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REVIEW ARTICLE

RNA and DNA complexes with hemin [Fe(III) heme] are efficient peroxidases and peroxygenases: how do they do it and what does it mean?

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Abstract

Guanine-rich RNAs and DNAs from chromosomal telomeres and elsewhere that fold into guanine quadruplexes (G-quadruplexes), are found to complex tightly with porphyrins such as N-methylmesoporphyrin IX (NMM) and hemin [Fe(III) heme]. By themselves, these DNAs and RNAs are found to be efficient catalysts for porphyrin metallation. When complexed with hemin, under physiological conditions, these nucleic acids display robust peroxidase (one-electron oxidation), as well as peroxygenase (two-electron oxidation, or oxygen transfer) activity. These surprising catalytic properties, that frequently match the catalytic performance of natural peroxidase and P450 monooxygenase enzymes, have been the subject of significant mechanistic analysis, as well as having found utility in a wide range of biosensing and other applications. This review summarizes recent insights into a surprising yet fundamental property of many RNAs and DNAs, a property with undoubted ramifications for cellular oxidative disease, *de novo* hemoenzyme design, and our understanding of the evolution of early biocatalytic systems.

Keywords: DNA, RNA, G-quadruplex, hemin, Fe(III) heme, ferrochelatase, peroxidase, peroxygenase, P450 monooxygenases, ribozyme, DNAzyme

Introduction

Iron-protoporphyrin IX, in both its Fe(II) oxidation state ("heme") or Fe(III) state ("hemin"–Figure 1A) is a ubiquitous metabolic cofactor, used as a prosthetic group by large families of proteins of diverse function (reviewed in Chance *et al.*, 1966). Some of these cellular functions, in which heme plays a crucial role, include electron transfer, the transport and sensing of diatomic gases, and various kinds of oxidative catalysis. Different classes of heme proteins (hemoproteins) contribute in different ways to the activation and/or function of their heme prosthetic group; however, the environment of the bound heme tends to share certain features (Figure 1B). Beyond the four-fold coordination afforded the iron moiety by the porphyrin ring itself, the apoprotein invariably supplies an axial (5th) coordination to the iron, often involving imidazole, thiol, or hydroxyl ligands. This key axial coordination helps control the reactivity of the iron. The distal face of the heme then becomes the binding site for transient ligands (for instance, diatomic gases, water, or the peroxide anion), and the amino acid residues near the distal face play important roles in facilitating specific transformations that may be required of the transient ligands (reviewed in Chance *et al.*, 1966).

Heme enzymes (hemoenzymes) that catalyze oxidative reactions include the peroxidases, P450 monooxygenases, and dioxygenases (Figure 2). A catalytic continuum exists between the various peroxidases and P450 monooxygenases. So-called "classical" peroxidases, including horseradish peroxidase (HRP), catalyze mainly 1-electron oxidations; whereas, a "non-classical" peroxidase like chloroperoxidase (CPO) has significant capability for oxidizing both 1- and 2-electron oxidations. The P450 monooxygenases, activated by dioxygen *in vivo*, catalyze 2-electron oxidations, which feature oxygen atom transfer to substrates (reviewed in Marnett and Kennedy, 1995; Bröring, 2008). *In vitro*, however, the monooxygenases can

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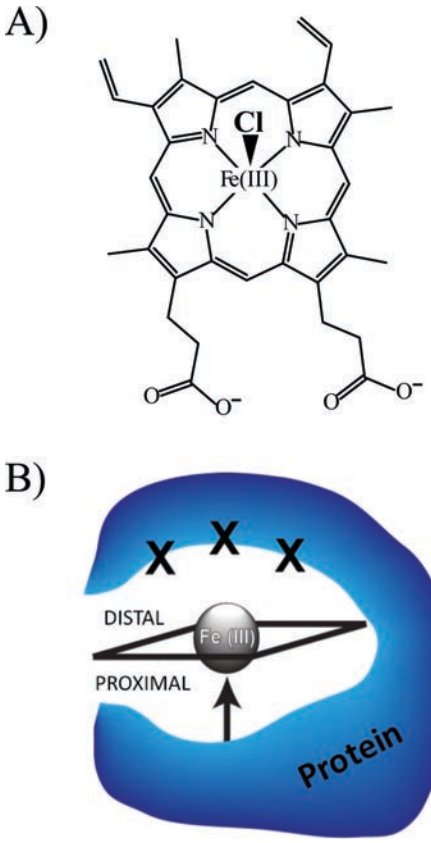

