DNA-catalyzed serine side chain reactivity and selectivity†

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New deoxyribozymes are shown to catalyze reactions of serine side chains, forming nucleopeptide linkages and discriminating between serine and tyrosine or between two competing serines.

Since the identification of the first artificial deoxyribozyme, which catalyzes RNA phosphodiester bond cleavage,[1] many DNA enzymes have been found to catalyze reactions that typically involve oligonucleotide substrates.[2–4] A major challenge is to incorporate small molecules, proteins, and other substrates into the repertoire of DNA catalysis. Towards this goal, we recently reported the Tyr1 deoxyribozyme, which catalyzes the nucleophilic attack of a tyrosine phenolic OH group into 5′-triphosphate-RNA, leading to the formation of a nucleopeptide linkage.[5] Covalent linkages between nucleic acids and proteins are integral to the mechanisms of topoisomerases[6] and recombinases[7] and are found in many other biological contexts.[8]

The Tyr1 deoxyribozyme was identified by *in vitro* selection, which iteratively searches through $\sim 10^{14}$ random DNA sequences to find those with catalytic activity.[9] During the Tyr1 selection process, tyrosine was presented at the intersection of a three-helix junction (3HJ) formed from candidate deoxyribozyme sequences and two nucleic acid strands. One of these strands comprised DNA containing the embedded tyrosine, whereas the other strand was 5′-triphosphate-RNA (Fig. 1a). The 3HJ architecture, inspired by the discovery of 7S11 and related deoxyribozymes that catalyze formation of 2′,5′-branched RNA,[10] spatially juxtaposes a nucleophile (*e.g.*, tyrosine OH group) and electrophile (5′-triphosphate). This design allows interrogation of DNA sequences while focusing primarily on their intrinsic ability to catalyze the desired chemical reaction, without requiring that the DNA also bind a separate free substrate molecule.

Alongside the successful identification of Tyr1, parallel selection experiments were previously performed in which a single serine residue (rather than tyrosine) was placed at the 3HJ intersection (Fig. 1a), but essentially no active deoxyribozymes were identified.[5] The serine aliphatic hydroxyl group is less reactive than the tyrosine phenolic hydroxyl group, providing a greater catalytic challenge. In the present report, for the first time we have achieved robust DNA-catalyzed reactivity of the serine side chain, by expanding the structural context from a single amino acid to

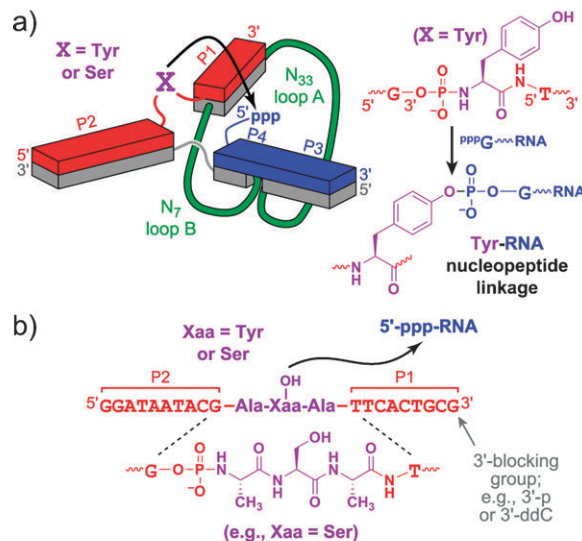

Fig. 1 Architecture used to investigate DNA-catalyzed amino acid side chain reactivity. (a) Three-helix-junction (3HJ) architecture brings together the amino acid side chain and the electrophilic 5′-triphosphate. The previously reported Tyr1 deoxyribozyme forms a tyrosine–RNA nucleopeptide linkage (X = Tyr), but DNA catalysis was not found when X = Ser.[5] (b) In the present study, X was expanded to a tripeptide Ala-Xaa-Ala (Xaa = Ser or Tyr; Ala as negative control), to pursue new DNA-catalyzed reactivity of the serine side chain. Full nucleic acid sequences and enzyme–substrate complex architecture are shown in Fig. S1.†

an Ala-Ser-Ala tripeptide while retaining the 3HJ architecture (Fig. 1b). We additionally investigated the chemical selectivity of DNA catalysis involving the Ser side chain, with promising results both for discriminating Ser *versus* Tyr and for favoring one of several competing serines within the same substrate.

