Phosphoester-transfer mechanism of an RNA-cleaving acidic deoxyribozyme revealed by radioactivity tracking and enzymatic digestion†

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A convenient method involving ^{32}P-labeling of an RNA substrate at the cleavage site and subsequent enzymatic digestion of cleavage products *via* phosphatases reveals that pH4DZ1—an RNA-cleaving deoxyribozyme with optimal activity at pH 4—forms a 5′-cleavage fragment with 2′,3′-cyclic phosphate group and a 3′-cleavage fragment with 5′-OH group.

Many catalysts made of RNA (ribozymes) and DNA (deoxyribozymes) that can specifically cleave RNA substrates have been discovered in nature or created in laboratories.[1,2] In recent years, these enzymatic molecules have found increasing use in a broad range of chemical and biological applications, including gene therapeutics,[3] biosensing,[4] and DNA based devices.[5]

We recently initiated a research program to isolate deoxyribozymes that are able to cleave an RNA/DNA chimeric substrate in which a lone RNA linkage (ribo-A, written as R in the substrate sequence in Fig. 1(a)), is flanked immediately by two deoxyribothymidines modified with a fluorescein-fluorophore (F) and a DABCYL-quencher (Q), respectively.[1,4c,e] Arranging a fluorophore and a quencher within a short physical distance in the same molecule results in efficient fluorescence quenching. Hence, upon cleavage of the lone RNA linkage, the fluorophore is separated from the quencher and generates a strong fluorescence signal. With this synchronized catalysis-signaling ability, these special deoxyribozymes offer an excellent opportunity for designing deoxyribozyme-based biosensors. Our efforts have led to the creation of several signaling deoxyribozymes with robust catalytic activities, diverse metal-ion specificities, and variable pH sensitivities.[1,4c,e] Among them is a highly unusual deoxyribozyme denoted pH4DZ1 which, unlike most other counterparts, has an optimal activity at pH 3.8. Although the original pH4DZ1 is a 123-nt *cis*-acting catalyst, a bimolecular *trans*-acting system consisting of 35-nt substrate (**S**) and 73-nt deoxyribozyme (**E**) (Fig. 1(a)) has recently been designed.[6] As part of our quest to understand how this deoxyribozyme achieves a k_{obs} of $\sim$1 min^{-1} and a rate enhancement of $\sim$10^{6}-fold at pH 3.8, we sought to characterize its cleavage products and define its reaction mechanism. To do this, we have developed a new technique that can accurately trace

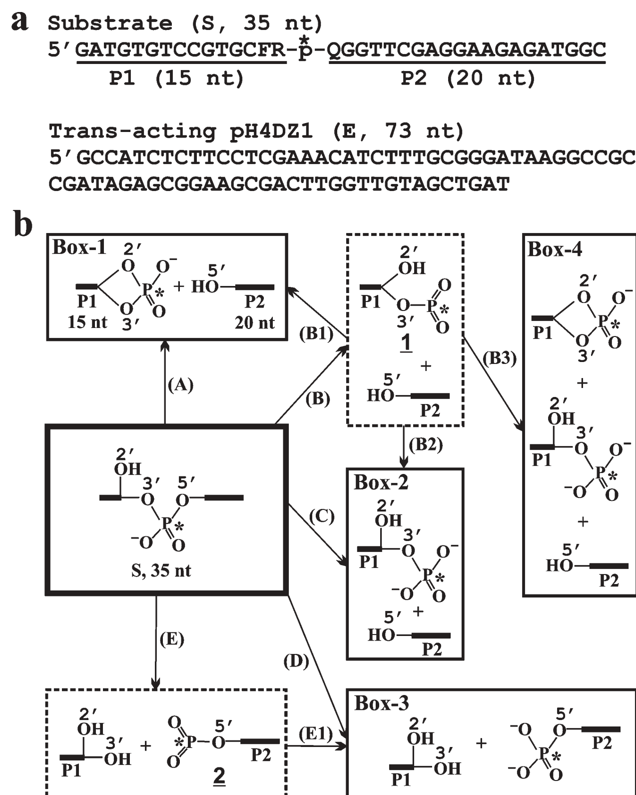

Fig. 1 (a) Sequences of the substrate (**S**) and the *trans*-acting pH4DZ1 (**E**). The radioactive phosphorous atom (^{32}P) is depicted as p* in all figures in this article. (b) Proposed schemes to pinpoint the possible reaction pathway of RNA cleavage through radioactivity tracing of the movement of ^{32}P at the cleavage site. Note that the mechanistic pathways illustrated here are simplified for clarity.

the fate of the phosphorous atom situated in the phosphodiester linkage at the cleavage site in just a few experimental steps.

