified oligonucleotide carrying the cleaver (represented by the arrow) is the lower sequence in bold italic.

studies, the rates were generally modest, although excess or equimolar amounts of cleaver were used and no real turnover was shown.

In our efforts to create oligonucleotide based artificial nucleases (OBANs), 2′-*O*-methyloligoribonucleotides (2′-*O*-MeOBANs)[14−16,20] carrying Zn(II)-2,9-dimethylphenanthroline as cleaving agent were developed. A particular feature of these artificial ribonucleases is that upon Watson−Crick binding to the RNA target a nonpaired bulge region is formed in which the target is cleaved (Figure 1). A similar design but with an aza-crown zinc chelate was developed by Niittymäki et.al.[17,18] The reactions catalyzed by the above systems occur at what could be described as a usable rate (multihour half-life), with catalytic turnover and with no cleavage in the base paired region, but only with, at best, partial site-selectivity within the bulge.[14,20,21]

DNA and 2′-*O*-methyloligoribonucleotides fragments are useful as scaffolds to obtain sequence recognition of the target RNA, but these scaffolds are also limited in that they are quite susceptible to degradation by natural nucleases when used in biological fluids. On the other hand, peptide nucleic acids (PNA)[26] are virtually nondegradable under such conditions. Together with other desirable qualities,[27] this makes PNA an attractive backbone choice for further development of artificial ribonucleases. Our previously reported peptide nucleic acid based artificial nucleases (PNAzymes)[21,28] display rates of RNA cleavage similar to those of the corresponding 2′-*O*-MeOBANs,[20] give catalytic turnover, and have so far given ca. 50% site-selectivity at multiple-turnover conditions.

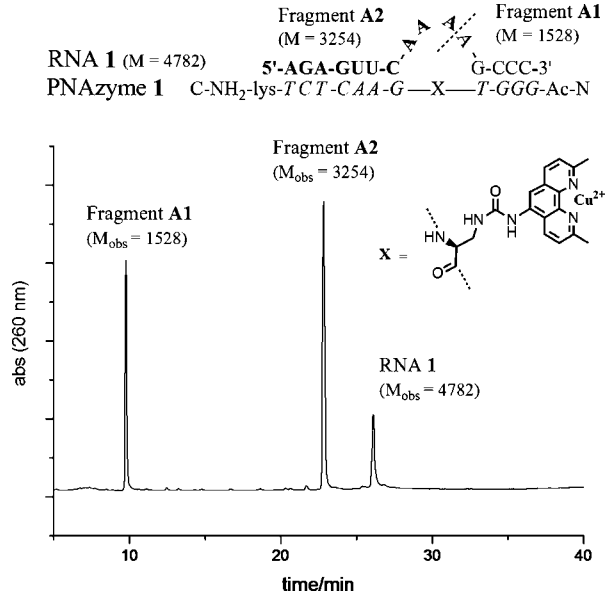

Figure 2. The complex between PNAzyme **1** and substrate RNA **1**, as well as the IE-HPLC chromatogram displaying selective cleavage of the target RNA (3 h incubation time, 1:1 PNAzyme **1**/RNA **1** 4 μM of each, 10 μM Cu^{2+}, pH 7.4, 37 °C). The cleavage site is indicated by the dashed line. M designates calculated molecular weight and M_{obs} molecular weights of isolated fragments as measured by mass spectrometry (ESI-TOF).

It seemed challenging to try to evolve these stable PNAzymes into enzymes with higher rates of cleavage and with high site-specificity. It was already at an early stage clear that the proximity/linker plays a role in determining the rate of cleavage as well as for the site selectivity.[14,15] It is, however, a quite cumbersome approach to try to optimize the linker and it is also likely that the metal ion plays a major role in anchoring to the phosphate at the cleavage site. To utilize a metal ion with higher acidity and different ligand geometry preferences than zinc ions seemed like a possibility to affect both rate of cleavage and selectivity. Cu^{2+} ions fulfill this requirement but create solubility difficulties if the concentration and pH is similar to the concentration of Zn^{2+} ions used for both OBANs[14,15,20] and PNAzymes.[21,28] However, the complex constant for binding to 2,9-dimethylphenanthroline is substantially higher[29] which enables use of a lower concentration of copper ions, and thus makes it possible to explore Cu^{2+}-based PNAzymes.

Results and Discussion

The peptide nucleic acid conjugates were PNA sequences with an internally placed L(*S*)-diaminopropionic acid unit, that serves as attachment point for the chelate (Figure 2).[21] The PNA-2,9-dimethylphenanthroline conjugates were synthesized as described earlier.[30] The first studies were carried out with equimolar amounts of PNA−conjugate and RNA substrate (Figure 2) in the presence of 10 μM Cu^{2+}, which corresponds to 2.5 fold excess of the metal ion, which was chosen to ensure more complete saturation of the neocuproine moiety (see below).

