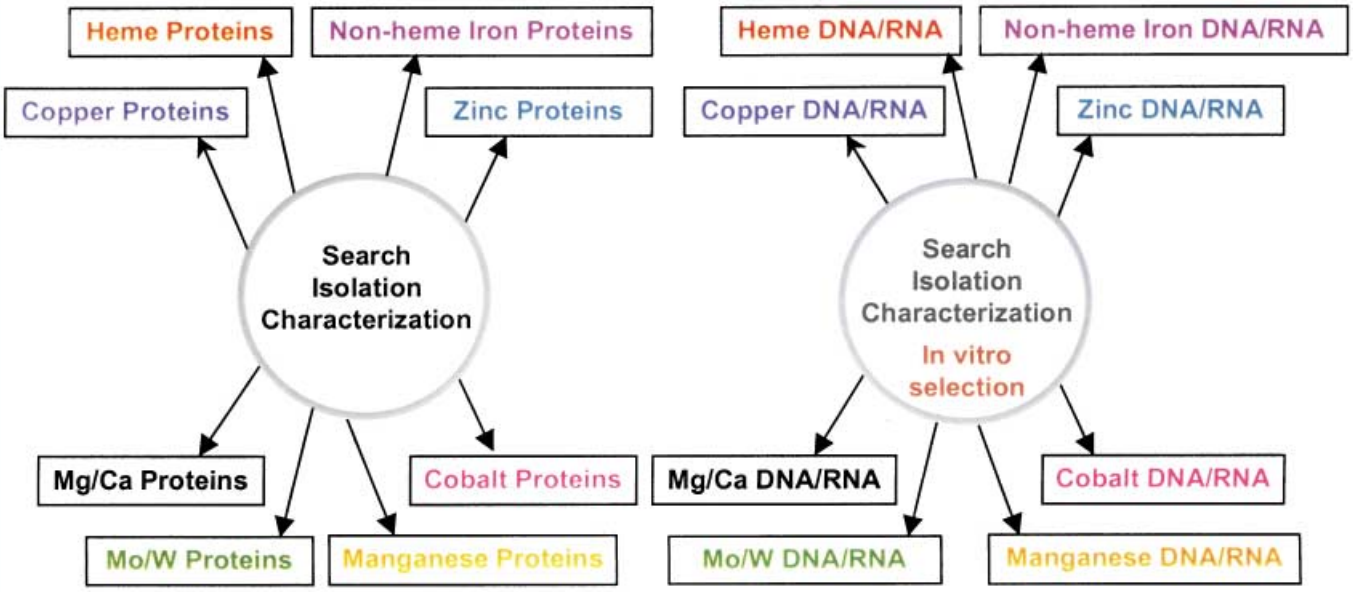
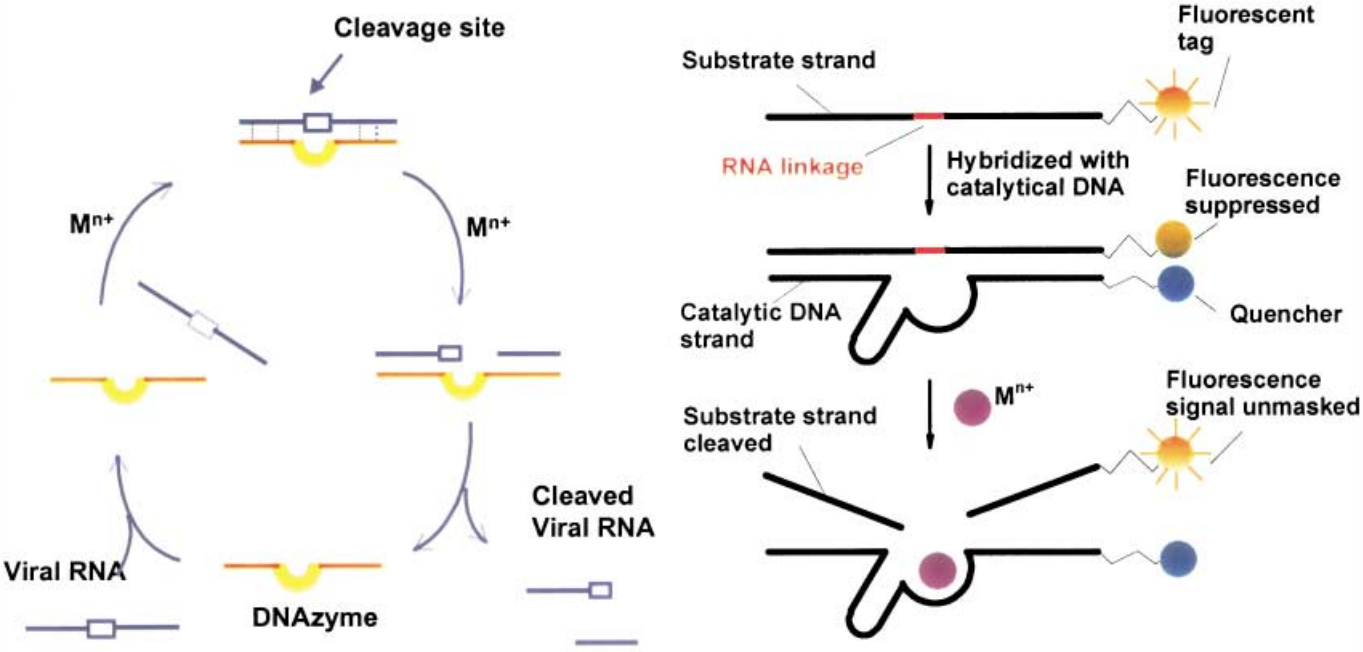
New Transition-Metal-Dependent DNAzymes

