

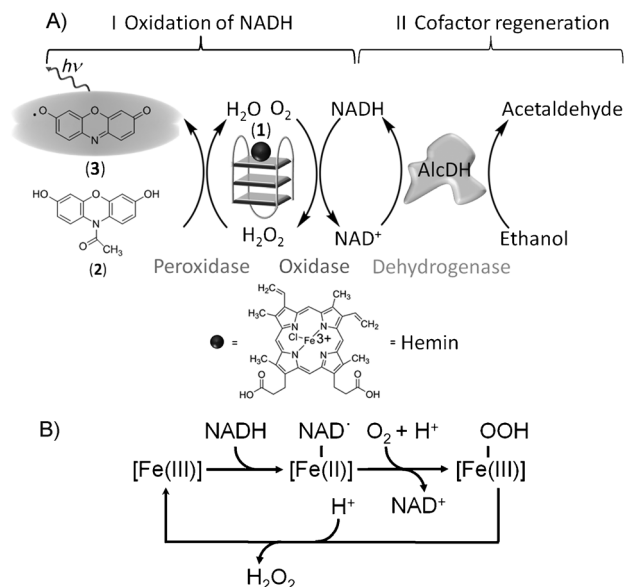
DNAzymes

A Hemin/G-Quadruplex Acts as an NADH Oxidase and NADH Peroxidase Mimicking DNAzyme**

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Appropriately ordered guanosine bases in DNA chains form G-quartets stabilized by interbase hydrogen bonds. These G-quartets are further stacked, in the presence of ions, into a G-quadruplex structure.^[1] Different organic molecules are known to interact with G-quadruplex structures.^[2] Specifically, hemin was found to bind to a G-quadruplex, and the resulting complex revealed horseradish peroxidase mimicking activities.^[3] For example, the hemin/G-quadruplex catalyzes the H₂O₂-mediated oxidation of the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate dianion) (ABTS²⁻) to the colored product ABTS^{•-}^[3] or leads to the generation of chemiluminescence^[4] in the presence of H₂O₂/luminol. Also, the hemin/G-quadruplex structure linked to electrodes was reported to act as an electrocatalyst for the reduction of H₂O₂,^[5] and the hemin/G-quadruplex associated with semiconductor quantum dots was found to quench the luminescence of the quantum dots.^[6] These properties enabled the use of the hemin/G-quadruplex as a versatile label for numerous sensors including enzyme-,^[7] DNA-,^[8] and aptamer-based^[9] sensors. In the present study, we demonstrate that the hemin/G-quadruplex acts not only as a horseradish peroxidase mimicking DNAzyme, but, also, as an NADH oxidase and NADH peroxidase mimicking DNAzyme. The regeneration of the nicotinamide adenine dinucleotide, NAD⁺, cofactor attracted substantial interest for biotechnological applications, and NAD⁺-dependent enzymes have been widely used for chemical transformations.^[10] Our results pave the way to use the hemin/G-quadruplex as a biocatalyst for the regeneration of the NAD⁺ cofactor and to apply the DNAzyme as a catalyst for enzyme-driven transformations.

NADH oxidase catalyzes the oxidation of NADH by O₂ with the concomitant formation of H₂O₂.^[11] The present system consists of reduced nicotinamide adenine dinucleotide (NADH), hemin/G-quadruplex (1), and Amplex Red (2), as a fluorescent reporter dye (Scheme 1A). Under aerobic conditions, NADH is oxidized to NAD⁺ (see below), while 2 is oxidized to resorufin (3), which generates fluorescence at λ_{max} = 581 nm. Figure 1A, curve (a), shows the time-dependent increase in the fluorescence generated by the system. Control experiments reveal minor background fluorescence generated under an inert argon atmosphere (curve (e)), or



