G-Quadruplexes

International Edition: DOI: 10.1002/anie.201708964
German Edition: DOI: 10.1002/ange.201708964

A Thermophilic Tetramolecular G-Quadruplex/Hemin DNAzyme

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Abstract: *The quadruplex-based DNAzyme system is one of the most useful artificial enzymes or catalysts; their unique properties make them reliable alternatives to proteins for performing catalytic transformation. The first prototype of a thermally stable DNAzyme system is presented. This thermophilic DNAzyme is capable of oxidizing substrates at high temperatures (up to 95 °C) and long reaction times (up to 18 h at 75 °C). The catalytic activity of the DNAzymes were investigated with the standard peroxidase-mimicking oxidation of 2,2′-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid) (ABTS) by H_2O_2. The step-by-step design of this unique heat-activated G-quadruplex/hemin catalyst, including the modification of adenines at both ends of G-tracts, the choice of cation, and its concentration for DNAzyme stabilization, is described. This work investigates thoroughly the molecular basis of these catalytic properties and provides an example of an industrially relevant application.*

G-quadruplex-DNA/hemin complexes, usually referred to as a class of DNAzymes, display interesting catalytic properties that notably mimic horseradish peroxidase (HRP).[1,2] Compared to the catalytic efficiency of natural enzymes under routine experimental conditions (for example, ambient temperature),[3–10] DNAzymes possess practical advantages.[6–8,11,12] They can thus be promising candidates for practical applications,[12–15] but their relatively low catalytic proficiency must be addressed. To this end, various strategies have been proposed including modification of DNA sequences,[8,10,16] fine-tuning of the experimental conditions,[17] and addition of catalytic enhancers.[7,9,18] However, the improvements, often modest, occur at the expense of the simplicity of the DNAzyme protocols and can be further optimized. Notably, the thermal stability, as one of the general advantages of artificial enzyme mimics, has not yet received enough attention, which provides room for improvement and innovation.

Natural proteinaceous peroxidases possess high catalytic activity but are sensitive to thermal denaturation.[19,20] Although different nanomaterials have been developed to catalyze peroxidase-type reactions at elevated temperatures, the improvements of catalytic activity and thermal stability did not meet initial expectations.[19,20] In this study, a thermally stable and highly competent DNAzyme, so called thermophilic DNAzyme, with efficient peroxidase activity at high temperature is reported through a series of model reactions and the rapid oxidation of hazardous organic pollutants (Figure 1). Although tetramolecular G-quadruplexes are kinetically inert and highly thermally stable once formed,[21,22] and have been used for cation detection,[23] the catalytic activity of tetramolecular G-quadruplex/hemin complexes has not attracted much attention. Herein, we chose them to systematically evaluate the thermal stability and the effects of flanking bases on the catalytic activity and heat-resistant property of hemin-mediated catalytic competences.

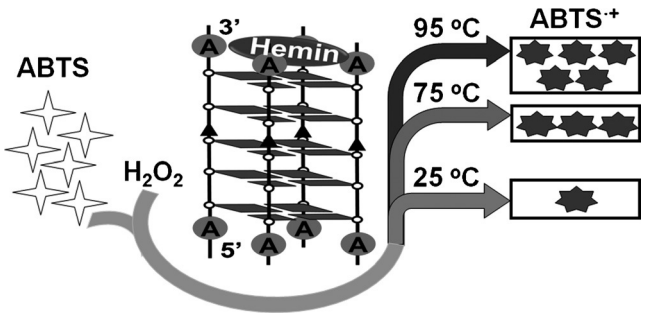

Figure 1. Representation of the catalytic activity of thermophilic tetramolecular G-quadruplex/hemin DNAzyme at different temperatures.