1. From Metalloproteins to Metallo-DNA/RNAzymes: *A New paradigm in Chemistry and Biology?*



2. Two promising applications: *as efficient endonucleases and as selective metal biosensors*



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New Transition-Metal-Dependent DNAzymes as Efficient Endonucleases and as Selective Metal Biosensors

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Abstract: Like proteins and RNA molecules, many DNA molecules have now been shown to catalyze a variety of reactions and are thus called DNAzymes. With limited building blocks, DNAzymes need to recruit other cofactors in order to match other enzymes in terms of reaction diversity and catalytic efficiency. Several unique properties make transition-metal ions an ideal choice of cofactor for DNAzymes. Indeed, new DNAzymes that bind transition-metal ions with high affinity and selectivity have been obtained through the use of a powerful combinatorial biology tool called in vitro selection. This accomplishment now makes it possible to obtain different classes of metallo-DNAzymes in the laboratory within a short period of time. It also offers a rare opportunity to compare and contrast structural and functional properties of metal-binding sites in proteins and in DNAzymes. The resulting transition-metal-dependent DNAzymes have displayed high activity toward cleavage of DNA and RNA and thus hold promise for their biochemical and pharmaceutical applications. Finally, the use of DNAzymes as a new class of highly sensitive and selective biosensors for metal ions has been demonstrated recently.

Keywords: biosensors · deoxyribozymes · in vitro selection · metalloenzymes · nucleases

DNAzymes—A New Class of Enzymes with Promise in Biochemical, Pharmaceutical, and Biotechnological Applications

Long considered as strictly a genetic material, DNA was shown in 1994[1] to carry out catalytic functions, and thus became the newest member of the enzyme family after proteins and RNA. Since then, the DNA molecules (called DNAzymes here, also called deoxyribozymes, DNA enzymes, or catalytic DNA elsewhere) have been shown to catalyze many of the same reactions as RNAzymes (also called ribozymes) or protein enzymes,[2–5] including RNA/DNA-cleavage,[1, 6–13] ligation,[14, 15] phosphorylation,[15, 16] cleavage of phosphoramidate bonds,[17] and porphyrin metallation[18] (Figure 1). The catalytic efficiency and reaction mechanism of DNAzymes are often similar to those of RNAzymes and protein enzymes. In one notable case, the catalytic efficiency of $10^9\,\text{M}^{-1}\,\text{min}^{-1}$ observed for the "10–23" DNAzyme[10, 19] rivals that of the protein enzyme ribonuclease. Therefore, understanding the structure and function of DNAzymes is a new frontier in chemistry and biology.

Among the functions displayed by DNAzymes, the endonuclease activity has been explored the most. DNAzymes have been used to cut, process, and map RNA molecules in biochemical studies.[20] Similar to that of RNAzymes,[21] the endonuclease activity makes DNAzymes promising anti-viral pharmaceutical agents, against diseases such as AIDS and leukemia (Figure 2).[22–27] For example, the "10–23" DNAzyme destroys hepatitis B viral RNA,[22] abnormal *BCR-ABL* fusion mRNA,[23, 24] *c-myc* RNA,[25, 26] and Egr-1 mRNA.[28] Recently, the use of DNAzymes as highly sensitive and selective metal-ion sensors has also been demonstrated.[29]

Metal Ions as Important Cofactors in DNAzymes

For DNAzymes to be effective in biochemical and clinical applications, they must be able to compete with other catalysts for efficiency and diversity. In contrast to protein enzymes constructed from 20 natural amino acids, structural repertoires of DNA/RNAzymes are limited due to the availability of only four different nucleotide building blocks. The lack of a 2′-OH functional group in DNAzymes in comparison to RNAzymes makes DNAzymes even more limited. To overcome this limitation, several groups have made progress toward introducing modified bases into DNAzymes.[30] In biology, one common solution to the lack of efficiency and diversity is the employment of cofactors, such as NADH, porphyrins, and, especially, metal ions. Protein enzymes are known to recruit these cofactors to broaden the scope of reactions, to increase catalytic efficiency, and to fine-tune the reactivity for difficult reactions.[31–34] With

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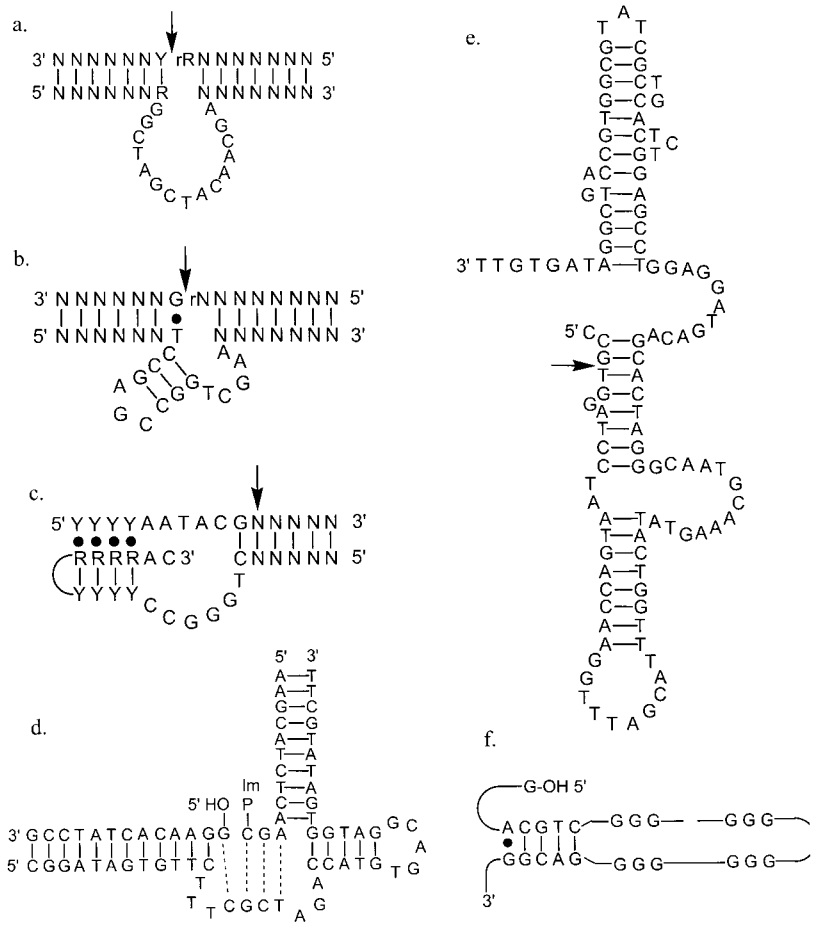