Scheme 1. A) Frame I: Hemin/G-quadruplex-catalyzed oxidation of NADH by O₂ to NAD⁺ and H₂O₂, respectively, and the concomitant oxidation of Amplex Red (2) to resorufin (3) by H₂O₂. Frame II: Coupled DNAzyme–alcohol dehydrogenase biocatalytic system for the formation of NADH and the concomitant regeneration of the NAD⁺ cofactor by the hemin/G-quadruplex. B) Suggested mechanism for the DNAzyme-catalyzed oxidation of NADH.

upon exclusion of hemin from the system (curve (f)). Also, only a low fluorescence intensity is generated in the presence of only hemin and in the absence of 1 (curve (d)), or in the presence of hemin and a foreign nucleic acid (4) that cannot form the respective G-quadruplex (curve (c)). These results may suggest that the hemin/G-quadruplex-catalyzed oxidation of NADH by O₂ yields H₂O₂, and the generated fluorescence occurs by the well-established hemin/G-quadruplex DNAzyme-catalyzed oxidation of 2 by H₂O₂.^[12] Indeed, the intermediate formation of H₂O₂ in the system was confirmed by the addition of catalase. Figure 1A, curve (g), confirms that in the presence of catalase the fluorescence is blocked, consistent with the decomposition of the generated H₂O₂. Thus, in the first step, the hemin/G-quadruplex DNAzyme acts as an NADH oxidase, where NADH is oxidized by O₂ to form NAD⁺. It should be noted that the fluorescent resorufin (3) is formed also in the absence of NADH (Figure 1A, curve (b)). Presumably, Amplex Red by itself has a residual donor activity that participates in the activation of O₂ (substituting NADH). The low fluorescence intensity changes should be considered as the background signal of the system.

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[**] This study was supported in part by the Israel Science Foundation and the Volkswagen Foundation (Germany).