Hypothetically, four possible sets of cleavage products (illustrated in Box-1 to Box-4 in Fig. 1(b)) could be produced when the phosphodiester bond in **S** (thick-lined box in Fig. 1(b)) is cleaved. Briefly, Box-1 products consist of **P1** (the 5′-cleavage fragment) carrying a 2′,3′-cyclic phosphate and **P2** (3′-cleavage fragment) with a 5′-hydroxyl group. They can be generated either by pathway A (an S_N2 reaction where the 2′-OH acts as the nucleophile to attack the adjacent phosphorous center of the scissile phosphodiester linkage) or by pathway B→B1 (an S_N1 reaction in which intermediate **1** is first produced and then reacts

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with the adjacent 2′-OH).[7] Box-2 products comprise of **P1** carrying a 3′-phosphate and **P2** having a 5′-hydroxyl. These two products can be generated either by pathway C (an S_N2 reaction in which water acts as the nucleophile and the 5′-bridging oxygen as the leaving group) or by pathway B→B2 (where **1** reacts with water). Box-3 products are composed of **P1** possessing a 3′-hydroxyl and **P2** with a 5′-phosphate. These two products can also be obtained *via* two routes: pathway D (an S_N2 reaction in which water acts as the nucleophile and the 3′-bridging oxygen as the leaving group) or pathway E→E1 (an S_N1 reaction where intermediate **2** reacts with water). Finally, Box-4 products are a mixture of **P1** carrying a 3′-phosphate, **P1** bearing a 2′,3′-cyclic phosphate and **P2** with a 5′-hydroxyl. This group of products is expected when **1** reacts with both water and the 2′-OH adjacent to the phosphorous center. It is clear that different reaction pathways will generate cleavage fragments with distinct phosphorylated and nonphosphorylated termini, and therefore, correct identification of these fragments can facilitate the understanding of the mechanism of any RNA-cleavage reaction. We describe below in detail an approach that can easily achieve this purpose.

First, we generated a radioactive **S** by ligating a 5′-^{32}P-labeled oligonucleotide having the sequence of **P2** to a 3′-ribo-terminated oligonucleotide having the sequence of **P1**. A radioactive **S** with ^{32}P-labeled phosphodiester linkage at the cleavage site allows us to keep track of the movement of the concerned phosphorus atom by radioactivity detection. Specifically for pH4DZ1, both pathways A, B (including B1, B2 and B3) and C would generate a visible **P1** (15 nt, radioactive) and an invisible **P2** (20 nt, non-radioactive) on the autoradiogram following denaturing PAGE analysis of the reaction mixture. Pathways D and E, in contrast, would produce a visible **P2** and an undetectable **P1**.

When 0.5 μM of the radioactive **S** was incubated with 25 μM of **E** for various time points (Fig. 2), increasing amount of radioactive **P1** was produced while no band corresponding to **P2** was observed. This result showed that pH4DZ1 followed pathway A or B or C, but not pathway D and E.

Although the results presented in Fig. 2 confirmed that **P1** contained a terminal phosphate at the 3′-end, it did not reveal whether it was in the form of a 2′,3′-cyclic phosphate or 2′ and/or 3′ monophosphate. Though chromatographic technique such as HPLC[8] and spectroscopic techniques such as NMR and MALDI-TOF[9] have been reported to characterize the phosphate terminus, these techniques are often applicable only to mononucleotides or short oligonucleotide fragments and require highly pure samples. The separation of short RNA fragments containing 2′,3′-cyclic phosphate and 2′- or 3′-monophosphate by mild acid hydrolysis followed by 20% PAGE is also a frequently used technique.[10]

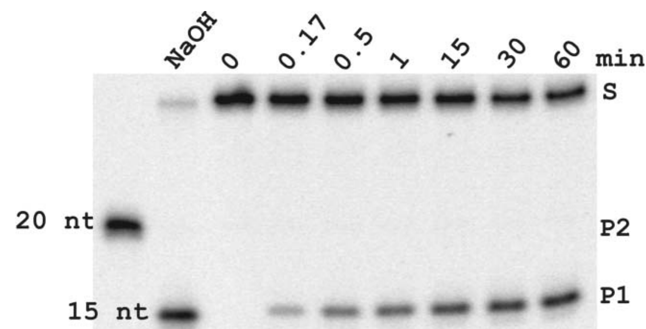

Fig. 2 Evidence for pH4DZ1-mediated phosphoester-transfer mechanism. Lanes: 1, 5′-^{32}P-labeled **P2**; 2, **P1** generated by cleavage of **S** with 0.2 M NaOH at 90 °C for 10 min; 3, **S** alone in the reaction mixture; 4–9, **S** incubated with **E** for the indicated times.