Figure 1. Examples of DNAzymes: a) "10–23" DNAzyme with RNA nuclease activity;[10, 19] b) "8–17" DNAzyme with RNA nuclease activity;[10, 11, 13] c) DNAzyme with DNA nuclease activity; d) DNAzyme with ligase activity;[14] e) DNAzyme with kinase activity;[16] f) DNAzyme with 3′-5′-phosphoramidate bond cleavage activity.[17]

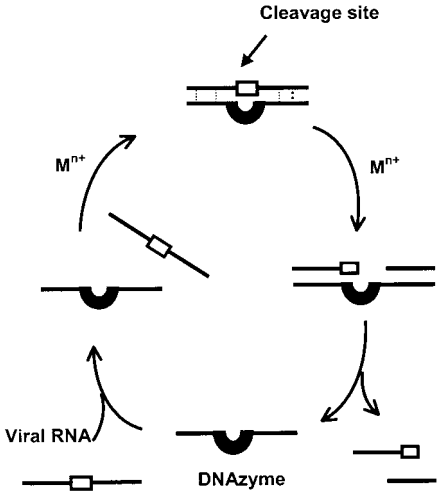

Figure 2. Concept of DNA/RNAzymes as endonucleases to target viral RNA. DNA/RNAzymes can be designed to bind specifically to the target viral RNA through Watson-Crick base pairs, form a unique three-dimensional structure, and perform catalytic function by cleaving the viral RNA. After the RNA cleavage, the DNA/RNAzymes can, in principle, diffuse away, bind to another viral RNA, and perform another catalytic cycle. Metal ions may play essential roles in at least two steps, the folding and formation of active structure and the viral RNA cleavage steps. Adapted from reference [21].

more limited building blocks, the need for cofactors is even greater for DNAzymes.

Metal ions are arguably the most important class of cofactors for enzymes because of their ability to broaden and fine-tune the reactivity of the enzymes.[31–34] Indeed, despite the recent reports that some DNA/RNAzymes are active in the presence of monovalent ions[8] or amino acid cofactors,[9] divalent metal ions such as Mg^{II}, Mn^{II}, or Ca^{II}, are essential for the catalytic function of the majority of DNA/RNAzymes under physiological conditions.[35–38] However, DNA/RNAzymes found to date cannot match protein enzymes in terms of either the variety of metal ions they employ or the specificity of the metal-binding sites. For example, while protein enzymes use metal ions from almost all groups of metals, including even the second- and third-row transition-metal ions such as Mo and W, DNA/RNAzymes only utilize a limited number of metal ions such as Mg^{II}, Ca^{II}, and Mn^{II}. Furthermore, it is well known that protein enzymes possess remarkable metal-binding affinity and specificity, and they are commonly classified by the metal ions they specifically bind (e.g., copper proteins or zinc proteins). In contrast, DNA/RNAzymes tend to work with several metal ions equally efficiently.[35–38] For example, hammerhead ribozymes are known to be active not only with Mg^{II}, but also with Mn^{II} and Co^{II}.[39] Furthermore, metal-binding affinity of DNA/RNAzymes is generally much weaker than that of protein enzymes. These observations lead to the question of whether the lack of variety and specificity of metal ions employed by DNA/RNAzymes is the result of the inherent structural limitation of these enzymes, or is because DNA/RNAzymes with high specificity for a diverse group of metal ions have yet to be discovered.

The Search for New Metallo-DNA/RNAzymes with Broad Diversity and High Specificity

To answer the question of whether the lack of variety and specificity of metal ions employed by DNA/RNAzymes is an inherent limitation of these enzymes, or is due to the fact that those DNA/RNAzymes with broad diversity and high specificity have yet to be discovered, one can search for more

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examples of DNA/RNAzymes in nature through isolation and characterization of DNA/RNAzymes in different cellular environments. This process can take a long time. However, the development of combinatorial biology techniques such as systematic evolution of ligands by exponential enrichment (SELEX) of aptamers or in vitro selection of DNA/RNAzymes can accelerate this process dramatically.[40–44] In the in vitro selection method (Figure 3), a small population of DNA/RNAzymes with the desired properties is selected and

