#### 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 Gquartets are further stacked, in the presence of ions, into a Gquadruplex structure.<sup>[1]</sup> Different organic molecules are known to interact with G-quadruplex structures.<sup>[2]</sup> Specifically, hemin was found to bind to a G-quadruplex, and the resulting complex revealed horseradish peroxidise mimicking activities.<sup>[3]</sup> For example, the hemin/G-quadruplex catalyzes the H<sub>2</sub>O<sub>2</sub>-mediated oxidation of the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate dianion) (ABTS<sup>2-</sup>) to the colored product ABTS<sup>-[3]</sup> or leads to the generation of chemiluminescence<sup>[4]</sup> in the presence of H<sub>2</sub>O<sub>2</sub>/luminol. Also, the hemin/ G-quadruplex structure linked to electrodes was reported to act as an electrocatalyst for the reduction of  $H_2O_2$ <sup>[5]</sup> and the hemin/G-quadruplex associated with semiconductor quantum dots was found to quench the luminescence of the quantum dots.<sup>[6]</sup> These properties enabled the use of the hemin/Gquadruplex as a versatile label for numerous sensors including enzyme-,<sup>[7]</sup> DNA-,<sup>[8]</sup> and aptamer-based<sup>[9]</sup> 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<sup>+</sup>, cofactor attracted substantial interest for biotechnological applications, and NAD+-dependent enzymes have been widely used for chemical transformations.<sup>[10]</sup> Our results pave the way to use the hemin/Gquadruplex as a biocatalyst for the regeneration of the NAD<sup>+</sup> cofactor and to apply the DNAzyme as a catalyst for enzymedriven transformations.

NADH oxidase catalyzes the oxidation of NADH by  $O_2$ with the concomitant formation of  $H_2O_2$ .<sup>[11]</sup> The present system consists of reduced nicotinamide adenine dinucleotide (NADH), hemin/G-quadruplex (1), and Amplex Red (2), as a fluorescent reporter dye (Scheme 1 A). Under aerobic conditions, NADH is oxidized to NAD<sup>+</sup> (see below), while 2 is oxidized to resorufin (3), which generates fluorescence at  $\lambda_{max} = 581$  nm. Figure 1 A, 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

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**Scheme 1.** A) Frame I: Hemin/G-quadruplex-catalyzed oxidation of NADH by  $O_2$  to NAD<sup>+</sup> and  $H_2O_2$ , respectively, and the concomitant oxidation of Amplex Red (**2**) to resorufin (**3**) by  $H_2O_2$ . Frame II: Coupled DNAzyme–alcohol dehydrogenase biocatalytic system for the formation of NADH and the concomitant regeneration of the NAD<sup>+</sup> 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 H<sub>2</sub>O<sub>2</sub>.<sup>[12]</sup> Indeed, the intermediate formation of H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub>. Thus, in the first step, the hemin/Gquadruplex DNAzyme acts as an NADH oxidase, where NADH is oxidized by  $O_2$  to form NAD<sup>+</sup>. It should be noted that the fluorescent resorufin (3) is formed also in the absence of NADH (Figure 1 A, curve (b)), Presumably, Amplex Red by itself has a residual donor activity that participates in the activation of O<sub>2</sub> (substituting NADH). The low fluorescence intensity changes should be considered as the background signal of the system.

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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 Gquadruplex sequence (1) and NADH, but in the absence of hemin, g) catalase, 38 U, was added to the system described in (a). B) Timedependent 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: а) 1 mм, b) 0.5 mм, c) 0.1 mм, d) 0.01 mм. 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 mм, b) 0.5 mм, c) 0.05 mм, d) 0.01 mм, e) 0 mм. 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 mм, pH 7.2 in the presence of NH<sub>4</sub>OAc, 300 mм, hemin, 1  $\mu$ M, the G-quadruplex sequence 1 (or foreign DNA (4)), 1 µм, and Amplex Red (2), 100 µм (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 1A, curve (g)). Nonetheless, the primary process of oxidation of NADH to NAD<sup>+</sup> should still proceed in the presence of catalase. Indeed, this process was characterized spectroscopically, by following the depletion of NADH (Figure 1B). 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 1B), implying that the oxidation of NADH by O<sub>2</sub> is independent of Amplex Red. From the time-dependent depletion of NADH at different concentrations of this cofactor, the values of  $k_{\rm cat} = (1.1 \pm 0.06) \times 10^{-4} \, {\rm s}^{-1}$  and  $K_{\rm m} = (5.7 \pm 0.3) \times 10^{-5} \, {\rm M}$  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  $K_{\text{m}} = 4.1 \times$  $10^{-6}$  M.<sup>[11]</sup> 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  $1 \times 10^{-4}$  M the fluorescence changes of the catalytic process reach a saturation value. Since the mechanism of the hemin-catalyzed reductive activation of O<sub>2</sub> to H<sub>2</sub>O<sub>2</sub> was extensively studied,<sup>[13]</sup> we suggest the following sequence of reactions, where NADH is the electron source, as the possible route for the formation of H<sub>2</sub>O<sub>2</sub> (Scheme 1B). In this context it should be noted that horseradish peroxidase, exhibiting NADH oxidase functions,<sup>[14]</sup> 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<sup>+</sup> cofactor is a fundamental process utilizing NAD+-dependent enzymes and NAD+dependent enzyme-driven biotransformations.<sup>[15]</sup> Different regeneration schemes that use secondary enzymes,<sup>[16]</sup> electrochemical processes,<sup>[17]</sup> or photochemical methods<sup>[18]</sup> have been used to regenerate the NAD<sup>+</sup> cofactor. One basic limitation of this process involves the oxidation of the NADH to the NAD radical, which dimerizes into the biologically inactive substance (NAD)<sub>2</sub>. 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<sup>+</sup>, 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<sup>+</sup> cofactor as a primary component. The NADH cofactor is generated through the alcohol dehydrogenase mediated reduction of NAD<sup>+</sup> by ethanol, and the resulting NADH cofactor is recycled to NAD<sup>+</sup> by the DNAzymecatalyzed oxidation of NADH while forming H<sub>2</sub>O<sub>2</sub>. The process is, then, monitored by the DNAzyme-catalyzed generation of the fluorescence of 3. Figure 2A shows the