Short G-rich sequences, containing four, five, and six successive guanines, were named as G4 (dGGGG), G5 (dGGGGG), and G6 (dGGGGGG), respectively. To study the effect of flanking bases on the performance of formed complexes, adenine (dA), cytosine (dC), and thymine (dT) were added at the 5′ and/or 3′ end of a tetramolecular G-quadruplex structures (Supporting Information, Table S1). Firstly, we verified by circular dichroism (CD) analyses that all sequences adopted the so-called parallel G-quadruplex (or type I) topology as shown by positive CD signals around 264 nm and negative peaks at 243 nm (Supporting Information, Figure S1).[8,24,25] Next, the catalytic activities of the resulting DNAzymes were investigated, via the standard peroxidase-mimicking oxidation of 2,2′-azino-bis(3-ethylbenzothiozoline-6-sulfonic acid) (ABTS) by H_2O_2 in the presence of hemin, which can be conveniently monitored since it generates a green-colored oxidized product $ABTS^{\cdot+}$ with

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a characteristic absorbance maximum around 420 nm.[9,26] We therefore assessed the catalytic efficiency of the DNAzyme systems through the measurement of absorbance at 420 nm as a function of time (Supporting Information, Figure S2), from which the initial velocity V_0, defined as the concentration of $ABTS^{\bullet+}$ generated as a function of time (V_0 expressed in $nM\,s^{-1}$; of note, data were subtracted from data obtained without pre-catalyst),[26] could be determined to quantify the catalytic activity of DNAzymes.

G4, G5, and G6 without flanking nucleotides exhibited very low peroxidase activity (less than 20 $nM\,s^{-1}$; Figure 2).

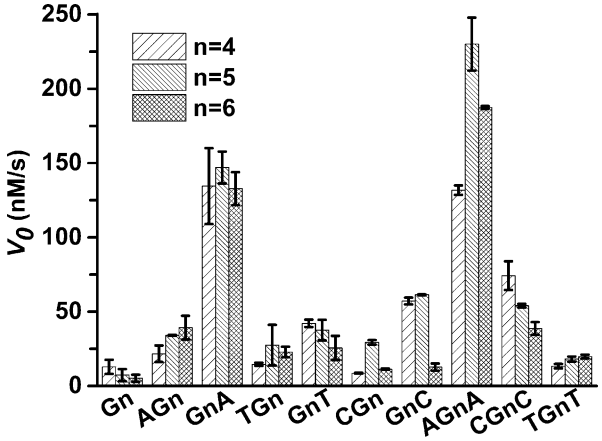

Figure 2. V_0 values of DNAzymes formed by four, five, and six G-tetrads with dA, dT, or dC at either or both ends.

The presence of dA at either 3′ end or both ends of G4 (that is, G4A, dGGGGA, and AG4A, dAGGGGA) resulted in a >10-fold increase in catalytic activity compared to G4. G5A and AG5A had 20- and 31-fold higher activities than G5, respectively, and G6A and AG6A were 25- and 36-fold more active than G6, respectively. Obviously, the increase efficiency of catalytic activity depended on the number of G-tetrads. When dA was introduced at the 5′ ends of these sequences (AG4, AG5, and AG6), the activity enhancements were much lower than those modified at 3′ end, indicating that the dA effect is specific for the 3′ end. The 10- to 30-fold enhancement obtained here was higher than the enhancement factor of approximately 5 in previous report,[10] which could be attributed to the higher number of G-tetrads and the concomitant presence of four dAs on the 3′ end of tetramolecular G-quadruplexes vs. single dA in the intramolecular form. Of note, the presence of either dC or dT on the 3′ end or at both ends of G4, G5, and G6 improved peroxidase activities to a much lower extent than dA, highlighting the specificity of dA as a catalytic activator. Interestingly, the base spacer (one or two dTs or dCs) inserted between dA and G-quartet core of G5A was detrimental to the peroxidase activity. Moreover, 2-aminopurine (2-AP) modified G5 series did not exhibit altered peroxidase activities, except a slight increase for that with 2-AP at the 5′ end of G5 (Supporting Information, Figure S3).