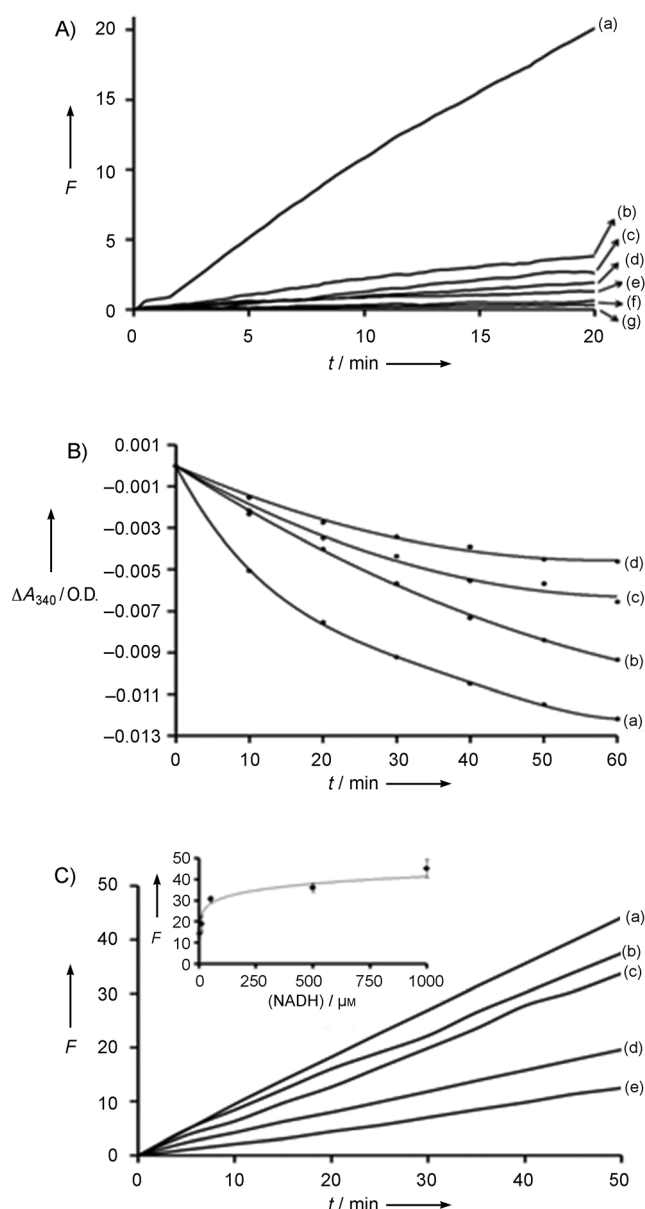


Figure 1. A) Time-dependent fluorescence changes as a result of the catalyzed oxidation of Amplex Red (**2**) to resorufin (**3**) with: a) hemin/G-quadruplex and 1 mM NADH, b) hemin/G-quadruplex, no NADH, c) foreign DNA (**4**) in the presence of hemin and NADH, d) only hemin in the presence of NADH, e) hemin/G-quadruplex under an inert atmosphere, in the presence of NADH, f) in the presence of G-quadruplex sequence (**1**) and NADH, but in the absence of hemin, g) catalase, 38 U, was added to the system described in (a). B) Time-dependent absorbance changes at 340 nm as a result of the catalyzed oxidation of NADH by the hemin/G-quadruplex DNAzyme in the presence of catalase (38 U) and various concentrations of NADH: a) 1 mM, b) 0.5 mM, c) 0.1 mM, d) 0.01 mM. C) Time-dependent fluorescence changes as a result of the catalyzed oxidation of Amplex Red by the hemin/G-quadruplex DNAzyme in the presence of various concentrations of NADH: a) 1 mM, b) 0.5 mM, c) 0.05 mM, d) 0.01 mM, e) 0 mM. Inset: Derived calibration curve corresponding to the fluorescence changes of **3** after 50 min, induced by the hemin/G-quadruplex and NADH. All experiments were prepared in a HEPES buffer solution, 5 mM, pH 7.2 in the presence of NH_4OAc , 300 mM, hemin, 1 μM , the G-quadruplex sequence **1** (or foreign DNA (**4**)), 1 μM , and Amplex Red (**2**), 100 μM (in Figure 1 B, **2** was not added).

The generation of the fluorescence of **3** is blocked upon the addition of catalase, due to the decomposition of H_2O_2 (Figure 1 A, curve (g)). Nonetheless, the primary process of oxidation of NADH to NAD^+ should still proceed in the presence of catalase. Indeed, this process was characterized spectroscopically, by following the depletion of NADH (Figure 1 B). As the concentration of NADH increases the DNAzyme-catalyzed oxidation of NADH is enhanced. It should be noted that the hemin/G-quadruplex-catalyzed oxidation of NADH proceeds, also, in the absence of Amplex Red (**2**), while forming H_2O_2 (cf. Figure 1 B), implying that the oxidation of NADH by O_2 is independent of Amplex Red. From the time-dependent depletion of NADH at different concentrations of this cofactor, the values of $k_{\text{cat}} = (1.1 \pm 0.06) \times 10^{-4} \text{ s}^{-1}$ and $K_m = (5.7 \pm 0.3) \times 10^{-5} \text{ M}$ for the hemin/G-quadruplex NADH oxidase were calculated. For comparison, the flavin adenine dinucleotide (FAD) dependent NADH oxidase exhibits $k_{\text{cat}} = 5.1 \text{ s}^{-1}$ and $K_m = 4.1 \times 10^{-6} \text{ M}$.^[11] Similarly, the fluorescence intensities of **3** are controlled by the concentrations of NADH in the system (Figure 1 C). As the concentration of NADH increases, the time-dependent fluorescence intensities of **3** are intensified. Figure 1 C, inset, depicts the resulting calibration curve, which demonstrates that at a concentration of NADH corresponding to $1 \times 10^{-4} \text{ M}$ the fluorescence changes of the catalytic process reach a saturation value. Since the mechanism of the hemin-catalyzed reductive activation of O_2 to H_2O_2 was extensively studied,^[13] we suggest the following sequence of reactions, where NADH is the electron source, as the possible route for the formation of H_2O_2 (Scheme 1 B). In this context it should be noted that horseradish peroxidase, exhibiting NADH oxidase functions,^[14] requires the ligation of the fifth axial histidine ligand to generate the active site. Presumably, the guanosine units in the G-quadruplex acts as a fifth activating ligand of the hemin to generate the NADH oxidase activity.