What was immediately clear from the initial kinetics experiments is that this PNAzyme which utilizes Cu^{2+} as cofactor shows a remarkable selectivity in the cleavage reaction producing only two fragments when cleaving the

(14) Åström, H.; Williams, N. H.; Strömberg, R. *Org. Biomol. Chem.* **2003**, *1* (9), 1461–1465.
(15) Åström, H.; Strömberg, R. *Org. Biomol. Chem.* **2004**, *2* (13), 1901–1907.
(16) Åström, H.; Strömberg, R. *Nucleosides, Nucleotides Nucleic Acids* **2001**, *20* (4−7), 1385–1388.
(17) Niittymaeki, T.; Loennberg, H. *Bioconjugate Chem.* **2004**, *15* (6), 1275–1280.
(18) Niittymaeki, T.; Virta, P.; Ketomaeki, K.; Loennberg, H. *Bioconjugate Chem.* **2007**, *18* (5), 1583–1592.
(19) Kalek, M.; Benediktson, P.; Vester, B.; Wengel, J. *Chem. Commun.* **2008**, (6), 762–764.
(20) Murtola, M.; Strömberg, R. *ARKIVOC* **2009**, 4–94.
(21) Murtola, M.; Strömberg, R. *Org. Biomol. Chem.* **2008**, *6* (20), 3837–3842.
(22) Magda, D.; Miller, R. A.; Sessler, J. L.; Iverson, B. L. *J. Am. Chem. Soc.* **1994**, *116* (16), 7439–7440. Magda, D; Crofts, S.; Lin, A.; Miles, D.; Wright, M.; Sessler, J. L. *J. Am. Chem. Soc.* **1997**, *119* (9), 2293–2294.
(23) Matsuda, S.; Ishikubo, A.; Kuzuya, A.; Yashiro, M.; Komiyama, M. *Angew. Chem., Int. Ed.* **1998**, *37*, 3284–3286.
(24) Shi, Y.; Kuzuya, A.; Machida, K.; Komiyama, M. *Tetrahedron Lett.* **2004**, *45* (19), 3703–3706. Kuzuya, A.; Shi, Y.; Sasayama, T.; Komiyama, M. *JBIC, J. Biol. Inorg. Chem.* **2005**, *10* (3), 270–274. Kuzuya, A.; Shi, Y.; Tanaka, K.; Machida, K.; Komiyama, M. *Chem. Lett.* **2009**, *385*, 432–433.
(25) Gnaccarini, C.; Peter, S.; Scheffer, U.; Vonhoff, S.; Klussmann, S.; Goebel, M. W. *J. Am. Chem. Soc.* **2006**, *128* (24), 8063–8067.
(26) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254* (5037), 1497–500.
(27) Lundin, K. E.; Good, L.; Strömberg, R.; Graslund, A.; Smith, C. I. *Adv Genet* **2006**, *56*, 1–51.
(28) Murtola, M.; Ossipov, D.; Sandbrink, J.; Strömberg, R. *Nucleosides, Nucleotides Nucleic Acids* **2007**, *26* (10−12), 1479–83.
(29) Irving, H.; Mellor, D. H. *J.Chem. Soc.* **1962**, 5237–5245.
(30) Sandbrink, J.; Murtola, M.; Strömberg, R. *Nucleosides, Nucleotides Nucleic Acids* **2007**, *26* (10−12), 1485–1489.

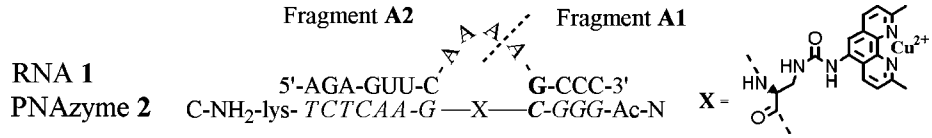

Figure 3. The PNAzyme **2**/RNA **1** complex. The cleavage site is indicated by the dashed line and the Watson−Crick pairing replacing the wobble pair at position 4 (from the N-terminal of the PNAzyme) is indicated by bold letters.

target RNA **1**. The target (**1**) is cleaved uniquely at one phosphodiester linkage that is situated within the RNA-bulge created by hybridization to PNAzyme **1**. HPLC analysis (Figure 2) reveals that virtually only two products are formed and MS-analysis of the fragments show that these are 5′-HO-AGCCC-3′-OH and the 5′-HO-AGAGUUCAAA-3′-cyclic phosphate. This also is evidence that there is virtually no oxidative cleavage (which also is not to be expected for RNA). In addition, the rate of cleavage with Cu^{2+} system is nearly 10-fold higher when compared with the analogous Zn^{2+} based system giving $t_{1/2} \approx 1$ h for the PNAzyme−Cu^{2+} complex and $t_{1/2} \approx 11$ h for the PNAzyme−Zn^{2+} complex (the half-life represents depletion of substrate irrespective of number of cleavage sites, which are multiple for the Zn complex. The half-life for cleavage only at the same site, as for the Cu catalyst, would be approximately 22 h for the Zn system since 50% cleavage takes place at this position).