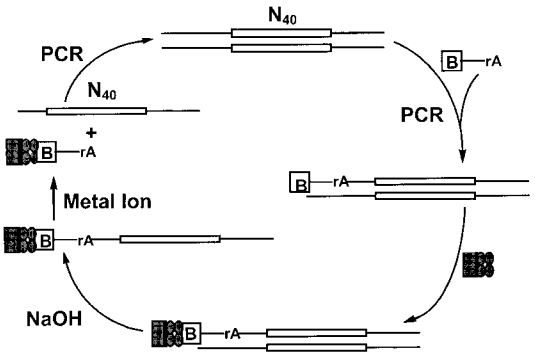

Figure 3. An example of in vitro selection of DNAzymes with RNA endonuclease activity. The initial selection pool (top left) contains a random sequence domain of 40 nucleotides (shown as a bar) flanked by two conserved primer-binding regions (shown as single lines). After one polymerase chain reaction (PCR) to amplify the DNA pool, a second PCR is performed in which one of the PCR primers contains a biotin moiety (**B**) at the 5′-end, and a ribonucleic adenosine (**rA**) embedded in the 5′-conserved sequence region. The **rA** is intended to be the cleavage site due to the relative lability of the RNA bond toward hydrolytic cleavage. The DNA pool is then immobilized on an avidin column through the biotin moiety on the 5′ of the DNA. Since single-stranded DNA molecules are most likely to form complex three-dimensional structure necessary for DNAzyme function, the double-stranded DNA molecules are denatured by NaOH, and the DNA strand without biotin can be washed away from the column. Addition of metal ions to the column containing the remaining single-stranded DNA under defined conditions (time, pH, temperature) and subsequent elution from the column allows selection of DNAzymes that undergo cleavage at the internal RNA bond in the presence of the metal ion of choice. The selected DNAzymes can be amplified through PCR, and used to seed the following round of selection. The activity of the selected enzymes can be improved by gradually using more stringent conditions (such as shorter incubation times or lower temperatures) in each subsequent round of selection. The metal-binding affinity of the enzymes may also be improved by gradually decreasing the concentration of the metal ion. The selection continues until the generation at which improvement of activity stops. The DNAzymes can then be cloned and sequenced. Adapted from reference [6].

amplified from a pool of up to 10^{15} DNA/RNA molecules with random sequences. The selected enzymes are then subjected to further rounds of mutation, amplification, and selection, often with more stringent selection conditions. Unlike random mutagenesis or evolution of proteins, in vitro selection of DNA/RNAzymes can integrate mutation and amplification, which are both genotype-related, with selection, which is phenotype-related. This integration is advantageous because it assures that the same molecule that performs desired functions can be selected, amplified, and de-coded without intermediate molecules. It also makes the methodology simple and cost-effective.

It has now been demonstrated that both SELEX of aptamers and in vitro selection of DNA/RNAzymes can lead to new DNA/RNA that are specific for a target metal ion. For example, new Zn^{II}-binding RNA aptamers have been obtained.[45–47] In vitro selected variants of the group I intron[48] and the RNase P ribozyme[49] have shown greatly improved activity with Ca^{II}, which is not an active metal ion cofactor for the native ribozyme. The Mg^{II} concentration required for optimal hammerhead ribozyme activity has been lowered by using in vitro selection to improve the enzyme performance under physiological conditions.[50, 51] Similarly, DNA/RNAzymes that are highly specific for Pb^{II},[1, 52] Cu^{II},[7, 14] and Zn^{II}[13, 53] have been obtained.

Metal-Binding Selectivity Issue

While in vitro selection makes it possible to search for new DNA/RNAzymes that are specific for selected metal ions, the selections sometimes result in DNA/RNA molecules which are active not only in the presence of the metal ion of choice, but also in other metal ions. In a few cases, the activity in the presence of the desired metal ion is lower than the activity in certain other metal ions under comparable conditions. For example, the Zn^{II}-binding aptamers bind several other divalent metal ions, such as Ni^{II} and Co^{II}, equally well.[45–47] Although the in vitro selected variants of the group I intron[48] and RNase P[49] have gained Ca^{II}-dependent activity, their Mg^{II}-dependent activity remains high. Furthermore, even though the "10–23" DNAzyme was selected from a solution containing Mg^{II}, it displays higher activity with Mn^{II}.[10, 19, 54] Finally, the same "8–17" motif was obtained by three different in vitro selection processes involving 10 mM Mg^{II},[10] 0.5 mM Mg^{II}/50 mM histidine,[11] or 100 μM Zn^{II},[13] with metal-dependent activity in the order of $Zn^{II} \gg Ca^{II} > Mg^{II}$ under similar conditions.[10–13, 55] Further assays of this enzyme indicate highly Pb^{II}-dependent activity with $k_{obs} = 6.5\ min^{-1}$ at pH 6.0.[29] The apparent K_d values for Pb^{II}, Zn^{II}, and Mg^{II} are 13.5 μM (at pH 6.0), 0.97 mM (at pH 6.0), and 10.5 mM (at pH 7.0), respectively.[29] This lack of specificity from the selection process is problematic if the selected DNA/RNA molecules are to be used either as model systems for elucidation of structural and functional features of metal-specific DNA/RNA or as metal-ion sensors.

To improve the metal ion specificity during the in vitro selection process, a negative selection strategy was implemented whereby the nucleic acid pool was subjected to a solution containing competing metal ions. As a result, those nucleic acids that were active in those metal ions were discarded. To demonstrate the effectiveness of the negative selection strategy, two parallel in vitro selections of Co^{II}-dependent DNAzymes were carried out (Figure 4).[56] When no negative selection was used in the selection process, the resulting catalytic DNA molecules were more active in Zn^{II} and Pb^{II} than in Co^{II}. On the other hand, when the negative selection steps were inserted between the normal positive selection steps, the resulting catalytic DNA molecules were much more active in Co^{II} than in other metal ions including Zn^{II} and Pb^{II}. No detectable cleavage activity was observed with several other metal ions investigated including Ca^{II}, Mg^{II}, and Cd^{II}, and cleavage activity in the presence of Mn^{II} was