The new experiments used our previously established selection strategy[11] to identify catalytic DNA sequences. At the outset of each selection round, the 5′-triphosphate-RNA was ligated *via* its 3′-terminus to the 5′-end of the deoxyribozyme pool strand. Any DNA sequence that successfully joins the amino acid side chain of the tripeptide-containing substrate to the 5′-triphosphate-RNA as shown in Fig. 1 becomes separable from even a substantial excess of catalytically inactive DNA sequences by polyacrylamide gel electrophoresis (PAGE). During the key selection step of each round, the incubation conditions were 50 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM KCl, 20 mM $MnCl_2$, and 40 mM $MgCl_2$ at 37 °C for 2 h. Both Mn^{2+} and Mg^{2+} were included because both divalent metal ions have supported various DNA-catalyzed reactions.[2,3] In addition to a selection experiment involving the Ser side chain, a selection experiment in which the substrate included the tripeptide Ala-Tyr-Ala was

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performed as a positive control, because Tyr reactivity was anticipated to emerge on the basis of our previous report.[5]‡

We originally placed a 3′-phosphate at the 3′-end of each tripeptide-containing substrate, to block unwanted nucleophilic reactivity of the DNA 3′-hydroxyl group (Fig. 1b). In selection round 4, detectable catalytic activity ($\geq 1\%$) was observed in each of three separate selection experiments with Ala-Xaa-Ala tripeptide-containing substrates, where Xaa = Tyr, Ser, or, as a control lacking any nucleophilic side chain, Ala.† Activity was observed both for intramolecular (in *cis*) reaction, in which the substrate was covalently ligated to the DNA pool, and intermolecular (in *trans*) reaction. The PAGE migration rate of each product formed in *trans* was compared with the migration rate of an appropriate synthetic standard (a branched DNA–RNA conjugate), whose migration rate should correspond closely to that expected upon nucleopeptide linkage formation. For both the Xaa = Tyr and Ser selections, the product indeed appeared to result from reaction of the amino acid side chain. In contrast, for the Ala selection the DNA 3′-phosphate rather than the amino acid side chain acted as the nucleophile, forming a pyrophosphate-linked product, as revealed by the distinctive PAGE migration rate and by lack of reactivity when the 3′-phosphate was absent (Fig. S2a).† The selections were continued for three additional rounds. In round 7, the product from 3′-phosphate reactivity now predominated in all three selections, although for each of the Tyr and Ser selections, a small fraction (3–7%) of the total product still appeared to result from the intended reaction at the amino acid (Fig. S2b, ESI†).

To redirect the selection outcomes[12] towards the desired reactivity of the amino acid side chains, we resumed the selections from round 8 using tripeptide-containing substrates that were terminated with an additional 3′-ddC nucleotide, which lacks any strong nucleophile. In round 10 for the Tyr and Ser selections, substantial catalytic activity of each pool was observed (9% and 32%). On the basis of PAGE migration rates of the products formed in *trans* and lack of reactivity of the Xaa = Ala substrate, the products now appeared to be the intended nucleopeptide linkages (Fig. S3, ESI†).

Individual round 10 deoxyribozymes were cloned (Table S1, ESI†) and characterized (Fig. 2).† From the Tyr selection, a single new sequence named TyrB1 was identified. From the Ser selection, four distinct sequences named SerB1–SerB4 were found. None of the new deoxyribozymes are active with Ala or lysine (Lys) substrates (Fig. S4, ESI†), and all of the new deoxyribozymes require Mn^{2+} but not Mg^{2+} for catalysis (Fig. S5, ESI†). At 20 mM Mn^{2+} with 40 mM Mg^{2+}, TyrB1 functions with k_{obs} = 0.30 h^{-1}, 75% yield, and at least 600-fold selectivity for reaction of the Tyr side chain over Ser (Fig. 2); the $K_{d,app}$ for Mn^{2+} is 17 ± 3 mM (Fig. S6, ESI†). Under the same conditions, SerB1 has k_{obs} = 0.24 h^{-1} and 82% yield, and the selectivity for Ser over Tyr is only 12-fold. SerB2 through SerB4 are all very similar in reactivity, with k_{obs} = 0.4 h^{-1}, ~80% yield, and merely two-fold selectivity for Ser over Tyr (Fig. 2). For all four of SerB1–SerB4, the $K_{d,app}$ for Mn^{2+} is >10 mM (Fig. S6, ESI†). The product identities were verified by MALDI mass spectrometry and by biochemical cleavage assays (Fig. S7 and S8, ESI†). These