The regeneration of the NAD^+ cofactor is a fundamental process utilizing NAD^+ -dependent enzymes and NAD^+ -dependent enzyme-driven biotransformations.^[15] Different regeneration schemes that use secondary enzymes,^[16] electrochemical processes,^[17] or photochemical methods^[18] have been used to regenerate the NAD^+ cofactor. One basic limitation of this process involves the oxidation of the NADH to the NAD^\bullet radical, which dimerizes into the biologically inactive substance $(\text{NAD}^\bullet)_2$. Thus, for demonstrating the ability of the hemin/G-quadruplex DNAzyme as a catalyst for biotechnological applications it is essential to prove the conversion of NADH into NAD^+ , and the regeneration of the cofactor within an enzyme-driven process. Toward this end, the hemin/G-quadruplex DNAzyme system was coupled to the alcohol dehydrogenase (AlcDH) catalyzed oxidation of ethanol (Scheme 1 A, frame II). In this system we place the NAD^+ cofactor as a primary component. The NADH cofactor is generated through the alcohol dehydrogenase mediated reduction of NAD^+ by ethanol, and the resulting NADH cofactor is recycled to NAD^+ by the DNAzyme-catalyzed oxidation of NADH while forming H_2O_2 . The process is, then, monitored by the DNAzyme-catalyzed generation of the fluorescence of **3**. Figure 2 A shows the

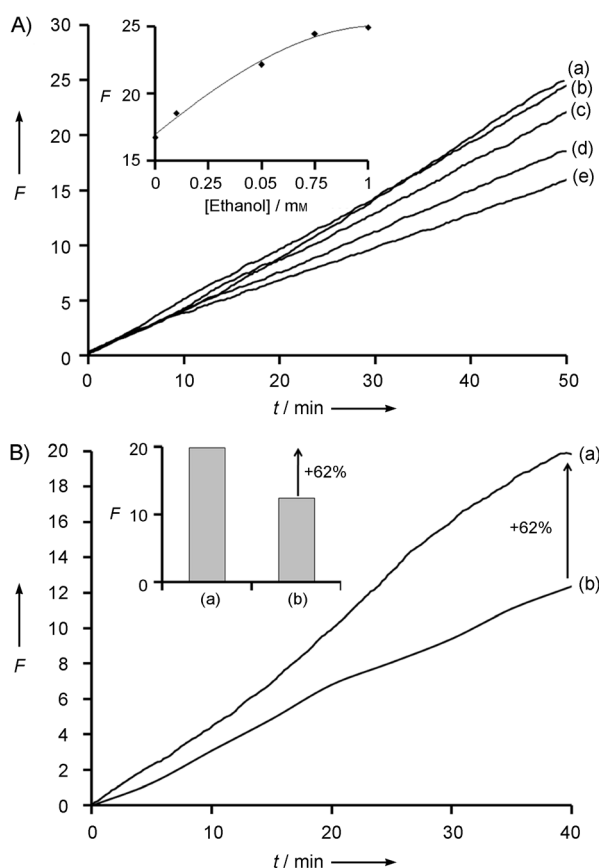
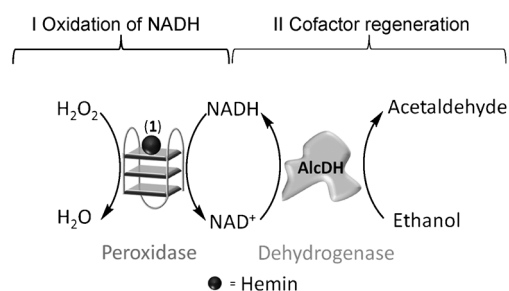