The enhanced catalytic activity owing to the presence of dA at 3′ end of G-quadruplex sequences was further verified through analyses of oxidation of two additional substrates, 3,3′,5,5′-tetramethylbenzidine (TMB)[27] and β-nicotinamide adenine dinucleotide (NADH)[3] by H_2O_2, respectively (Supporting Information, Figures S4 and S5). Thus, this phenomenon can be considered as a common function to improve the catalytic activity of DNAzymes.

To gain more insights into the hemin binding mode, the stoichiometries of hemin/G-quadruplex associations were assessed using the continuous variation analysis (Job plots) method[28–30] with two series of G-quadruplexes, one catalytically competent (AG4A, AG5A, and AG6A), the other incompetent (TG4T, TG5T, and TG6T). For the same number of G-tetrads no significant difference in hemin binding stoichiometry (ca. 1 privileged hemin binding site per quadruplex) was found (Supporting Information, Figure S6 and Table S2), implying that the difference in the catalytic activities of AGnA and TGnT series did not result from the stoichiometry of the hemin fixation per se.

The fluorescence emission spectra of AG5A labeled with 6-carboxyfluorescein (FAM) at the 5′ or the 3′ end (Supporting Information, Table S1) in the presence and absence of hemin were further used to characterized the binding site of G-quadruplex with hemin. The 3′ end labeled AG5A showed greater fluorescence quenching than 5′ end labelled one upon addition of hemin (Supporting Information, Figure S7), indicating that the 3′ end of the G-quadruplex was the preferred hemin binding site.

To further evaluate the catalytic activity of AGnA DNAzymes, apparent activation energies (E_a) of G5, AG5A, CG5C, and TG5T DNAzyme were calculated based on the Arrhenius equation. The E_a of ABTS oxidation using AG5A as pre-catalyst was comparable to the E_a values of CG5C and TG5T (25.90, 25.33, and 28.37 $kJ\,mol^{-1}$, respectively; Supporting Information, Figure S8), implying that these three pre-catalysts provide comparable G-tetrad interaction interface for hemin.[31] However, the activation energy of the oxidation performed with G5 was significantly higher ($E_a = 43.60\ kJ\,mol^{-1}$), which might result from the formation of higher-order G-quadruplex structures (also termed quadruplex multimerization) through end-to-end stacking as demonstrated previously by NMR[32,33] and polyacrylamide gel electrophoresis (PAGE) analyses.[34,35] The PAGE analysis showed similar result (Supporting Information, Figure S9), implying that the quadruplex multimerization could prevent proper hemin binding. More significantly, the pre-exponential factor, defined as the *y*-intercept of the Arrhenius plot, for AG5A was higher than those for CG5C and TG5T DNAzymes (5.47 s^{-1} versus 1.33 and 0.73 s^{-1}, respectively). This suggested that the better performance of AG5A DNAzymes was mainly due to the influence of the flanking nucleotides (dA vs. dC or dT) on hemin fixation and activation,[36] further verified by analysis of the catalysis pH dependencies (Supporting Information, Figure S10).

We next sought to determine how dA facilitates the oxidation process. From a mechanistic point of view, the DNAzyme-promoted oxidation of ABTS can be divided into three main steps (Figure 3 A).[9] First, the hemin (or porph·Fe^{III}) readily reacts with H_2O_2 to form the highly reactive radical cation porph·Fe^{IV}=$O^{\bullet+}$ (Compound 1), which then