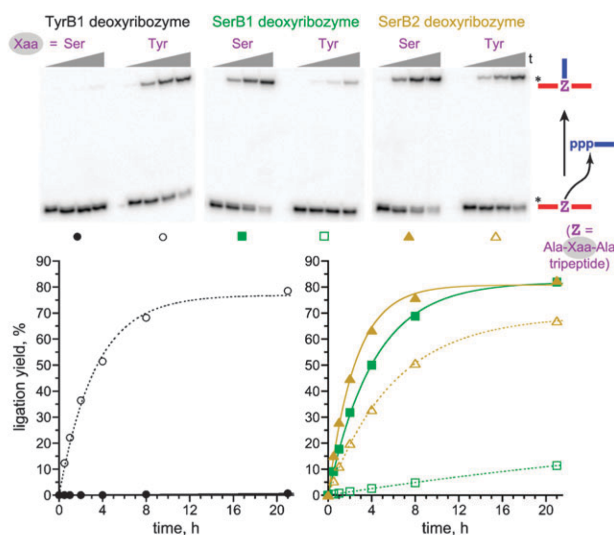

Fig. 2 Catalytic activities of new deoxyribozymes for Tyr and Ser side chain reactivity. (a) TyrB1 deoxyribozyme. In the tripeptide substrate, Xaa = Tyr (○) or Ser (●). The ligation yield with Xaa = Ser at 21 h was <1%. The k_{obs} value with Tyr was 0.30 h^{-1}. (b) SerB1 and SerB2 deoxyribozymes, with Xaa = Ser (■, SerB1; ▲, SerB2) or Tyr (□, SerB1; △, SerB2). k_{obs} values: SerB1, 0.24 h^{-1} with Ser and 0.020 h^{-1} with Tyr; SerB2, 0.40 h^{-1} with Ser and 0.17 h^{-1} with Tyr. Data for SerB3 and SerB4 were similar to that for SerB2. For all gel images, t = 0, 1, 5, and 21 h. See Fig. S4 (ESI†) for SerB3 and SerB4 data.

findings constitute the first example of DNA catalysis involving the Ser aliphatic hydroxyl side chain.

The rather modest discrimination by SerB1–SerB4 between Ser and Tyr side chains (when presented separately at the same amino acid position within the substrate) is reminiscent of a previous observation with the 7S11 deoxyribozyme that forms 2′,5′-branched RNA.[13] When 7S11 is presented with a 5′-triphosphate-RNA substrate that has its 5′-terminus lengthened by one or more nucleotides, reactivity of the branch-site adenosine 2′-hydroxyl group of the other RNA substrate can be observed at either (i) the relatively reactive 5′-triphosphate, now displaced spatially from its original location, or (ii) the original electrophilic phosphorus atom, in its original spatial location but now as a much less reactive internucleotide phosphodiester. For SerB1–SerB4, the less nucleophilic Ser hydroxyl group is at the 'correct' spatial location because Ser was used during the selection process, whereas the more nucleophilic Tyr hydroxyl group is at an 'incorrect' spatial location. Therefore, both Ser and Tyr can react in some balance as dictated by the presently unknown details of deoxyribozyme structure. The balance for SerB1 favors Ser over Tyr by a factor of 12, whereas the balance for SerB2–SerB4 favors Ser over Tyr by only a factor of 2. In contrast, the previously reported Tyr1 and new TyrB1 deoxyribozymes are highly (at least 350- and 600-fold) selective for Tyr over Ser, because Tyr is both properly positioned and inherently more reactive than Ser.