Figure 2. A) Time-dependent fluorescence changes as a result of the catalyzed oxidation of Amplex Red (**2**) to resorufin (**3**) by the hemin/G-quadruplex DNAzyme in the presence of NAD^+ , 1×10^{-3} M, AlcDH (50 U), and various concentrations of ethanol: a) 1 mM, b) 0.75 mM, c) 0.5 mM, d) 0.1 mM, e) 0 mM. Inset: Derived calibration curve corresponding to the fluorescence changes of resorufin after 50 min. B) Time-dependent fluorescence changes as a result of the catalyzed oxidation of Amplex Red (**2**) to resorufin (**3**) by the hemin/G-quadruplex DNAzyme in the presence of a) 50 mM ethanol, NAD^+ , 5×10^{-6} M, and AlcDH (50 U), b) in the presence of NADH, 5×10^{-6} M, AlcDH (50 U), and in the absence of ethanol. The buffer solution and the composition of the components in the system are as outlined in Figure 1.

time-dependent generation of fluorescence at different concentrations of ethanol. As the concentration of ethanol increases, the fluorescence intensities increase as well, consistent with the enhanced formation of the NADH cofactor in the system. From the calibration curve correlating the fluorescence intensities generated by the system at different concentrations of NADH (Figure 1C, inset), one may estimate the concentration of NADH formed by the NAD^+ . For example, the fluorescence intensity generated by the system in the presence of ethanol, 1 mM, after 50 min of reaction translates to a NADH concentration that corresponds to 5×10^{-5} M. However, this value does not confirm that the NAD^+ cofactor was regenerated in the system (since a high initial concentration of NAD^+ , corresponding to 1 mM, was used). To prove that the NADH was, indeed, transformed to NAD^+ , we constructed a system that consisted of the hemin/G-quadruplex, 1×10^{-6} M, ethanol/alcohol dehydrogenase, and

included the NAD^+ cofactor at a low concentration of 5×10^{-6} M. Figure 2B, curve (a) depicts the time-dependent fluorescence changes observed in the system. For comparison, we followed the time-dependent fluorescence changes of the system in the absence of ethanol and AlcDH, but in the presence of the NADH cofactor at a low concentration (5×10^{-6} M; Figure 2B, curve (b)). The resulting fluorescence changes of the system in the presence of ethanol are substantially higher than the molar depletion of the NADH at this concentration, in the absence of ethanol, indicating that the NADH was regenerated by the ethanol/alcohol dehydrogenase. By using the fluorescence intensity generated by the system after 40 min, and applying the calibration curve shown in Figure 1C, inset, we estimate that the fluorescence generated in the presence of the ethanol/alcohol dehydrogenase regenerating system corresponds to the fluorescence intensity changes generated by the DNAzyme in the presence of a NADH concentration of $(3.3 \pm 0.32) \times 10^{-5}$ M. This value is (6 ± 0.3) -fold higher than the added concentration of NAD^+ , implying that the cofactor was regenerated in the system and that the DNAzyme oxidation of NADH yielded the biologically active NAD^+ cofactor.