Figure 1. (A) The structure of hemin. Chloride is shown as an axial ligand, which exchanges with water in aqueous solution. (B) A schematic drawing of a Fe(III) hemoprotein. The "X" signs on the distal face of the active site, in oxidative enzymes, represent residues that participate in promoting the formation of activated Fe(III) heme species, such as Compound I.

be activated by common oxidants, such as hydrogen peroxide (H_2O_2). Indeed, some P450 enzymes in organisms such as *Sphingomonas paucimobilis* and *Bacillus subtilis* use H_2O_2 as their activating oxidant (Matsunaga and Shiro, 2004), and can therefore be classified as peroxygenases. A mechanistic feature shared by these oxidative hemoenzymes is that they are initially activated to an oxo-iron species called Compound I, in which the heme iron has a formal oxidation state of +5 (although the dominant resonance contributor has the iron as Fe^{4+}, and the remaining oxidizing equivalent is delocalized in the porphyrin or localized on specific amino acid side chains of the apoprotein). For those hemoenzymes that catalyze 2-electron oxidations, two distinct mechanisms have been invoked: (i) direct transfer of the ferryl oxygen of Compound I to the substrate, and (ii) two successive 1-electron oxidations that involve a substrate radical intermediate. The latter mechanism is referred to as the "oxygen rebound", and is thought to operate in most heme enzymes (reviewed in Marnett and Kennedy, 1995; Bröring, 2008)

$$2\ \text{Substrate-H} + H_2O_2 \xrightarrow{\text{peroxidase}} 2\ \text{Substrate}^{\bullet} + 2\ H_2O$$

$$\text{Substrate} + O_2 + H_2X \xrightarrow{\text{monooxygenase}} \text{Substrate-O} + H_2O + X$$

$$\text{Substrate} + H_2O_2 \xrightarrow{\text{peroxygenase}} \text{Substrate-O} + H_2O$$

$$\text{Substrate} + O_2 \xrightarrow{\text{dioxygenase}} \text{Substrate-}O_2$$

Figure 2. A list of oxidation reactions catalyzed by heme enzymes.

Given the outstanding oxidative properties of hemoenzymes, there has been a significant interest in using these enzymes, or chemical model systems inspired by them, in industrial scale oxidative processes. In their cellular milieu, of course, hemoenzymes function under mild, aqueous conditions, often with enviable regio- and stereo-selectivity. The large numbers of heme- and metalloporphyrin-containing model compounds that have been inspired by hemoenzymes, by contrast, often function well in organic solvents, as well as at high temperatures, with facile activation by such oxidants as meta-chloroperoxybenzoic acid (mCPBA), hydrogen peroxide (H_2O_2), iodosylbenzene (PhIO), and sodium hypochlorite (NaOCl). These abovementioned model compounds lie outside the scope of this review; however, excellent reviews on them have been written by Mansuy *et al.* (1994) and Ricoux *et al.* (2007).

The recent imperatives of "green" chemistry, however, have refocused the interests of bulk-scale substrate oxidation back to aqueous systems. Of the hemoenzyme models that do function well in an aqueous milieu, there are two, worth considering in some depth. The first is microperoxidase, a "minimal" system that preserves the key features of an oxidative heme enzyme (reviewed by Lombardi *et al.*, 2001). The other is a set of catalytic antibodies, which bind heme to their antigen-binding sites and catalyze peroxidase activity (Cochran and Schultz, 1990b). What makes the catalytic antibodies interesting is that, selected as they are out of the 10^{10}–10^{12}-strong antibody repertoire of the mouse immune system, they constitute successful solutions of *de novo* design for a hemoenzyme (some details of such catalytic antibodies are given below). The microperoxidases (MP8-MP11; Figure 3 shows MP11), on the other hand, are protein fragments, consisting of the heme prosthetic group of horse cytochrome c along with an appended 8–11 amino acid fragment of the protein. From this 8–11 amino acid vestige, a histidine (His 18) serves as the fifth (proximal) axial ligand to the heme iron. Microperoxidases catalyze both 1-electron and 2-electron oxidations, and therefore, represent minimized members of the peroxidase-monooxygenase/peroxygenase enzyme spectrum. At pH values <9.0, the heme iron's sixth (distal) coordination position in microperoxidases is taken up by a water molecule. This water is a weak ligand, and can exchange rapidly with H_2O_2, which in turn leads to the formation of Compound I, and oxidative catalysis. The microperoxidases, however,

lack the usual polar residues (Arg and His) on the heme's distal side, which in peroxidases contribute to the rapid activation of iron-bound H_2O_2. However, the small size of microperoxidases creates a relatively "open" active site, which in turn offers excellent access to the activated heme by exogenous substrates, and so enables MP8's catalysis of both peroxidase (1-electron) and oxygen transfer (2-electron) reactions (reviewed by Dallacosta *et al.*, 2004; Perry *et al.*, 2002).