A particular feature of the complex formed (PNAzyme **1**/RNA **1**) is that it contains a wobble-base pair at position 4 from the 3′-end of the RNA target. Indications from previous studies suggest that this wobble-base pair influences the action of the artificial nucleases on the bulge (unpublished data). To investigate this further, we constructed another PNA−conjugate, PNAzyme **2**, containing a cytidine instead of a thymidine at position 4 from the N-terminal of the PNAzyme, thus, creating a Watson−Crick (W−C) base pair with RNA **1** instead of the wobble base pair (Figure 3).

After 1 h reaction time, 18% of RNA **1** is cleaved by PNAzyme **2**. Cleavage selectivity is essentially maintained (see Supporting Information Figure S1) but reaction is more than 2.5 times slower than with the original wobble base pair. The wobble pair thus influences the bulge in a way that either makes it more susceptible to cleavage or alters the geometry to allow better positioning of the copper chelate. To gather more information on the influence of the choice of nucleosides at the same position, RNAs **2**−**4** (Table 1) were subjected to reaction with PNAzyme **1**. RNAs **2** and **3** form a mismatch with PNAzyme **1** at position 4 (from the N-terminal) and RNA **4** generates another Watson−Crick pairing at the same position but with an AT pair instead of the GC formed between RNA **1** and PNAzyme **2**. With the AT pair, the RNA cleavage proceeds at a rate that is even lower than with the GC pair, and with the mismatches, RNA cleavage is barely detectable even after 7 h incubation. This outcome points out that a mismatch at this site is enough to essentially kill the catalytic activity of this artificial enzyme and that a Watson−Crick pair instead of a wobble pair severely retards the rate of the cleavage reaction.

The base sequence of linear single-stranded RNAs has been reported to substantially influence the reactivity of the phosphodiester linkages, where transesterification reactions within single-stranded RNA molecules are retarded by stacking interactions between the neighboring nucleic acid bases.[31,32] An interesting question is if the base sequence within the relatively flexible RNA-bulge would influence the rate of cleavage in the PNAzyme/RNA complexes. Another major issue is if the virtually specific cleavage site selectivity would be retained upon change of the bulge sequence.

To investigate the influence of the bulge sequence, 18 additional RNA sequences (RNA **5**−**22**, Table 1.) were treated with PNAzyme **1**. The cleavages of the target RNAs were investigated using equimolar amounts of PNAzyme and RNA. Aliquots were withdrawn from the reaction mixtures at 1, 3, and 7 h.

By systematically varying the 4-adenosine bulge in the substrate by replacing one adenine by one of the other three natural bases (RNA **5**−**16**), a strong dependence of rate of cleavage on the bulge composition is revealed (Table 1). All sequences displaying enough cleavage to evaluate site selectivity exhibit almost exclusive scission between nucleosides 5 and 6 counted from the 3′-end of the RNA (as in Figure 2). There is, however, some correlation with rate in that the systems with lower rates in general give a somewhat less selective cleavage (with up to 10% cleavage at another sites, see Supporting Information Figures S2−5), while with the fastest systems, it is hard to detect any other scission sites than between nucleosides 5 and 6.

Replacing the adenine neighboring the GT wobble pair (RNA **5**−**7**) substantially decreases the rate of cleavage, and while a guanine gives a lengthening in half-life to about 7 h, a pyrimidine base virtually kills the activity. Replacement of the adenosine in position 6 (RNA **8**−**10**), that provides the 2′-hydroxyl nucleophile in the reaction, with guanine hardly disturbs the catalytic activity, but drastic drops in cleavage rates are obtained if the base is replaced by a pyrimidine. If, on the other hand, the A in position 7 (RNA **11**−**13**) is changed for a pyrimidine, the rate of cleavage goes up leading to the bulge sequence -AUAA- that gives the highest rate observed. Replacing the A in position 8 (RNA **14**−**16**) appears acceptable but does lead to lower rates of transesterification. As could be expected from the results with RNA **10**,**11**,**13**, and **15**, RNAs **20**−**22** are cleaved at rates within the same range. The overall most prominent sequence preferences are summarized in a schematic fashion in Figure 4.

As mentioned, most reactions display relatively high site selection which suggests that there is no major interaction between bases and the Cu^{2+}-neocuproine that affects the selectivity. The rate is, on the other hand, quite sensitive to the sequence which indicates that the structure of the bulge plays a major role for the accessibility and/or proximity to the cleaved phosphate. The G wobble is preferred and the bases neighboring the cleaved phosphate should not be pyrimidines for effective transesterification. This would correlate with the fact that stacking between the bases 4−6 promotes cleavage. Interestingly, such stacking is seen in molecular dynamics studies of a

(31) Kaukinen, U.; Lyytikainen, S.; Mikkola, S.; Lönnberg, H. *Nucleic Acids Res.* **2002**, *30* (2), 468–474.