We further find that the hemin/G-quadruplex acts as a NADH peroxidase under anaerobic conditions, and that the DNAzyme catalyzes the oxidation of NADH by H_2O_2 (Scheme 2). It should be noted that only under anaerobic



Scheme 2. Frame I: The anaerobic hemin/G-quadruplex-catalyzed oxidation of NADH by H_2O_2 to NAD^+ and H_2O , respectively. Frame II: Coupled DNAzyme–alcohol dehydrogenase biocatalytic system for the regeneration of NADH.

conditions, where the NADH oxidase activity is prohibited, can the oxidation of NADH by H_2O_2 as the oxidant proceed. Figure 3A shows the time-dependent absorbance changes of NADH in the presence of H_2O_2 at various concentrations. As the concentration of H_2O_2 increases, the rate of the NADH oxidation is enhanced. Control experiments reveal that no absorbance changes occur in the absence of the H_2O_2 (curve (h)), or that minute absorbance changes are observed in the presence of hemin only or hemin in the presence of a foreign nucleic acid (**4**; curves (g) and (f), respectively). These control experiments clearly demonstrate that the hemin/G-quadruplex DNAzyme acts as the catalyst for the oxidation of NADH and that H_2O_2 is the oxidant that stimulates the process. From these experiments the values of $k_{\text{cat}} = (4.6 \pm 0.2) \times 10^{-3} \text{ s}^{-1}$ and $K_{\text{m}} = (6.0 \pm 0.3) \times 10^{-6} \text{ M}$ were calculated. For comparison, the FAD-dependent NADH peroxidase

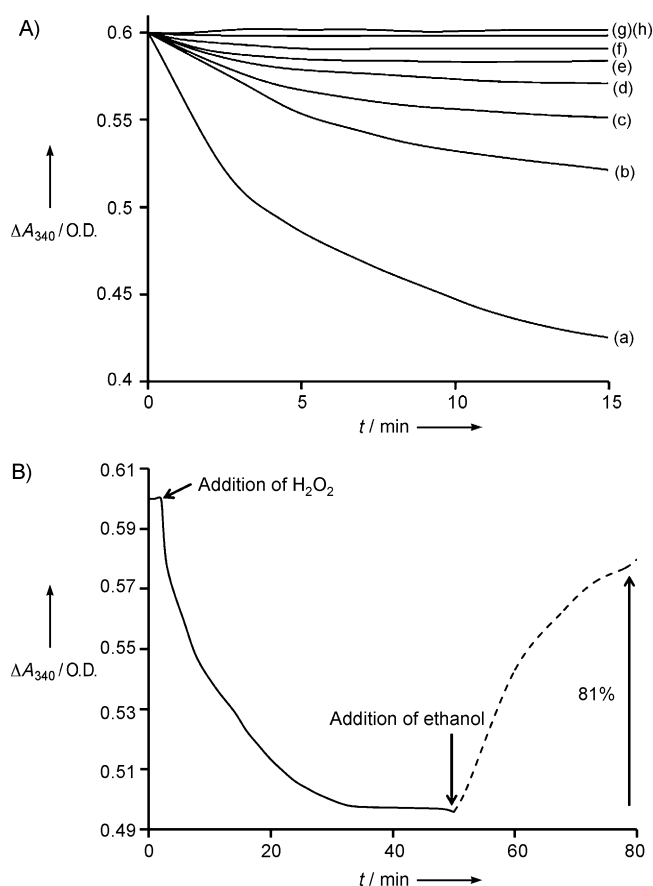


Figure 3. A) Time-dependent absorbance changes at 340 nm as a result of the catalyzed oxidation of NADH, 0.1 mM, by the hemin/G-quadruplex DNAzyme under inert conditions in the presence of various concentrations of H_2O_2 : a) 50 μM , b) 20 μM , c) 10 μM , d) 6 μM , e) 3 μM , f) 20 μM , in the presence of a foreign DNA (4) (g) 20 μM , in the presence of hemin only, (h) no H_2O_2 . B) Time-dependent absorbance changes at 340 nm: Solid line: Absorption changes as a result of the catalyzed oxidation of NADH, 0.1 mM, by the hemin/G-quadruplex DNAzyme in the presence of 20 μM H_2O_2 . Dotted line: Absorption changes as a result of the regeneration of NADH in the presence of 50 U alcohol dehydrogenase and 40 mM ethanol. All experiments were performed in a HEPES buffer solution, 5 mM, pH 7.2, in the presence of NH_4OAc , 300 mM, hemin, 1 μM , and the G-quadruplex sequence 1, 1 μM .