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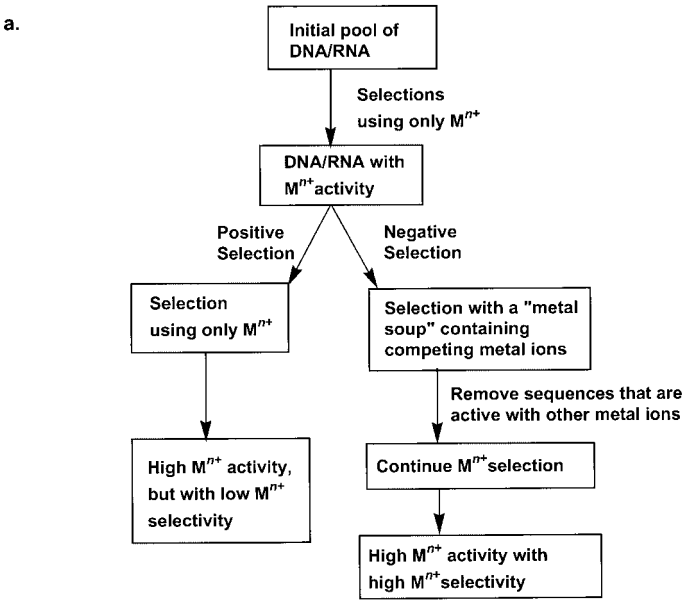

b.

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Clone 18   ATCTC TTGTATTAGCTACACTGTTAGTGGATCGGGTCTAATCTCG GTGAC
Clone 11   ATCTC TTGTATTAGCTACACTGTTAGTGCATCGTTTTTAATCTCG GTGAC
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Figure 4. a) Strategies in improving metal-ion selectivity during in vitro selection. b) DNAzyme sequences from an in vitro selection of Co^{II}-dependent DNAzymes. Clone 18, selected without the negative selection strategy, prefers Zn^{II} and Pb^{II} over Co^{II}. However, Clone 11, selected after using the negative selection strategy, prefers Co^{II} over Zn^{II} and Pb^{II}. Adapted from reference [56].

minimal. These results suggest strongly that in vitro selection can be used to obtain highly specific transition metal ion-dependent DNA/RNAzymes, which hold great promise as versatile and efficient endonucleases as well as sensitive and selective metal-ion sensors.

Advantages of Transition-Metal-Ion-Dependent DNAzymes

Most RNAzymes isolated from nature utilize alkaline-earth metal ions such as Mg^{II} and Ca^{II} and, in some cases, Mn^{II}.[35–38] One exception is the hammerhead ribozyme, which is also active in the presence of the transition-metal ions Co^{II}, Zn^{II}, and Cd^{II}, although spermine is required for Zn^{II}-catalyzed reactions.[39] Therefore, most efforts have been devoted to the study of Mg^{II}- and Ca^{II}-dependent DNA/RNAzymes. However, transition-metal-dependent DNAzymes are equally attractive as alkaline-earth-metal-dependent DNAzymes for study and application for several reasons. First, the different transition-metal ions with a wide range of properties can help broaden the functional repertoire of DNAzymes, just like they can for protein enzymes. Second, given the same reaction, such as phosphodiester transfer or cleavage, transition- or pseudo-transition-metal ions, such as Co^{II} or Zn^{II}, may be able to catalyze the reaction more efficiently than Mg^{II} or Ca^{II}, because Co^{II} or Zn^{II} are better Lewis acids and their metal-bound water possesses a lower pK_a, both of which may be important for the reaction. Indeed, several transition/lanthanide-metal complexes have been shown to be quite effective in DNA/RNA cleavage.[57–63] This argument is also supported by the existence of many Zn^{II}-dependent hydrolytic protein enzymes such as carboxypeptidase, phosphotriesterase, and alkaline phosphatase.[31–34] Third, a clear understanding of the metal-binding site and its structural changes during catalysis is necessary for designing better nucleic acid enzymes. Transition-metal ions have much richer spectroscopic features than alkaline-earth-metal ions, making the study of coordination spheres and reaction mechanisms of metal-binding sites in DNAzymes much more fruitful. This advantage has been nicely demonstrated in the study of Mn^{II} derivatives of hammerhead ribozymes and related model systems.[64–68] To provide structural information in solution, metal ions such as a high-affinity metal inhibitor Tb^{III}[69] or highly thiophillic Hg^{II} in combination with a phosphorothioate at the cleavage site, had to be used in the past.[70] Finally, the search for and understanding of different transition-metal-ion-dependent DNAzymes make it possible to design better metal biosensors and to use a DNAzyme array for simultaneous detection of metal ions.

Transition-Metal-Dependent DNAzymes as Efficient Endonucleases

Recent results have shown that both RNAzymes and DNAzymes can utilize transition-metal ions and that the resulting enzyme may be more efficient than Mg^{II}- and Ca^{II}-dependent enzymes.[1–3, 7, 13, 14, 18, 53, 71] For example, a group of highly efficient transition-metal-dependent RNA-cleaving DNAzymes has been obtained through in vitro selection.[13] It was found that this commonly selected motif (called the 8–17 motif, see Figure 1b)[10–13, 55] can efficiently and specifically cleave both RNA and DNA/RNA chimeric substrates. It can cleave any unpaired ribonucleotide followed by a G-T wobble pair. The pH profile and reaction products of the DNAzyme are similar to those reported for the hammerhead ribozyme.[72] This DNAzyme has higher activity in the presence of transition-metal ions than in the presence of alkaline-earth-metal ions. At saturating concentrations of Zn^{II}, the cleavage rate is 1.35 min^{-1} at pH 6.0 and is estimated to be $\approx$50 min^{-1} at pH 7.5, at which most assays of Mg^{II}-dependent DNA/RNAzymes are carried out.