(32) Kaukinen, U.; Venalainen, T.; Lönnberg, H.; Perakyla, M. *Org. Biomol. Chem.* **2003**, *1* (14), 2439–2447.

Table 1. Extent of Cleavage of the Different RNA Sequences **1**−**22** in the Presence of PNAzyme **1**[a]

PNAzyme **1**	H_2NlysT C T C A A G-**X**-TGGGNAc	% Cleavage		
RNA	Sequence	1h	3h	7h
1	5'-*AGAGUUC*- AAAA -*GCCC*-3'	50	80	
2	- AAAA -***U***	-	-	4
3	- AAAA -***C***	-	< 3	< 3
4	- AAAA -***A***	-	16	28
5	- AAA**U** -	-	< 3	< 3
6	- AAA**C** -	-	-	< 3
7	- AAA**G** -	-	25	49
8	- AA**U**A -	-	7	10
9	- AA**C**A -	-	7	11
10	- AA**G**A -	45	80	
11	- A**U**AA -	82		
12	- A**G**AA -	36	72	
13	- A**C**AA -	62		
14	- **U**AAA -	25	64	
15	- **C**AAA -	22	46	
16	- **G**AAA -	23	52	
17	- AA**UU** -	-	-	< 3
18	- AA**GG** -	-	15	41
19	- AA**CC** -	-	-	-
20	- A**UG**A -	56	88	
21	- A**CG**A -	67		
22	- **CUG**A -	46	74	

[a] For sequences **2**−**22**, only the varied RNA-bulge is indicated except for sequences **2**−**4** where the base replacing the G neighboring the bulge is also shown. Reactions were carried out with a 1:1 ratio of PNAzyme **1** and RNA target, (4 μM each) at pH 7.4, 37 °C and in the presence of 10 μM Cu^{2+} (-: below detection limit).

Figure 4. A schematic presentation of how the rate of cleavage in the fastest systems is dependent on the sequence in the central bulge of the RNA target, and the neighboring nucleoside on the 3′-side, upon cleavage with PNAzyme **1**.

similar bulge (different stem sequences but an A_4 bulge neighboring a GU wobble pair).[33] In the mentioned model, there is also stacking between A-8 and the opposite strand which reduces the flexibility of the bulge. That an A is the most preferred base at this position, for obtaining efficient cleavage, suggests that this reduced mobility could be contributing toward the scission site being kept closer to the catalyst. That position 7 preferably should be a pyrimidine could perhaps be explained by the need for the nucleoside 6 to make a conformational change upon transesterification and that stacking with a purine at position 7 would raise the barrier to do so. In the modeling study mentioned, it was also found that A-7 over the time course studied did stack upon either of A-8 or A-6.[33] Overall, this indicates that even what may seem as slight changes in the bulge can alter the dynamics of the bulge in such a way that the rate of the PNAzyme catalyzed cleavage of the RNA target is substantially and sometimes dramatically affected. An important aspect of this with regard to target selectivity is that this means that apart from discriminating targets on the basis of Watson−

(33) Macchion, B. N.; Strömberg, R.; Nilsson, L. *J Biomol Struct Dyn.* **2008**, (2), 163–173.

Crick base pairing of the stems the PNAzymes will also give selectivity for variation in the bulge sequence due to kinetic resolution.

Intrigued by the possibilities, we decided to investigate the fastest system (PNAzyme **1**−RNA **11**) further. First-order kinetics is obeyed as in our previously studied systems (for observed rate constants k_{obs}, see Supporting Information Table S1 and Figures S6 and S7) which gave a half-life of 32 min. RNA scission is virtually site-specific, showing less than 1% off-site cleavage, and the cleavage position is the phosphodiester linkage between nucleotides 5 and 6 from the 3′-end. This is more than twice the rate of the second fastest system with the A_4-bulge (PNAzyme **1**−RNA **1**; $t_{1/2} = 70$ min). A slight excess (10 μM, 2.5 equiv) of Cu^{2+} was used for all sequences, and when a 1:1 ratio of metal ion and PNA−Chelate was used, the rate of cleavage is slightly lower (38% of RNA **1** instead of 50% and 63% of RNA **11** instead of 82% is cleaved after 1 h incubation at 4 μM (1 equiv) Cu^{2+} conc.; after 3 h incubation, the extent of cleavage for RNA **1** is 68% instead of 80%). This difference is relatively small and likely to be due to incomplete saturation of chelating phenanthroline derivative when only 1 equiv of Cu^{2+} is used. In addition the background, cleavage reaction of complexes between RNA and PNA, without neocuproine attached, in the presence of Cu^{2+} ions (10 μM, 2.5 equiv) is orders of magnitude slower and also nonselective with respect to cleavage site. The reactions of RNA **1** and RNA **11** in the presence of the neocuproine free PNAzyme precursor **1** gave half-lives longer than 1 month (see Supporting Information Figures S8 and S9). This shows that catalysis by free Cu^{2+} is highly inefficient at the reaction times and concentrations at which the PNAzyme study is conducted and can be neglected. From this data, we can also estimate that the rate of RNA cleavage catalyzed by PNAzyme **1** is at least $(2-3) \times 10^3$ times higher than with PNA-precursor **1** and free Cu^{2+} ions. The thermodynamic stabilities of these PNAconjugate/RNA complexes (without Cu^{2+} present) were determined as the UV thermal melting points (T_m). As expected, complexes had very similar melting points, that is, the sequence variation within the RNA-bulge did not affect the T_m substantially (T_m with RNA **1** = 55 °C, T_m with RNA **11** = 54 °C). The binding equilibrium should thus be almost completely shifted toward the complexes at the reaction conditions (at 37 °C, 1:1 ratio PNAzyme/RNA, 4 μM conc. of each).