Heme is a ubiquitous cofactor, found in organisms from all kingdoms of life. Ferrochelatase (protoheme ferrolyase) is the final enzyme of heme biosynthetic pathways (reviewed in Ferreira, 1999), and serves to catalyze the insertion of a ferrous (Fe^{2+}) ion into protoporphyrin IX, to generate protoheme IX (or Fe(II) heme B). The reaction transition state for ferrochelatase-catalyzed metal chelation is thought to involve a distortion of the porphyrin ring. Indeed, a resonance Raman spectroscopy study by Blackwood *et al.*, (1998) found that the binding of a substrate metal ion to the enzyme enabled an allosteric distortion of the bound porphyrin toward the chelation transition state. A naturally distorted porphyrin, N-methylmesoporphyrin IX (NMM–Figure 4) has been found to behave as a stable transition-state analogue for the chelation reaction, and is a strong inhibitor of ferrochelatases (Lavallee, 1985). Notably, immunization of mice with NMM has generated antibodies that are catalytic for porphyrin metallation (Figure 4) (Cochran and Schultz, 1990a). Moreover, these same antibodies have been found to bind heme (see above) and catalyze peroxidase reactions (Cochran and Schultz, 1990b). The history of artificial catalysts for porphyrin metallation and heme-based oxidative reactions are therefore, intertwined.

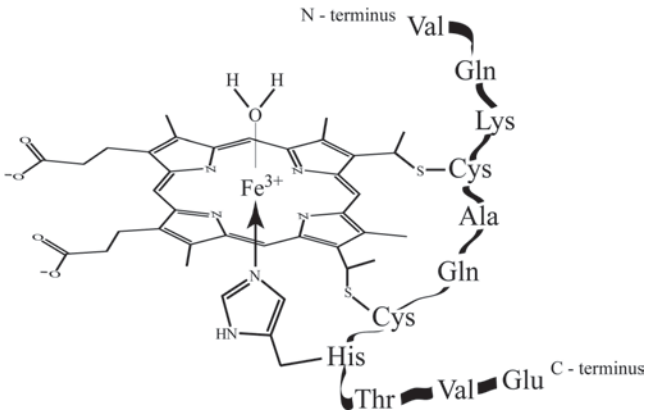

Figure 3. The structure of a microperoxidase, MP11. The drawing is adapted from Perry *et al.* (2002).

Ribozymes, the RNA World, and in vitro selection

In the 1980s the discovery of catalytic RNAs, or ribozymes, opened up new vistas on the scope of biological catalysis (reviewed by Doudna and Cech, 2002). The fact that RNA can manifest both genotype and phenotype (i.e. is able to function both as a medium for storage and propagation of genetic information, as well as as a catalyst) has encouraged the conception of a primordial "RNA world" (White, 1976; Gilbert, 1986; Benner *et al.*, 1989). In such