The use of transition-metal ions to expand the functional repertoire of DNAzymes has also been demonstrated. In

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contrast to RNA cleavage, DNA cleavage is much more difficult due to the inherent stability of DNA to hydrolysis. Therefore, no DNA/RNAzymes have been shown to carry out efficient DNA cleavage in the presence of alkaline-earth-metal ions such as Mg^{II}. However, through in vitro selection, Carmi and Breaker have been able to obtain Cu^{II}-dependent DNAzymes that can cleave DNA efficiently.[7, 71, 73] The DNAzyme was further minimized to 46-mer DNAzyme with conserved sequences defined (Figure 1c). This DNAzyme has a bell-shaped metal-binding curve; the enzyme activity increases with increasing concentration of Cu^{II} until it reaches 10 μM, then decreases with increasing concentration of Cu^{II} ions beyond 10 μM until the enzymes are completely inhibited in excess of 100 μM of Cu^{II}.[73] Interestingly, similar Cu^{II}-dependent activity was also observed in a DNAzyme with DNA ligase activity.[14] An even further extension of DNAzyme structural and function diversity was demonstrated by a successful in vitro selection of DNAzymes that catalyze the insertion of Cu^{II} or Zn^{II} into mesopoerphyrin IX.[18]

DNAzymes as Highly Sensitive and Selective Metal-Ion Sensors

The combination of transition-metal ions with DNA/RNAzymes has resulted in not only new DNA/RNAzymes with high catalytic efficiency and expanded functional diversity, but also in a new class of highly sensitive and selective metal-ion biosensors, as demonstrated recently.[29] This work is based on the observation that, since DNA/RNAzymes are capable of binding metal ions of choice with high affinity and specificity[1, 7, 13, 14, 53] through in vitro selection, the resulting enzymatic products can be used to monitor the identity and quantity of the specific metal ion involved in the reaction. The biosensor developed consists of a DNAzyme capable of base-pairing to a DNA substrate that contains a single ribonucleotide residue (Figure 5a).[29] When a fluorophore such as TAMRA is attached to the 5′-end of the substrate, the fluorescence signal at 580 nm is quenched by a fluorescence quencher, such as Dabcyl, at the nearby 3′-end of the DNAzyme (Figure 5b). In the presence of a metal ion, such as Pb^{II}, the fluorescence emission of TAMRA increases by