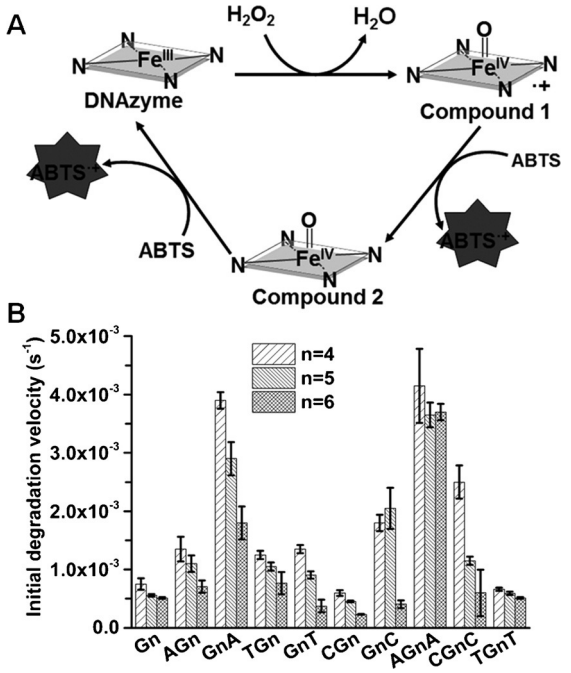

Figure 3. A) Proposed peroxidation cycle facilitated by G-quadruplex/hemin DNAzyme for oxidation of ABTS by H_2O_2. B) Initial degradation velocity of DNAzymes when exposed to H_2O_2.

withdraws one electron from ABTS to form the more stable ferryl-oxo porph·Fe^{IV}=O intermediate (Compound 2). This intermediate finally withdraws one electron from another ABTS molecule to provide the initial porph·Fe^{III} cofactor. Upon addition of H_2O_2, the dynamics of the absorbance change at 404 nm[8,37,38] demonstrated a fast degradation tendency within five minutes (Supporting Information, Figure S11), while G4, G5, and G6 DNAzyme were characterized by the low degradation rates (Figure 3B). Interestingly, G4A and AG4A DNAzyme showed similar degradation rates, which were higher than those of G4 modified at 5′ and/or 3′ ends with dT or dC. Similar trends were also observed in G5 and G6 DNAzyme series. Moreover, the G-quadruplex/hemin complexes with higher degradation rates had higher peroxidase activities (Figure 2 and Figure 3B). These results indicated that 3′ dA-mediated activation was possibly due to the accelerated formation of Compound 1-like intermediate, and was further verified with time-dependent adsorption spectra (Supporting Information, Figure S12).

The effects of cations such as Na^+, NH_4^+, and Pb^{2+} on the catalytic activity of resulting DNAzymes were also examined. Satisfyingly, the constructed tetramolecular G-quadruplex/hemin DNAzymes were applicable to different monovalent or bivalent cation solutions (Supporting Information, Figure S13), which lays a foundation for further applications. High concentration of potassium, which significantly stabilizes G-quadruplexes at high temperature,[21,22] was detrimental to the catalytic activity of DNAzymes (Supporting Information, Figure S14), for high K^+-contents promoted hemin deactivation through aggregation.[39] Of note, different cations may affect the stacking of tetramolecular G-quadruplex.[33] Fortunately, elevated ammonium concentration (up to 150 mM) did not inhibit the DNAzyme activity (Supporting Information, Figure S15). Therefore, the thermally stable DNAzymes was obtained in Tris-HCl buffer containing 100 mM NH_4^+. Under these conditions, AG5A had a melting temperature above 95 °C and CD experiments confirmed that the topology of the G-quadruplex was maintained for 24 h at 75 °C and for 4.5 h at 95 °C (Supporting Information, Figure S16).

The H_2O_2-promoted peroxidase activity of AG5A/hemin system exhibited high catalytic performance over a broad temperature range from 25 °C to 95 °C in 100 mM NH_4^+. The V_0 value at 25 °C was 360 nM s^{-1}. With increasing temperatures, the V_0 value for the oxidation reaction of ABTS by H_2O_2 increased and reached 1956 nM s^{-1} at 95 °C (Figures 4A and B). By contrast, at the same strand concentration, the V_0 value of AG3TA DNAzyme increased from 481 nM s^{-1} at 25 °C to 1383 nM s^{-1} at 75 °C, but decreased to 1110 nM s^{-1} at 95 °C (Figure 4B) due to the unfolding of AG3TA (T_m = 74 °C; Supporting Information, Figure S16A). The V_0 values of HRP progressively decreased at temperatures above 55 °C due to heat-induced deactivation of the protein (Supporting Information, Figure S17).