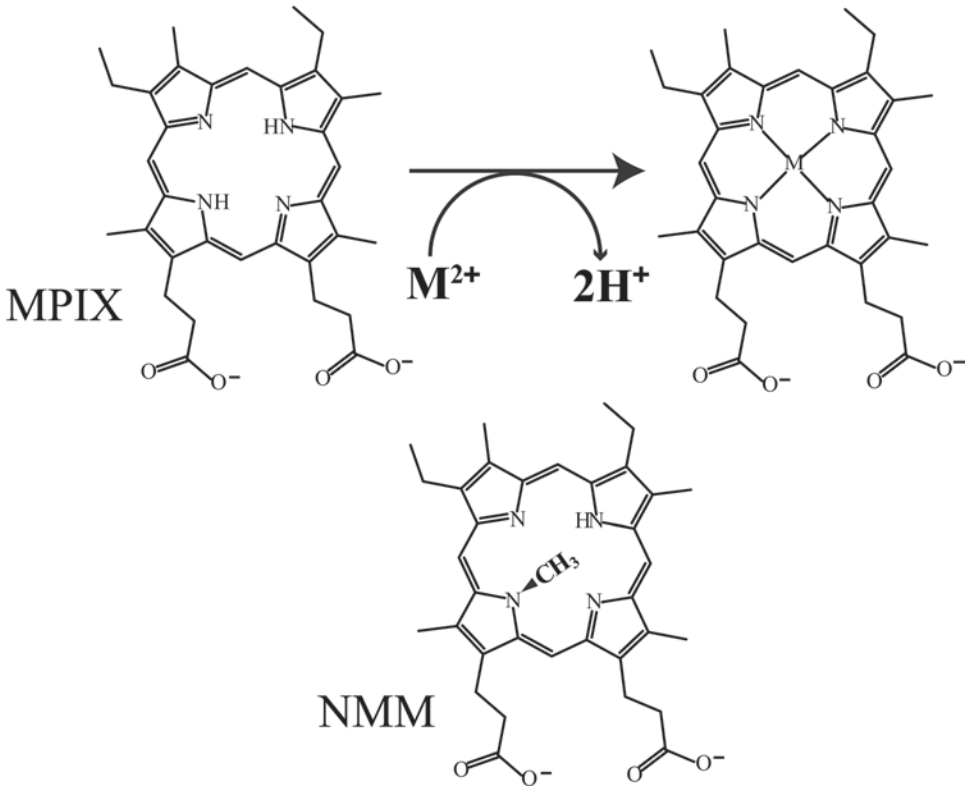

Figure 4. (*Upper*) The reaction scheme for mesoporphyrin IX (MPIX) metallation with a divalent cation, M^{2+}. (*Lower*) The structure of N-methylmesoporphyrin IX, a stable transition state analogue (TSA) for the MPIX metallation reaction.

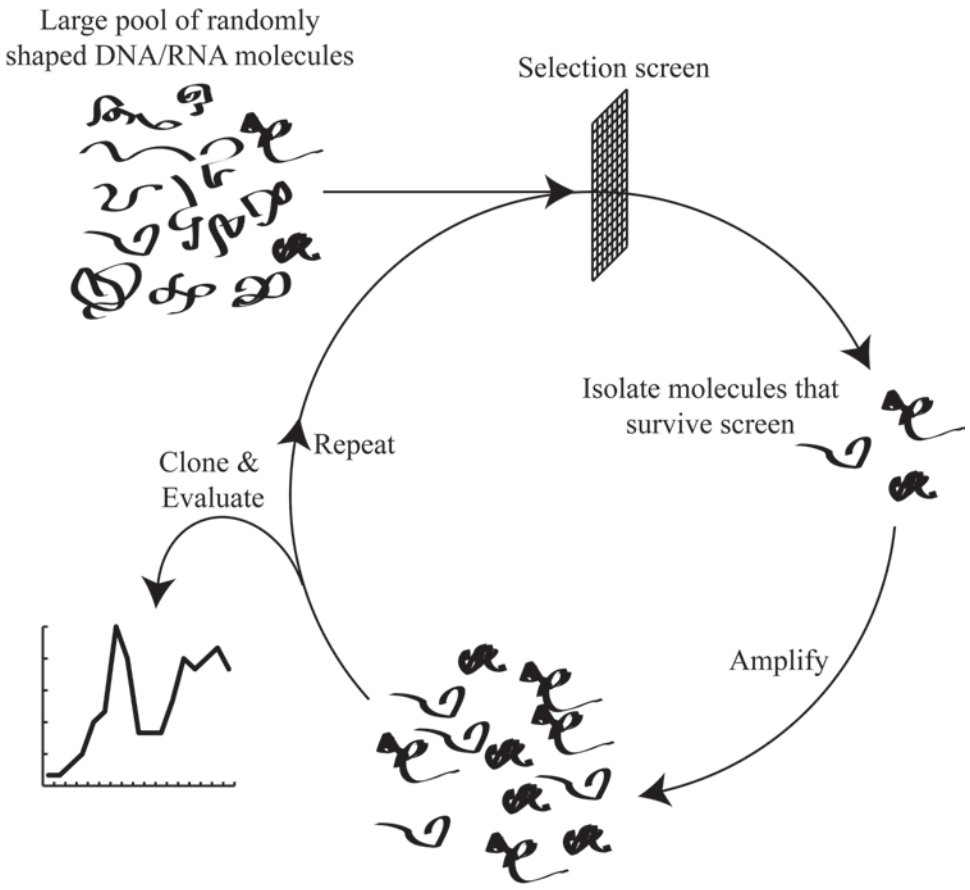

Figure 5. A schematic diagram for *in vitro* selection (SELEX).

an RNA world, ancestral primitive cells might have carried RNA genomes capable both of self-replication and the catalysis of key metabolic pathways.