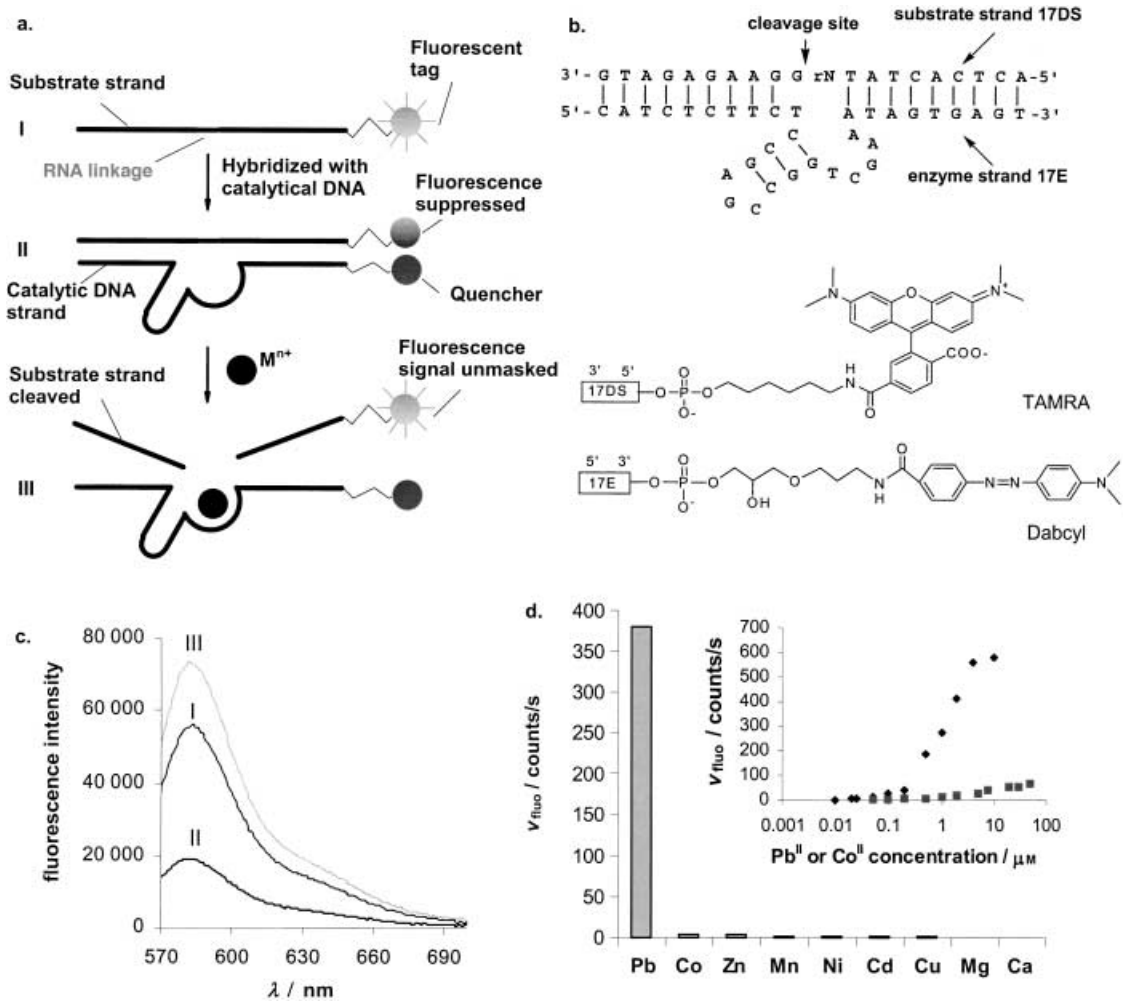

Figure 5. DNAzymes as a new class of metal ion biosensors. a) Concept and design of metal ion biosensors, using lead sensors as an example; adapted from *C&EN News*, **2000** (Oct. 30), 78(44), 9–10; b) the sequences and proposed secondary structure of the lead sensor (top), and examples of fluorescence tag (TAMRA) and quencher (Dabcyl); c) Steady-state fluorescence spectra of the substrate (Rh-17DS) alone (I), after annealing to the deoxyribozyme (17E-Dy) (II), and 15 min after adding 500 nM $Pb(OAc)_2$ (III); d) The fluorescence response rate (v_{fluo}) of sensor in the presence of 500 nM of different divalent metal ions in 50 mM HEPES (pH 7.5). The inset shows the variation of initial rate v_{fluo} with the concentration of Pb^{II} (◆) or Co^{II} (■). c) and d) are adapted from reference [29].

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400%, due to the cleavage of the substrate followed by product release (Figure 5c). This DNAzyme sensor is highly sensitive for Pb^{II}, with a quantifiable detection range from 10 nM to 4 μM (Figure 5d, inset). Even in the presence of equal concentrations of other metal ions (such as Mg^{II}, Ca^{II}, Mn^{II}, Co^{II}, Ni^{II}, Zn^{II}, Cd^{II}, and Cu^{II}) and under simulated physiological conditions, this biosensor displays remarkable sensitivity and selectivity (Figure 5d). The principles demonstrated in this work can be used to obtain DNAzyme sensors for other metal ions, cations or analytes.

The work described above complements those biosensors based on DNA/RNA aptamers and aptazymes (or allosteric DNA/RNAzymes),[74–79], with shared similarities and differences. DNA/RNA aptamer sensors are based on the high affinity of the sensors with target analytes through molecular recognition. Like antibodies, DNA/RNA aptamers can be selected to bind a variety of targets with high affinity and can be used as sensors for these analytes. Aptazyme or allosteric DNA/RNAzyme biosensors, on the other hand, contain both an aptamer and an enzyme unit with known activity, such as the hammerhead ribozyme. Structural changes associated with aptamer binding to the analytes are utilized as a way to trigger catalytic activity of the known enzymes.[80] In the aptazyme system, analytes to be sensed are usually not direct participants of enzymatic reactions, but rather exert their influence through allosteric structural changes. For example, a hammerhead ribozyme has been engineered into an aptazyme for Co^{II} through a combination of a Co^{II} aptamer and a hammerhead ribozyme that depends on Mg^{II} for activity.[80] Like aptamer and aptazyme biosensors, DNA/RNAzyme-based sensors utilize the ability of DNA/RNA to bind and recognize metal ions. However, the target analyte DNA/RNAzyme-based sensor is a direct participant of the enzyme reaction and plays an essential role in the enzymatic activity.

Advantages of DNAzymes in Pharmaceutical and Biotechnological Applications

Several features make DNAzymes an excellent choice for pharmaceutical and biotechnological applications. The first and perhaps biggest advantage of choosing DNAzymes is that they can be subjected to in vitro selection. When compared to other combinatorial methods based on organic chelators or peptides, in vitro selection can sample a larger pool of different molecules (up to 100 trillion), amplify the desired sequences by the polymerase chain reaction (PCR), and introduce mutations to improve performance by mutagenic PCR. For pharmaceutical applications, in vitro selection allows high flexibility in choosing target viral RNA sequences to cleave.[10, 23] For biotechnological application, in vitro selection makes it possible to fine-tune metal-binding affinity and selectivity of DNAzymes, through successive implementation of a negative selection strategy.[56] This approach can overcome our limited knowledge about the metal-binding affinity and selectivity of different organic- or biomolecules. A detailed study of the resulting metal-specific DNAzymes may provide insight into rational design of other metal sensors. Second, the in vitro selection can be carried out in short time and with limited cost (1–2 days and a few dollars per round of selection). Third, the synthesis of DNA is easier, and therefore less costly than the synthesis of RNA. Under physiological conditions, DNA is nearly 1,000-fold more stable to hydrolysis than proteins and nearly 100,000-fold more stable than RNA.[4] As seen from a recent crystal structure,[81] DNAzymes usually form a compact globular shape and are therefore not easily recognized by endo- or exonucleases; hence they are likely more resistant to nuclease attack than single or even double-stranded DNA/RNA.[82] When folded, the compact globular DNAzymes are also unlikely to bind biomolecules in the cells other than the single- or double-stranded DNA/RNA. Indeed, a properly designed "10–23" DNAzyme was shown to remain intact even after 48 hours of exposure to serum, and to be effective in targeting Egr-1 mRNA, resulting in inhibition of vascular smooth muscle proliferation and regrowth after injury.[28] Fourth, unlike proteins, most DNAzymes can be denatured and renatured many times without losing binding ability or activity. They can be used and stored under rather harsh conditions. Fifth, DNA is adaptable to fiber optic and microarray technology,[83, 84] which is important for on-site or remote sensing of multiple metal ions simultaneously. Finally, as demonstrated recently,[29] there are three additional advantages of DNAzyme fluorescent sensor systems. The metal sensing is achieved by both metal-binding and catalytic activity, allowing signal amplification through catalytic turnover. The fluorophores can be placed remotely from the binding and cleavage sites so that binding and sensing do not interfere with each other and can be optimized independently. The effective placement of the fluorophores can be accomplished with little knowledge of the three dimensional structure of the system.