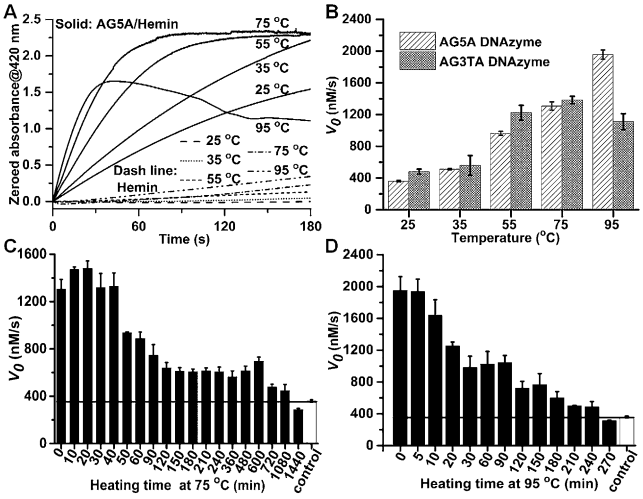

Figure 4. A) Plots of absorbance at 420 nm versus reaction time for AG5A/hemin complex and hemin and B) V_0 values for AG5A and AG3TA DNAzymes at different temperatures. V_0 values of AG5A DNAzyme after heated at C) 75 °C and D) 95 °C for indicated times and then added with 1.28 mM ABTS and 1.28 mM H_2O_2. Control is AG5A DNAzyme at 25 °C.

To verify the utility of thermophilic DNAzymes, we analyzed the DNAzyme-catalyzed reactions with TMB and NADH as substrates. The AG5A/hemin-complex-catalyzed oxidation of TMB by H_2O_2 was also dependent on temperature but showed the most efficient conversion at 55 °C (Supporting Information, Figures S18A,B). This phenomenon was ascribed to the fast formation of the second TMB oxidation product (diimine), the formation of which can be monitored at 450 nm.[9] The diimine product increased with the elevating temperature (Supporting Information, Figures S18C,D). Comparable increase of catalytic activity of AG5A/hemin complex upon elevating temperature was

observed with NADH (Supporting Information, Figure S19). These results highlighted the efficiency of the AG5A/hemin complex as a thermophilic DNAzyme system.

We further investigated the robustness of this prototype of thermophilic DNAzyme over a long reaction time. The AG5A-based catalyst was fully active for 40 min at 75 °C (Figure 4C). After 40 min, the catalytic activity progressively decreased, but the DNAzyme was still 53 % active after 10 h and 34 % active after 18 h. When the reaction was performed at 95 °C, the activity of AG5A DNAzyme began to decrease after 5 min. It was 54 % active after 1.5 h and 25 % active after 4 h (Figure 4D). Of note, after 18 h at 75 °C or 4 h at 95 °C, the enzymatic activity of DNAzyme was similar to that obtained at 25 °C, confirming its excellent thermophilic properties. The decline of catalytic activity might be due to the adverse effect of high temperature on π–π stacking interactions between hemin and G-quadruplexes (Supporting Information, Figure S20). In contrast, the activity of HRP was almost completely lost after heating at 75 °C for 2 min (Supporting Information, Figure S21A), and the activity of AG3TA DNAzyme fell to 23 % activity after 30 min at 95 °C (Supporting Information, Figure S21B). These results demonstrated that tetramolecular G-quadruplex DNAzymes display unique thermodynamic stability.

At temperatures lower than 75 °C, the degradation of $ABTS^{\bullet+}$ was not observed within 3 min, while the reaction temperature of 95 °C showed the intermediate degradation after 30 s (Figure 4A). So, adenosine triphosphate (ATP) was added to stabilize the $ABTS^{\bullet+}$ radical.[9] The $ABTS^{\bullet+}$ could be stabilized by 1.00 mM ATP at 95 °C (Supporting Information, Figure S22). Moreover, at the initial stage of the reaction, no obvious difference of absorbance in the presence of ATP up to 0.75 mM was observed (Supporting Information, Figure S22). Thus above V_0 values for ABTS-H_2O_2 reaction were reliable. Interestingly, similarly to a previous report,[40] the presence of 10 % DMF, 20 % formamide, or 30 % methanol did not obviously affect the catalytic activity of these tetramolecular G-quadruplex/hemin DNAzymes, even at high temperatures (Supporting Information, Figure S23).