The RNA world hypothesis has provided a strong stimulus for identifying new catalytic properties for the nucleic acids. RNA and DNA, as biopolymers, lack the rich repertoire of chemical functionalities that proteins have. One means for overcoming such a limitation is to harness the functional repertoire of cofactors, such as metal ions and organic and organometallic compounds, such as the various vitamins and heme. Indeed, these are made use of by numerous contemporary proteins to supplement their intrinsic functional groups. However, *de novo* design of folded RNA and DNA molecules that bind specified ligands, or carry out specific kinds of catalysis, is prohibitively difficult at present. Fortunately, the powerful methods of *in vitro* selection have been widely available and successfully used to select for RNA/DNA molecules that are either ligand binders (aptamers) or catalysts (new ribozymes and DNAzymes–reviewed by Höbartner and Silverman, 2007).

In vitro selection (SELEX) was first described in 1990 (Ellington and Szostak, 1990; Tuerk and Gold, 1990; Robertson and Joyce, 1990), and is shown conceptually in Figure 5. Briefly, large libraries (containing 10^{14}–10^{15} random sequences) of single-stranded DNA or RNA are subjected to some stringent selection screen, either for a binding or a catalytic activity. Individual DNA or RNA molecules that pass this test are amplified and re-subjected to it, iteratively, until a pool with a high enrichment of the desired sequences is obtained. These RNAs or DNAs are then cloned, sequenced, and subjected to the appropriate analysis (reviewed by Eckstein, 2007).

For the selection of novel ribozymes and DNAzymes using SELEX, two possible strategies are available. In the first, called "direct selection", the random RNA or DNA pool is challenged directly with the catalytic task in question. This strategy has been used to obtain the overwhelming majority of new ribozymes and DNAzymes reported to date. The second approach, called "indirect selection", mimics the strategy used for obtaining catalytic antibodies. Briefly, a DNA or RNA aptamer is selected for specific binding to a stable transition-state analogue (TSA) for a given reaction. Such an aptamer can then be expected to behave as an enzyme for catalyzing the reaction in question (reviewed by Burgstaller and Famulok, 1995; Sen and Geyer, 1998).

G-quadruplex forming DNAs catalyze porphyrin metallation

In 1996, Li *et al.* reported the selection of DNA aptamers that bound N-methylmesoporphyrin (NMM) with nanomolar affinity. NMM (shown in Figure 4), a porphyrin whose structure is distorted from planarity, is a potent inhibitor of naturally occurring ferrochelatase enzymes, which catalyze the insertion of Fe^{2+} ions into

Table 1. The sequences of DNAs and RNAs referred to in this review.

Name	Sequence
PS5.ST1	5′-TCG TGG GTC ATT GTG GGT GGG TGT GGC TGG TCC-3′
PS5.M	5′-GTG GGT CAT TGT GGG TGG GTG TGG-3′
PS2.M	5′-GTG GGT AGG GCG GGT TGG-3′
rPS2.M	5′-GUG GGU AGG GCG GGU UGG-3′
OXY4	5′-$(TTT TGG GG)_4$-3′
Bcl-2	5′-GGG CGC GGG AGG AAG GGG GCG GG-3′