Summary

DNAzymes are a new member of the enzyme family that holds great promise both as endonucleases for pharmaceutical applications and as metal-ion sensors for biotechnological applications. These abilities can be significantly enhanced when DNAzymes use transition-metal ions as cofactors for their enzymatic activities. Recent results demonstrated that in vitro selection of DNAzymes from a library of DNA is capable of obtaining new DNAzymes with high endonuclease activity as well as metal-ion selectivity. The close relationship between metal ions and DNAzymes has been nicely illustrated; metal ions can help DNAzymes to increase catalytic activity and broaden functional diversity, while at the same time the enzymatic activity, particularly the products, can be used to signal the presence and quantity of metal ions that catalyze the enzymatic reactions. Applications of these DNAzymes as effective anti-viral agents and as selective metal-ion sensors have been demonstrated.

Outlook I–from metalloproteins to metallo-DNA/RNAzymes, a new paradigm in chemistry and biology?: Through many years of research, different classes of metal-specific proteins, such as heme, copper or zinc proteins, are now

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known, including a comprehensive understanding of their sequence, structural, and functional features specific to each class of metalloproteins (Figure 6).[31–34] Similar information about metal-specific DNA/RNAzymes is virtually unknown. However, important progress has been made recently on the

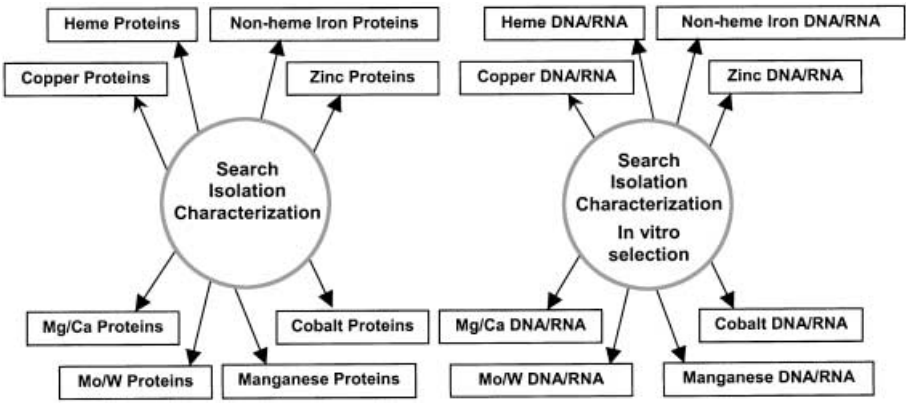

Figure 6. From metalloproteins to metallo-DNA/RNAzymes. While many years of searching, isolation, and characterization of metalloproteins have resulted in a thorough understanding of structural and function of metalloproteins (left), similar information about metal-binding sites in DNA/RNA is virtually unknown. Using in vitro selection, we can now can obtain new DNA/RNAzymes with specific metal-binding sites of our choice in a shorter period of time (right). Further study of these metallo-DNA/RNAzymes will allow us to provide equally detailed information of metal-binding sites in DNA/RNA, and to design highly efficient endonucleases and selective metal sensors for pharmaceutical and biotechnological applications.

biochemical and spectroscopic studies of metal-binding sites in Mg^{II}/Ca^{II}-dependent DNA/RNAzymes.[35–38] Furthermore, the implementation of in vitro selection and SELEX methods now allows us to obtain different DNA/RNAzymes with high specificity and affinity for a metal ion of choice and thus lays a foundation for a detailed and systematic study of metal-binding sites in DNA/RNAzymes. For example, the metal selectivity issue is not unique to DNA/RNAzymes. Heavy or thiophillic metal ions such as Hg^{II} can replace Cu^{II} in copper–thiolate proteins.[85] It is well known that Co^{II} and Zn^{II} have similar charges and ionic radii. Co^{II} can replace Zn^{II} in zinc proteins without any loss of activity.[86, 87] One may wonder if there is any difference between Co^{II}- and Zn^{II}-binding in proteins or DNA/RNA. Now using in vitro selection that incorporates a negative selection strategy, two classes of DNAzymes have been obtained, with one with a preference for Co^{II} over Zn^{II} and the other for Zn^{II} over Co^{II} (Figure 4b). Interestingly, they share similar sequence homology and yet posses subtle differences. Further biochemical and spectroscopic study of these and other DNA/RNAzymes will enrich our knowledge of metal-binding sites in DNA/RNAzymes so that similar level of understanding as in protein enzymes can be achieved.

Outlook II—in vitro selection of DNAzymes: a general and versatile method for obtaining metal-ion sensors?: The design of metal-ion sensors has long been a focus of research endeavors because it can provide portable, on-site, real-time detection and quantification of both beneficial and toxic metal ions in applications such as household and environmental monitoring (for drinking or lake water and soil), developmental biology, or clinical toxicology. For example, the use of Ca^{II} sensors in cells has revolutionized the study of cell physiology.[88] While remarkable progress has been made in developing metal sensors,[88–90] designing and synthesizing sensitive and selective metal-ion sensors remains a significant challenge. Since the knowledge of the design of metal-ion-specific sensors is limited, searching for sensors in a combinatorial way is particularly attractive. The concept and advantages of combinatorial searches for metal-ion sensors have been demonstrated recently by several groups.[29, 91, 92] Among these methods, in vitro selection of DNA/RNA from a library of $10^{14}-10^{15}$ random DNA/RNA sequences offers considerable possibilities.[40–44] A unique feature of in vitro selection of DNA/RNA (when compared with combinatorial selection of organic ligands or peptides) is its ability to sample a larger pool of sequences, amplifying the desired sequences by the PCR, and introducing mutations by mutagenic PCR to improve performance. The recent reports of the first DNAzyme biosensor for lead[29] and the ability to improve metal selectivity of the DNAzymes[56] are encouraging. Further employment and improvement of this method will allow us to find highly sensitive and selective sensors for not only any metal ions of choice, but also any oxidation state of a selected metal ion.

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