PNAzymes and 2′-*O*-MeOBANs utilizing Zn^{2+} as cofactors catalysis with turnover of substrate has been reported but with limited efficiency and selectivity and also disturbed by nonspecific background reactions.[14,20,21] With the new considerably faster and site selective Cu^{2+} based system (where also the background reaction of single stranded RNA is barely detectable, Supporting Information Figure S3), the turnover of excess of substrate can be pushed much closer to what natural enzymes can master. Thus, PNAzyme **1** (4 μM with $[Cu^{2+}] = 10$ μM) was incubated with a 100-fold excess of RNA substrate **11** (400 μM) and followed over 75 cycles of conversion (Figure 5). Thus, PNAzyme **1** is capable of multiple turnover of RNA **11**, the reaction is clean with little background reaction, and the cleavage reaction occurs with retained site-selectivity also with excess of substrate.

The PNAzyme **1** in presence of Cu^{2+} is the fastest site-selective artificial ribonuclease reported and it is also shown to be capable of multiple turnover reactions. It was then natural to investigate the ability of the PNAzyme to discriminate between targets with respect to mismatches, that is, would it

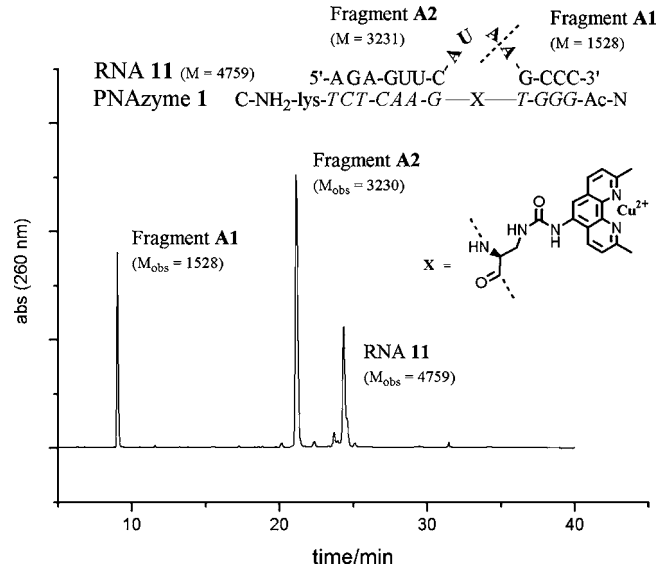

Figure 5. The complex between PNAzyme **1** and substrate RNA **11**, as well as the IE-HPLC chromatogram displaying selective cleavage of the target RNA under multiple turnover conditions showing retained selectivity in RNA cleavage after 75% conversion of the 100-fold excess of target RNA (168 h incubation time, [PNAzyme **1**] = 4 μM; initial [RNA **11**] = 400 μM; $[Cu^{2+}] = 10$ μM; pH = 7.4, T = 37 °C). The cleavage site is indicated by a dashed line. M designates calculated molecular weight and M_{obs} molecular weights of isolated fragments as measured by mass spectrometry (ESI-TOF).