The SerB1 and SerB2 deoxyribozymes were further investigated to determine their positional selectivities when presented with substrates that have more than one competing Ser side chain. A series of DNA–peptide–DNA substrates was

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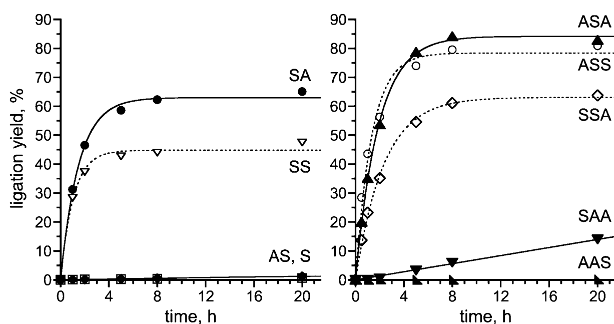

Fig. 3 Side chain positional selectivity for serine hydroxyl groups by the SerB2 deoxyribozyme. Each plot is labeled with the identity of the amino acid(s) located at the 3HJ intersection. Data for SerB1 were comparable (Fig. S9 (ESI†), which also shows data for tetrapeptide substrates). SerB2 is illustrated here because of its lesser selectivity between Ser and Tyr with the Ala-Xaa-Ala substrates (Fig. 2), thereby presenting a greater challenge for discriminating between serine side chains at different positions within the same substrate.

synthesized with various combinations of Ser (S) and Ala (A) residues. The peptide region was either a single amino acid (S); a dipeptide (AS, SA, or SS); a tripeptide (SAA, the parent ASA, AAS, SSA, or ASS); or a tetrapeptide (ASAA, AASA, or ASSA, each of which has S at either or both of the two middle positions). SerB1 and SerB2 showed similar positional selectivity profiles (Fig. 3 and Fig. S9, ESI†). Overall, for each deoxyribozyme, the reactivity depended on the length of the peptide region as well as the serine position(s). Including a single Ser residue led to only 0.7% ligation yield in 20 h, whereas certain dipeptide, tripeptide, and tetrapeptide sequences led to high yields. Each deoxyribozyme was able to discriminate between different peptide sequences that have the same length but different Ser positions. For the dipeptide substrates (Fig. 3), only SA and SS reacted well whereas AS reacted extremely poorly, demonstrating that DNA catalysts can distinguish between two spatially adjacent Ser side chains. Similarly, for the tripeptide substrates, substantial discrimination was observed (Fig. 3). The ASA substrate was utilized much better than the SAA substrate, whereas AAS was essentially unreactive. The tripeptide substrates with two adjacent Ser residues, SSA and ASS, each had high ligation rates and yields, consistent with reaction at either of the first two Ser residues and also consistent with no suppression of reactivity when Ser is located at the third position. Finally, the tetrapeptide substrates AASA and ASSA were used rather well whereas ASAA was used more poorly (Fig. S9, ESI†), showing tolerance of a peptide region larger than that present during the selection process. Future work will continue efforts to evaluate DNA catalysis using larger substrate peptide regions.

Deoxyribozymes initially identified from N_{40} random pools typically have suboptimal catalytic activity, because only 10^{-10} of sequence space is explored (*i.e.*, 10^{14} sequences out of $4^{40} \approx 10^{24}$ possibilities).[3] Therefore, the TyrB1, SerB1, and SerB2 deoxyribozymes were each partially randomized and subjected to reselection with the corresponding tripeptide-containing substrate. In each case, the resulting deoxyribozymes had comparable reactivity and selectivity characteristics as the corresponding parent sequence (data not shown), indicating that reselection did not change the intrinsic properties of these DNA catalysts.

In summary, we have shown for the first time that DNA can catalyze high-yield covalent modification reactions involving the serine side chain. Our previous efforts revealed DNA-catalyzed tyrosine reactivity, whereas serine proved refractory to catalysis.[5] Here, by relaxing the structural preorganization *via* placing a larger peptide region within the 3HJ architecture (Fig. 1), we obtained robust DNA-catalyzed reactivity of the much less reactive Ser side chain (Fig. 2). We also showed that deoxyribozymes can discriminate between Ser and Tyr when presented at the same amino acid position (Fig. 2), and we showed that deoxyribozymes can distinguish between multiple Ser side chains that are located close to each other in space (Fig. 3). The selectivities are incomplete, providing challenges that are being addressed in ongoing experiments by dispensing with the 3HJ architecture. These efforts are also important for pursuing DNA-catalyzed reactions of large protein substrates.

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Notes and references

‡ The previously identified Tyr1 deoxyribozyme,[5] which was selected with a substrate that contained tyrosine as the sole amino acid, gave only trace (∼0.7%) activity when tested with the Ala-Tyr-Ala tripeptide substrate.

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