DNA-based catalyst has been used for industrially relevant transformation of dibenzothiophene as a way to remove sulfur-containing compounds from petroleum feedstocks.[40] Here we chose methylene blue (MB), which is often found in contaminated groundwater and wastewater of textile and paper industries[41–43] and currently removed with Fenton-like reaction,[44] as an example to assess the practical application of the AG5A/hemin-based thermophilic DNAzyme system in the removal of toxic pollutants. As shown in Figure 5, the efficient MB oxidation occurred between 55 and 75 °C. The lower efficiency at 95 °C might be due to the decomposition of H_2O_2 for 10 min incubation rather than loss of DNAzyme efficacy. The removal efficiency could reach 94.3 % after 10 min at 75 °C when 1.32 μM AG5A/hemin DNAzyme was added in this system. This efficiency made the thermophilic DNAzyme fully competitive with Fenton-like and *Vitreoscilla* haemoglobin (VHb)-promoted reactions.[44,45]

In summary, this work demonstrates that adenine-capped tetramolecular G-quadruplexes have excellent HRP-like oxidizing activities that, when combined with their very high thermal stability, provides the first prototype of thermophilic DNAzyme. The results presented herein are promising but represent admittedly the first step towards larger-scale industrial applications of quadruplex-based catalysts. Many more examples of catalytic chemical transformations are now eagerly awaited, for both destructive (for example, decontamination) and constructive (for example, active compounds or intermediate synthesis) purposes. Thus deeper insights must also be gained into critical parameters that will represent the go/no-go decision for using oligonucleotide-based catalysts under real industrial constraints, notably in terms of cost, robustness, and recyclability. These results provide solid basis for launching research programs in quadruplex-based biotechnology.

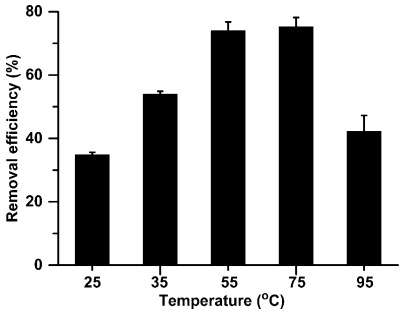

Figure 5. Oxidation removal efficiency of methylene blue, as measured by decolorization, by AG5A/hemin DNAzyme over a range of temperature in the presence of 0.86 μM AG5A, 1.72 μM hemin, 1.28 mM H_2O_2, and 10 mg L^{-1} MB.

Acknowledgements

We gratefully acknowledge J.-L. Mergny (France) for useful discussion, and the support from National Natural Science Foundation of China (21503229, 21635005, and 21361162002), Fundamental Research Funds for Central Universities (No. 020514380070, 020514380085, 020514380105), State Key Laboratory of Analytical Chemistry for Life Science (5431ZZXM 1711) and Program B for Outstanding Ph.D. Candidate of Nanjing University. D.M. thanks the Université de Bourgogne & Conseil Régional de Bourgogne (PARI) and the European Union (PO FEDER-FSE Bourgogne 2014/2020).

Conflict of interest

The authors declare no conflict of interest.

Keywords: DNAzyme · G-quadruplexes · hemin · peroxidase activity · thermophilicity

How to cite: *Angew. Chem. Int. Ed.* **2017**, *56*, 16636–16640
Angew. Chem. **2017**, *129*, 16863–16867

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Manuscript received: August 30, 2017
Revised manuscript received: October 24, 2017
Accepted manuscript online: November 6, 2017
Version of record online: November 30, 2017