Table 2. Extent of Cleavage in RNA−PNAzyme Complexes with a Watson−Crick Mismatch[a]

		% cleaved		
RNA	sequence	1 h	3 h	7 h
RNA **11**	5′-AGAGUUC-*AUAA*-GCCC-3′	82		
MIS **1**	5′-AGAGUUC-*AUAA*-GC**G**C-3′	-	4	7
MIS **2**	5′-AGAGU**A**C-*AUAA*-GCCC-3′	-	16	24
MIS **3**	5′-AGAGU**A**C-*AUAA*-GC**G**C-3′	-	-	-

[a] The RNA-bulge formed upon binding to PNAzyme **1** is indicated with italic letters and mismatch nucleotides are marked with bold letters. Reactions were carried out with a 1:1 ratio of PNAzyme **1** and RNA target, (4 μM each) at pH 7.4, 37 °C and in the presence of 10 μM Cu^{2+} (-, below detection limit).

cleave a target with a mismatch in the Watson−Crick base pairing and if so would it be much slower. As can be seen in Table 2, the rate of cleavage with a single mismatch is severely retarded and the cleavage products are below the detection limit after 1 h, at which time only 18% of the matched substrate remains, and with a mismatch in both stems, there is no reaction detectable even after 7 h reaction time (it is likely that the PNA does not even bind the substrate in this case). This shows that the PNAzyme has excellent mismatch rejection on the complementary part of the RNA chain and is thus likely to be highly sequence selective even when a similar bulge region can be formed.

As a further test of target selectivity, we constructed a longer RNA target sequence (RNA**11**MIS**1**, Figure 6) containing two potential cleavage sites, both with the potential to form an AUAA bulge vicinal to a G that form the wobble pair, but with one of the sites having a base substitution which would form a mismatch in one of the duplex stems when binding PNAzyme **1**. This situation mimics having longer RNA targets that can carry several possible competing target sites. In addition, we also constructed an RNA target (RNA**11**MIS**4**, Figure 7) with two potential cleavage sites differing only in the bulge sequence

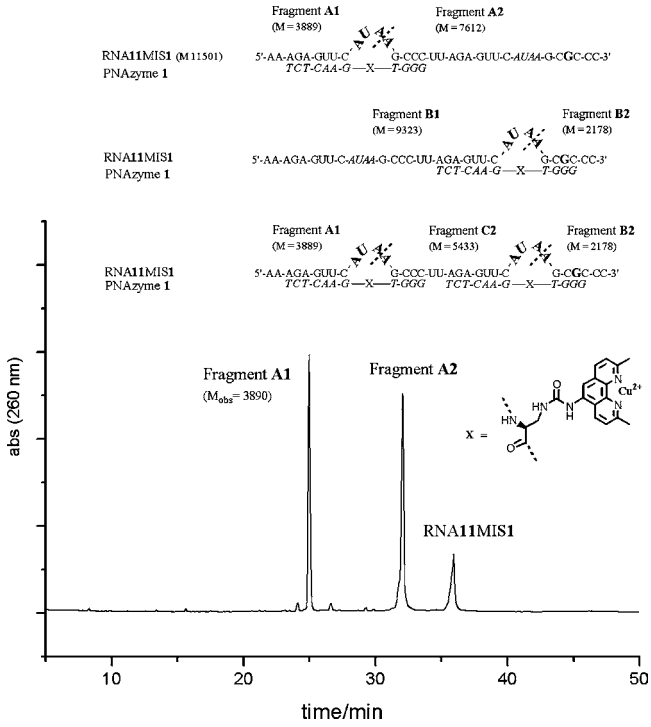

Figure 6. Possible complexes between RNA**11**MIS**1** and PNAzyme **1** indicating two potential binding and cleavage sites (dashed lines). The mismatch nucleotide is indicated by a bold letter. The IE-HPLC chromatogram displays selective cleavage of the target RNA after 84% conversion (5 h incubation time, 4 μM each of PNAzyme **1** and RNA**11**MIS**1**, 10 μM Cu^{2+}, pH 7.4, 37 °C). M designates calculated molecular weight and M_{obs} molecular weight of isolated fragment as measured by mass spectrometry (ESI-TOF).

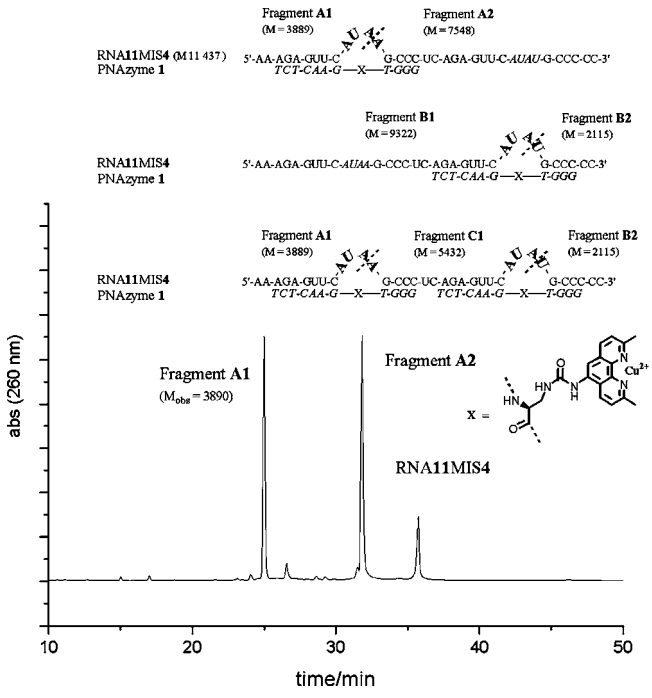

Figure 7. Possible complexes between RNA**11**MIS**4** and PNAzyme **1** indicating two potential binding and cleavage sites (dashed lines). The IE-HPLC chromatogram displays selective cleavage at one of the sites of the target RNA after 87% conversion (5 h incubation time, 4 μM each of PNAzyme **1** and RNA**11**MIS**4**, 10 μM Cu^{2+}, pH 7.4, 37 °C). M designates calculated molecular weight and M_{obs} molecular weight of isolated fragment as measured by mass spectrometry (ESI-TOF).