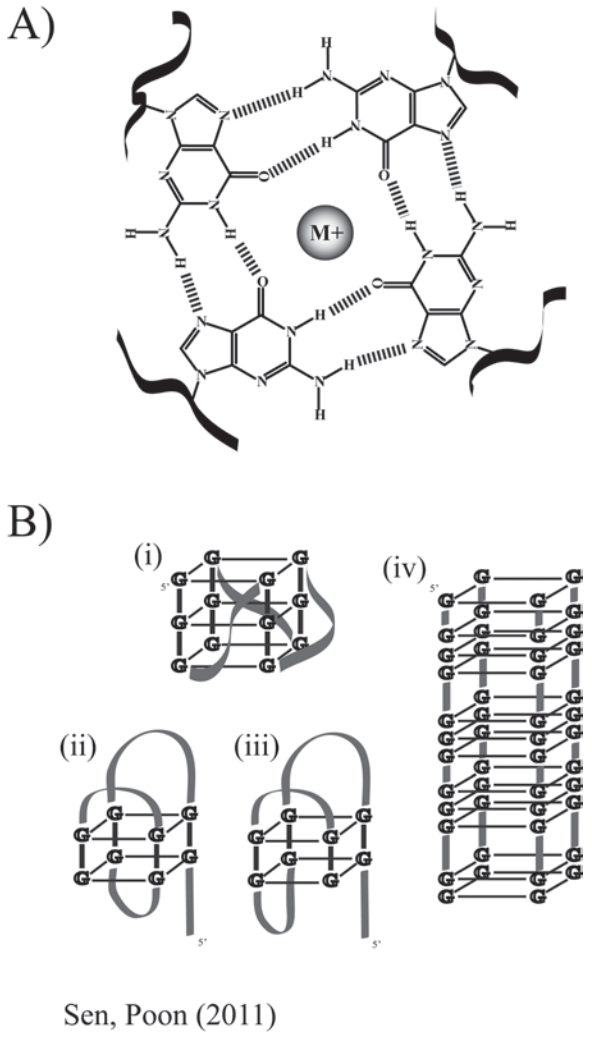

Figure 6. (A) The structure of a guanine base-quartet. M^+ represents a monovalent cation, such as K^+, that binds in the cavity between successive guanine quartets. (B) A range of guanine-quadruplexes: (i) intramolecular, parallel; (ii) and (iii) intramolecular, partially and wholly antiparallel, respectively; (iv) intermolecular, parallel.

protoporphyrin IX in the final step of heme biosynthesis (Lavallee, 1985). The highly effective inhibition of ferrochelatase by NMM is due to the latter's structural mimicking of the chelation transition state. Indeed, this property of NMM was utilized by Cochran and Schultz (1990a) to generate catalytic antibodies that mimicked ferrochelatase (reviewed by Schultz and Lerner, 1995). These antibodies catalyzed the insertion of Zn^{2+}, Co^{2+}, Mn^{2+}, and Cu^{2+} into the planar substrate mesoporphyrin IX (MPIX).

Li *et al.* (1996) asked the question: would polyanionic DNA/RNA aptamers distinguish between homologous porphyrins with subtle structural differences? This question was answered definitively, since PS5.ST1, one of several NMM-binding DNA aptamers characterized by Li *et al.* bound NMM ~100–1000-fold more strongly than it did MPIX (which differs by a single methyl group from NMM). Given this striking preference for binding a transition-state analogue over a substrate porphyrin, it was interesting to ask whether these NMM-binding aptamers would behave as enzymes for porphyrin metallation.

Li and Sen (1996) then reported that the 33-nucleotide, guanine-rich DNA aptamer, PS5.ST1, efficiently catalyzed metallation of MPIX by copper and zinc ions in an aqueous buffer. Table 1 lists the sequences of the various DNAs and RNAs referred to in this review. A 24-nucleotide fragment (PS5.M) from within PS5.ST1 was found to be both the minimal and optimal catalytic entity. Three structurally related porphyrins, MPIX, protoporphyrin IX, and deuteroporphyrin IX, were accepted as substrates by this new deoxyribozyme (DNAzyme). It was determined that unlike in protein chelatases, a catalytically relevant binding site for copper ions likely did not exist in PS5.M. The guanine-rich DNAzyme required potassium ions for its folding and catalytic function; this requirement was founded in the need to form guanine base-quartets (Figure 6A) by the folded PS5.M, as well as by the porphyrin-binding sequence within the folded PS5.ST1. Guanine-quadruplexes (Figure 6B) are stable folds formed by guanine-rich DNAs and RNAs (Sen and Gilbert, 1988; 1990). They are a highly polymorphic family of folded structures, varying in strand stoichiometry, orientation, and overall topology. The initial hypothesis of Li and Sen (1998) was that the active form of PS5.M was an intramolecular, antiparallel-stranded structure.

PS5.M and PS2.M (a shorter DNA rationally derived from a selected sequence) both obeyed Michaelis-Menten kinetics for the insertion of copper ions into MPIX, with respect to the MPIX substrate. Optimization of reaction and buffer conditions led the following catalytic parameters: k_{cat}: 1.3 min^{-1}; K_M: 40 μM. The corresponding parameters for a catalytic antibody characterized by Cochran and Schultz were: k_{cat}: 0.14 min^{-1}; K_M: 50 μM. Interestingly, concurrent with the report by Li *et al.* (1996) on DNA aptamers for NMM, Conn *et al.* (1996) reported an RNA aptamer for binding NMM. Furthermore, this RNA aptamer also catalyzed MPIX metallation with Cu^{2+} ions (k_{cat}: 2.0 min^{-1}; K_M: 16 μM). Porphyrin metallation was thus, perhaps the first reaction, where a three-way