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**Figure 2.** A) Time-dependent fluorescence changes as a result of the catalyzed oxidation of Amplex Red (2) to resorufin (3) by the hemin/Gquadruplex DNAzyme in the presence of NAD<sup>+</sup>,  $1 \times 10^{-3}$  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/Gquadruplex DNAzyme in the presence of a) 50 mM ethanol, NAD<sup>+</sup>,  $5 \times 10^{-6}$  M, and AlcDH (50 U), b) in the presence of NADH,  $5 \times 10^{-6}$  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<sup>+</sup>. 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  $5 \times$  $10^{-5}$  M. However, this value does not confirm that the NAD<sup>+</sup> cofactor was regenerated in the system (since a high initial concentration of NAD<sup>+</sup>, corresponding to 1 mm, was used). To prove that the NADH was, indeed, transformed to NAD<sup>+</sup>, we constructed a system that consisted of the hemin/Gquadruplex,  $1 \times 10^{-6}$  M, ethanol/alcohol dehydrogenase, and included the NAD<sup>+</sup> cofactor at a low concentration of  $5 \times$  $10^{-6}$  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  $\times$  $10^{-6}$  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  $(6 \pm 0.3)$ -fold higher than the added concentration of NAD<sup>+</sup>, implying that the cofactor was regenerated in the system and that the DNAzyme oxidation of NADH yielded the biologically active NAD<sup>+</sup> 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<sup>+</sup> 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/Gquadruplex 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  $k_{cat} = (4.6 \pm$  $0.2) \times 10^{-3} \text{ s}^{-1}$  and  $K_{\rm m} = (6.0 \pm 0.3) \times 10^{-6} \text{ M}$  were calculated. For comparison, the FAD-dependent NADH peroxidase

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**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<sub>2</sub>O<sub>2</sub>: 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<sub>2</sub>O<sub>2</sub>. 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<sub>2</sub>O<sub>2</sub>. 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<sub>4</sub>OAc, 300 mm, hemin, 1 μm, and the G-quadruplex sequence **1**, 1 μm.

exhibit  $k_{cat} = 3.0 \text{ s}^{-1}$  and  $K_m = 1.0 \times 10^{-6} \text{ M}$ .<sup>[19]</sup> A final aspect that needed to be confirmed was that the DNAzymecatalyzed oxidation of NADH by H<sub>2</sub>O<sub>2</sub>, indeed, yields the biologically active NAD<sup>+</sup> cofactor that is essential for the application of the process as a NAD<sup>+</sup> regenerating system. Toward this goal, the system consisting of  $H_2O_2$  (20×10<sup>-6</sup> M) and NADH  $(1 \times 10^{-4} \text{ M})$  was allowed to react with hemin/Gquadruplex  $(1 \times 10^{-6} \text{ M})$  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<sub>2</sub>O<sub>2</sub>, 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/Gquadruplex-catalyzed oxidation of NADH, indeed, yields the biologically active NAD<sup>+</sup> 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<sup>+</sup> 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<sup>[20]</sup> 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'-CGAACTCTGCAACATAAAAAA-3'

NADH oxidase: A HEPES buffer solution (5 mM, pH 7.2) containing 300 mM NH<sub>4</sub>OAc, 1  $\mu$ M **1**, 1  $\mu$ M hemin, and 100  $\mu$ M 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<sub>4</sub>OAc, 1  $\mu$ M 1, 1  $\mu$ M hemin, 50 U AlcDH, and 100  $\mu$ M Amplex Red was prepared and incubated at 25 °C for 30 min. To initiate the oxidation process, either NADH or NAD<sup>+</sup> were added, along with the appropriate amount of ethanol.

NADH peroxidase: A HEPES buffer solution (5 mM, pH 7.2) containing 300 mM NH<sub>4</sub>OAc, 1  $\mu$ M **1**, and 1  $\mu$ M 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<sub>2</sub>O<sub>2</sub> (under argon).

NADH peroxidase–alcohol dehydrogenase: A HEPES buffer solution (5 mm, pH 7.2) containing 300 mm NH<sub>4</sub>OAc, 1  $\mu$ m 1, and 1  $\mu$ m 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<sub>2</sub>O<sub>2</sub> (20  $\mu$ M), 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.

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



**Enzyme impressionist**: A DNAzyme consisting of hemin combined with a Gquadruplex was shown to mimic the activity of NADH oxidase under aerobic conditions and of NADH peroxidase under anaerobic conditions. Along with these new biocatalytic activities, the hemin/G-quadruplex complex is also capable of regenerating the biologically important NAD<sup>+</sup> cofactor in both cycles (see picture).

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