formed (AUAA vs AUAU) and with no mismatches in the Watson−Crick pairing.

In cleavage of RNA**11**MIS**1**, PNAzyme **1** exhibited excellent site-selectivity, cleaving the target virtually exclusively at the site with no mismatch and cleavage at the mismatch site was absent (Figure 6, the picture was similar also under turnover conditions with a ratio of 1:5 PNAzyme **1**:RNA**11**MIS**1**; see Supporting Information Figure S10). In cleavage of the target RNA**11**MIS**4**, excellent site selection was again observed and the target was cleaved at the AUAA bulge site with virtually no cleavage at the AUAU bulge site (Figure 7).

Conclusions

This new class of Cu^{2+} dependent PNA-based artificial ribonucleases (PNAzymes) gives reasonably high rates, displays multiple turnover, and gives highly site selective cleavage of the RNA targets. The Cu^{2+} based systems are considerably faster than their Zn^{2+} dependent counterparts, which partly is likely to be due the higher Brønsted acidity of copper(II) aquo ions. What is more remarkable is the much higher site selectivity. One could speculate on various interactions with the Cu^{2+} and different nucleobases and that this would direct the catalytic metal ion toward particular phosphate linkage. This is, however, not supported by the findings that base substitution in the targeted bulge or in the neighboring GU wobble base pair does not diminish the site selectivity substantially. The rate of reaction is, however, in many cases of base substitution greatly affected and this is likely to be due to structural changes in the bulge which affect its rigidity and probably also the likelyhood for the Cu^{2+} to reach a productive position. It is well-known that Cu^{2+} complexes in general have different geometry preferences than Zn^{2+} complexes. Therefore, it does not seem unlikely that distorsions in the geometry could be a reason to why the copper ions seem to be able to only reach a particular phosphate linkage while the zinc ions are less discriminating. At the moment, we do not have the structural resolution to suggest a detailed mechanism for the selectivity nor for the actual cleavage reaction. It seems plausible, however, that one contributing factor could be that the Cu^{2+} complex could anchor to the phosphate and Cu-bound water could protonate the leaving 5′-hydroxyl while the 2-hydroxyl is attacking, as suggested for metal aquo ion promoted cleavage of RNA.[34] It seems highly interesting to conduct further studies on both the detailed mechanism and on what contributes toward the site specificity.

In either case, the Cu^{2+} dependent PNAzymes presented here display rates corresponding to half-lives in the minute range which should enable this class of artifical enzymes to become useful tools. Moreover, the combination of single site cleavage, excellent mismatch rejection, discrimination of bulge sequence, and requirement of the wobble-base pair at the 3′-side of the RNA bulge makes PNAzyme **1** a highly sequence selective artificial ribonuclease that can be considered an RNA restriction enzyme. We have earlier shown that catalysis by oligonucleotide based artificial nucleases can be retained, although the Watson−Crick recognition part in the stems are changed[14,15,20,21] which suggests that the RNA restriction enzymes developed here can be tailor-made to target a desired sequence with the only requirement being that for optimum performance a central portion of the target should preferably be APyPuA-G (Py = Pyrimidine, Pu = Purine, see also Figure 4). These artificial

(34) Mikkola, S.; Stenman, E.; Nurmi, K.; Yousefi-Salakdeh, E.; Strömberg, R.; Lönnberg, H. *J. Chem. Soc., Perkin Trans. 2* **1999**, 1619–1625.

RNA restriction enzymes recognize a longer sequence than DNA restriction enzymes normally do, which in principle makes them even more selective in an RNA pool perspective. For some applications, it may be desired to obtain a larger number of cleavage sites, and for this, it may be necessary to either shorten one of the base pairing stems (it should be possible to remove one or two base pairs and still get sufficient binding at 37 °C) or alternatively use a mixture of PNAzymes with different base pairing regions.