exhibit $k_{cat} = 3.0 s^{-1}$ and $K_m = 1.0 \times 10^{-6} M$.^[19] A final aspect that needed to be confirmed was that the DNAzyme-catalyzed oxidation of NADH by H_2O_2 , indeed, yields the biologically active NAD^+ cofactor that is essential for the application of the process as a NAD^+ regenerating system. Toward this goal, the system consisting of H_2O_2 ($20 \times 10^{-6} M$) and NADH ($1 \times 10^{-4} M$) was allowed to react with hemin/G-quadruplex ($1 \times 10^{-6} M$) for 50 min. The resulting depletion of NADH through the oxidation process (Figure 3B, solid line) reaches a saturation value. The system was, then, treated with catalase to decompose any residual H_2O_2 , followed by the subsequent addition of ethanol and alcohol dehydrogenase. The absorbance of NADH was almost fully restored (Figure 3B, dotted line), indicating that the anaerobic hemin/G-quadruplex-catalyzed oxidation of NADH, indeed, yields the biologically active NAD^+ cofactor.

In conclusion, the present study has introduced new biocatalytic functions of the hemin/G-quadruplex that mimic NADH oxidase and NADH peroxidase activities. These systems pave the way to use the DNAzyme as a NAD^+ regeneration cycle for biocatalytic transformations and to implement the hemin/G-quadruplex as a catalyst for other chemical processes. Our systems complement the recently reported studies by Sen and co-workers^[20] that demonstrated that hemin linked to G-rich RNAs and DNAs catalyze oxygen transfer reactions. All these novel biocatalytic functions of hemin/G-quadruplex suggest that further new processes might be catalyzed by this nanostructure.

Experimental Section

The sequences of the oligonucleotides used in this study are:

(1) 5'-TTTGGGTAGGGCGGGTTGGG-3'

(4) 5'-CGAACTCTGCAACATAAAAA-3'

NADH oxidase: A HEPES buffer solution (5 mM, pH 7.2) containing 300 mM NH_4OAc , 1 μM 1, 1 μM hemin, and 100 μM Amplex Red was prepared and incubated at 25 °C for 30 min. To initiate the oxidation process, different concentrations of NADH were added.

NADH oxidase–alcohol dehydrogenase: A HEPES buffer solution (5 mM, pH 7.2) containing 300 mM NH_4OAc , 1 μM 1, 1 μM hemin, 50 U AlcDH, and 100 μM Amplex Red was prepared and incubated at 25 °C for 30 min. To initiate the oxidation process, either NADH or NAD^+ were added, along with the appropriate amount of ethanol.

NADH peroxidase: A HEPES buffer solution (5 mM, pH 7.2) containing 300 mM NH_4OAc , 1 μM 1, and 1 μM hemin was prepared in a quartz cuvette. The solution was treated for 30 min with argon and incubated at 25 °C for an additional 30 min, followed by the addition of NADH (0.1 mM) and H_2O_2 (under argon).

NADH peroxidase–alcohol dehydrogenase: A HEPES buffer solution (5 mM, pH 7.2) containing 300 mM NH_4OAc , 1 μM 1, and 1 μM hemin was prepared in a quartz cuvette. The solution was treated for 30 min with argon and incubated at 25 °C for an additional 30 min, followed by the addition of NADH (0.1 mM) and H_2O_2 (20 μM), under argon. The solution was allowed to react for 50 min; afterwards, catalase (38 U), AlcDH (50 U), and ethanol were added to initiate the regeneration of NADH.

Received: June 7, 2011

Revised: July 26, 2011

Published online: October 6, 2011

Keywords: DNAzymes · DNA structures · enzymes · G-quadruplexes

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