However, our attempts to separate these 5′-cleavage fragments by mild acid hydrolysis and 20% PAGE, considering that the phosphoester transfer might have resulted in the formation of 2′,3′-cyclic phosphate, were not successful (data not shown). This may be due to the presence of negatively charged and bulky fluorophore (F) on **P1** and its relatively large size. Therefore, we sought to explore three protein enzymes—T4 polynucleotide kinase (PNK), calf intestine alkaline phosphatase (CIAP) and 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase)—to characterize nature of the phosphate terminus of **P1**. PNK is known to have 2′,3′-cyclic nucleotide 3′-phosphodiesterase activity as well as 2′- and 3′-phosphatase activities.[11] CIAP, however, has only 2′- and 3′-phosphatase activities but no 2′,3′- cyclic nucleotide phosphodiesterase activity (Fig. 3(a)).[9b,12] CNPase hydrolyzes 2′,3′-cyclic phosphate solely to 2′-phosphate (Fig. 3(a)).[13]

The disappearance of radioactivity of **P1** when the RNA cleavage reaction mixture was incubated with PNK (lane 3, Fig. 3(b)) and the full retention of radioactivity (the ratio of the radioactivity of **P1** over that of **S** stayed unchanged) upon incubation with CIAP (lane 4) are indicative of the radioactive phosphate being a 2′,3′-cyclic phosphate but neither a 2′- nor 3′-phosphate. This notion was further supported by the disappearance of the **P1** band by first converting its 2′,3′-cyclic phosphate terminus to 2′-phosphate with CNPase, followed by the removal of monophosphate by PNK (lane 6) or CIAP (lane 7). As implicated from the fluorimage of the same gel, **P1** (which carries a fluorescein) migrated relatively slower if its phosphate terminus was removed (lanes 3, 6 and 7). We did not see, however, any difference in gel mobility between **P1** carrying a cyclic phosphate

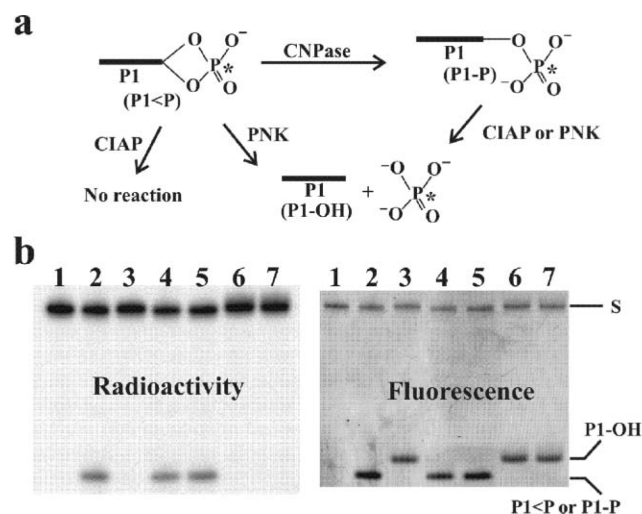

Fig. 3 (a) Scheme for verifying the radioactive 2′,3′-cyclic phosphate terminus of **P1** using PNK, CIAP and CNPase. (b) Treatment of **P1** by PNK, CIAP and CNPase. Lanes: 1, **S** alone; 2, **S** incubated with **E**; 3–7, **S** incubated with **E** followed by PNK (3), CIAP (4), CNPase (5), CNPase first then PNK (6), CNPase first then CIAP (7). The left and right images are the autoradiogram and the fluorescence scan of the same PAGE experiment, respectively.

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and **P1** carrying a monophosphate (lanes 2, 4 and 5), even under 20% denaturing PAGE. It is noteworthy that the radioactivity of uncleaved **S** remained unchanged, indicating that the disappearance of radioactivity in **P1** was not due to any plausible nuclease contaminants in each protein enzyme sample.

Using the above technique, we have shown that the cleavage products produced by pH4DZ1 consist of a 5′-cleavage fragment with a 2′,3′-cyclic phosphate and a 3′-cleavage fragment with a 5′-OH. It is interesting to note that same products are found for all naturally occurring self-cleaving ribozymes and all man-made RNA-cleaving nucleic acid enzymes whose mechanisms have been determined.[2e,14] Our results indicate that pH4DZ1 catalyzes RNA cleavage reaction *via* either pathway A or pathway B→B1. To further distinguish between these two pathways for pH4DZ1, additional experiments need to be conducted, which represents a future objective.

In summary, we report here a simple, yet effective method that can be conveniently used to characterize the cleavage products of RNA substrates by nucleic acid enzymes. It is simple and convenient in the sense that it only requires general lab equipment and reagents to set up the experiments for probing the reaction pathways, which is unlike other methods that necessitate NMR or mass-spectrometry facilities. It is also effective because this method is not constrained by the size of RNA substrates and (in principle) the type of oligonucleotide modifications such as chromophore labeling, which might be some of the limitations encountered by protocols that involve HPLC or solely denaturing PAGE to resolve short oligonucleotide fragments that carry 2′,3′-cyclic phosphates and oligonucleotides with 2′- or 3′-phosphate termini. With the advent of RNA-ligating deoxyribozymes that can facilitate the ligation between RNA strands more effectively than the conventional splint-directed ligation mediated by protein ligases,[15] we believe our method can also be applicable to all-RNA substrates and hasten the mechanistic study of yet to be discovered RNA-cleaving nucleic acid enzymes.

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