Experimental Section

Materials and Methods. All solvents and reagents used in purifications and kinetic experiments were of analytical quality (p.a.). Oligoribonucleotides were purchased from Dharmacon/Thermo Fisher Scientific and purified by reversed-phase HPLC equipped with an ODS Hypersil C18 (5 μm, 250 × 4.6 mm) column. A linear gradient of (A) 50 mM triethylammonium acetate in water (pH 6.5) and (B) 50 mM triethylammonium acetate in 50% aqueous acetonitrile (pH 6.5) was employed. A gradient of 0−40% buffer B over 40 min was used with a flow rate of 1 mL/min. Purifications were performed at 50 °C and UV detection was done at 260 nm. Purified oligoribonucleotides were lyophilized three times before use and stored as frozen solutions. Concentrations of oligoribonucleotides were determined by measuring UV absorption at 260 nm and calculation using extinction coefficients obtained by the nearest neighbor approximation.[35] Thermal melting analysis was obtained by measuring absorbance versus temperature profiles at 260 nm on a Cary 300 UV-VIS dual beam spectrophotometer (Varian) in 10 mM HEPES buffer containing 0.1 M NaCl and 0.1 mM EDTA, at pH 7.4 and with 4 μM each of substrate RNA and PNAzyme. Mass spectrometry of oligonucleotides was performed in acetonitrile−water 1:1 (v/v) solutions on a Micromass LCT electrospray time-of-flight (ESI-TOF) mass spectrometer. Molecular weights of the oligoribonucleotides were reconstructed from the *m*/*z* values of the multiply deprotonated molecules using the mass deconvolution program of the instrument (Mass Lynx software package). PNAzymes were synthesized and purified as previously reported.[21,28,30] ESI-TOF: mass calculated for PNAzyme **1** $C_{145}H_{180}N_{72}O_{38}$ [M], 3540; found, 3540; PNAzyme **2** $C_{144}H_{179}N_{73}O_{37}$ [M], 3525; found, 3524.

Assay for RNA Cleavage Reactions: Single- and Multiple-Turnover Analyses. RNA Cleavage Reactions. All kinetic reactions were carried out in sealed tubes immersed in a water bath (t = 37 °C). Experiments were performed in 10 mM HEPES buffer containing 10 μM Cu^{2+} and 0.1 M NaCl at pH 7.4. Reactions with equimolar concentrations of substrate RNA and PNAzyme were performed using 4 μM of each component. Multiple-turnover experiments were done keeping the PNAzyme concentration at 4 μM and adding appropriate amounts of target RNA. For single-turnover experiments, 1.25 times; and for multiple-turnover experiments, 10 times more concentrated buffer stock solutions were used. Appropriate amounts of RNA, buffer stock solution, Cu^{2+}-solution, and water were added to achieve the appropriate concentrations after which the reaction vials were incubated 15 min at 37 °C. Reactions were started by adding the PNAzyme in water which gave the final concentrations of all components (i.e., 10 μM Cu^{2+}, 10 mM HEPES, 0.1 M NaCl, pH 7.4). Immediately after the addition of PNAzymes and at appropriate time intervals, 40 μL aliquots were withdrawn from the reactions mixture and aliquots were quenched by adding 70 μL of a solution of 600 μM EDTA in 30% MeCN-water Aliquots were frozen and stored in −20 °C before IE-HPLC analysis.

IE-HPLC Analysis. The aliquots were analyzed by anion exchange HPLC (IE-HPLC) equipped with a Dionex NucleoPac PA-100 (4 × 250 mm) column. A linear gradient of (A) 20 mM NaOAc in 30% aqueous acetonitrile and (B) 20 mM NaOAc, 0.4 M $LiClO_4$ in 30% aqueous acetonitrile was employed. A gradient of 0−50% buffer B over 35 min was used with a flow rate of 1.5 mL/min at 60 °C. UV detection was carried out at 260 nm. First-order rate constants for the cleavage of RNA substrates were obtained by quantification of the remaining RNA and the sum of all the formed fragments detected in the HPLC analysis. The natural logarithm of the inverse of the remaining fraction of substrate RNA was plotted against time (see Supporting Information Figures S6 and S7 for examples) and the observed rate constants (k_{obs}) were obtained by least-squares linear fitting.

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Supporting Information Available: (1) IE-HPLC chromatograms showing the cleavage of RNA **1** with the nonwobble base pairing PNAzyme **2**. (2) IE-HPLC chromatograms showing examples of variation of extent of cleavage selectivity within the RNA-bulge for different RNA targets and lack of background cleavage for a single stranded RNA in the absence of PNAzyme. (3) Determination of thermal meltings for complexes of RNA **1** and RNA **11** with copper free PNAzyme **1**, and observed rate constants for cleavage of RNA **1** and RNA **11** by PNAzyme **1**.(4) IE-HPLC chromatograms showing background cleavage of RNA in the complexes between PNAzyme precursor **1** (without attached neocuproine) and RNA **1** and RNA **11**. (5) IE-HPLC chromatogram showing site selective cleavage of RNA**11**MIS**1** at multiple turnover conditions with PNAzyme **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(35) Puglisi, J. D.; Tinoco, I., Jr. *Methods Enzymol.* **1989**, *180*, 304–325.