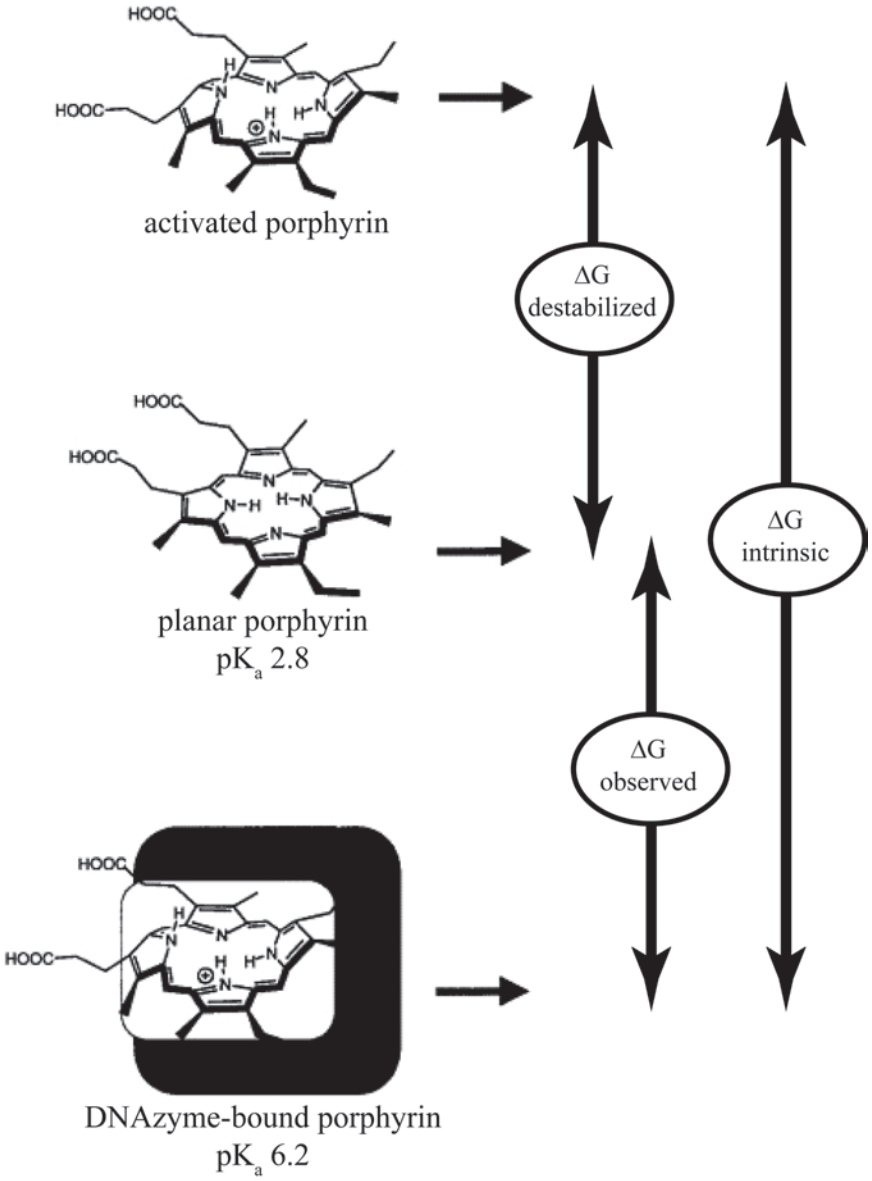

Figure 7. A schematic diagram showing the relationship between the intrinsic binding energy and the substrate destabilization energy for catalysis or porphyrin metallation by the DNAzyme, PS5.M. The figure is adapted from Geyer and Sen (2000).

comparison was possible for the relative abilities of DNA, RNA, and protein catalysts to catalyze the reaction. It was evident, first, that RNA and DNA performed comparably to each other; and, second, that their performance was not inferior to that of the protein. It is important to note in this context that a catalytic antibody, obtained by "*in vivo* selection", is the most appropriate point of comparison for *in vitro*-selected ribozymes and DNAzymes. All are "naïve" catalysts, in that unlike natural ferrochelatase enzymes they have not been subjected to long-term optimization, by natural selection, within living organisms. Nevertheless, comparison of these various "naïve" enzymes with natural ferrochelatases does highlight the rather surprising finding that the "naïve" enzymes are not significantly poorer catalysts (for instance, k_{cat} for the insertion of Zn^{2+}into MPIX by a natural ferrochelatase is 7.2 min^{-1}; and K_M is 11.8 μM (Okuda *et al.*, 1994).

PS5.M appears to fit all the standard criteria of what an enzyme should be. It catalyzes multiple turnovers of porphyrin metallation, and it shows saturation kinetics with respect to the porphyrin substrate. Furthermore, it is subject to competitive inhibition by non-substrate metalloporphyrins, such as heme (see below). An energetic analysis of porphyrin metallation catalyzed by a DNA and a protein enzyme (Narlikar & Herschlag, 1997) revealed striking similarities in the way that binding energy was used by both types of enzyme to carry out catalysis (Geyer and Sen, 2000). This was a demonstration of the "Circe effect", as postulated by Jencks (1975), which posits that an enzyme uses a part of its "intrinsic" binding energy (total energy of the enzyme-substrate interaction) to distort and/or destabilize the substrate in the direction of the transition state. Geyer and Sen (2000) found that PS5.M did precisely that (shown schematically in (Figure 7).

With PS5.M, an interesting consequence of distortion of the bound substrate is a substantial change in the porphyrin's basicity, which undoubtedly contributes to its observed catalysis. Li and Sen (1998) used UV-vis absorption and fluorescence spectroscopy to make the observation that PS5.M-MPIX's absorption spectra more closely resembled the spectra of PS5.M complexed with the transition state analogue, NMM, than that of uncomplexed MPIX. The pH titration experiments revealed that complexation to PS5.M raised the pK for protonation and thus, likely, also for metallation, of MPIX by as much as 3–4 pH units. Changes in the porphyrin's basicity may be brought about by at least two mechanisms: structural distortion of the planar core of MPIX to resemble that of the transition-state analogue, NMM; or, stabilization (by the negative charges of the DNA phosphate backbone) of the growing positive charge in the protonation and metallation transition states (Sen and Geyer, 1998). Perhaps, a combination of the two mechanisms, as well as other contributing factors is operational. Although the mechanistic details of the RNA catalyst for porphyrin metallation (Conn *et al.*, 1996) have not been reported, the chemical similarities between DNA and RNA, and the propensity of both to fold into G-quadruplexes, suggest that the ribozyme achieves its catalytic enhancement using the same strategies as PS5.M (reviewed in Sen and Geyer, 1998).

DNA/RNA-hemin complexes show enhanced peroxidase activity

As described above, hemin was found to be an excellent competitive inhibitor of porphyrin metallation by the DNAzymes PS2.M and PS5.M (Li and Sen, 1996). Like NMM, the structure of hemin is "non-planar" in as much as hemin's ferric moiety sits approximately 0.5 Å above the porphyrin ring (Koenig, 1955). The strong hemin-binding of PS2.M and PS5.M meant that they could be viewed as aptamers for hemin, and not just for NMM.

A natural question stemming from the demonstration of tightly bound DNA-hemin complexes was: could they possibly show any of the catalytic properties characteristic of hemoproteins? *A priori*, there was no reason for thinking that they might, given that they had not been selected for any such activity. Furthermore, no biological role for putative heme-nucleic acid complexes had been hypothesized up to that point; nor had it been demonstrated that a stable complex of heme with DNA or RNA was possible. Figure 8 shows a general schematic for the peroxidase (1-electron oxidation) catalytic cycle. Travascio *et al.* (1998) investigated whether PS5.M- and PS2.M-hemin were capable of accelerating a peroxidase reaction, relative to hemin alone or hemin mixed with a control DNA that did not complex with it. Hydrogen peroxide was used to activate the heme, and a chromogenic substrate, ABTS [2.2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] was used as the substrate. Remarkably, both aptamer-hemin complexes showed significantly higher peroxidase activity than the two controls (Travascio et al., 1998). The

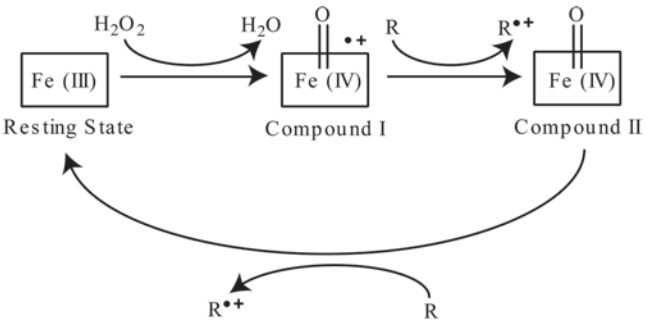

Figure 8. The peroxidase catalytic cycle.

observed velocity (V_{obs}) of PS2.M-hemin, under the most optimal reaction conditions (see below), was ~250-fold greater than that of hemin alone, and was superior to that of a previously reported catalytic antibody-hemin complex (Cochran and Schultz, 1990b).

UV-vis spectroscopic analysis of the aptamer-hemin complex showed some key differences from the spectra of hemin alone or hemin mixed with a non-hemin binding DNA (Figure 9A). While noteworthy differences were seen in the hemin visible spectrum (480–700 nm wavelength), the most striking feature was a ~2-fold hyperchromicity (and slight red-shift) of the prominent Soret absorption band of hemin (at ~400 nm wavelength). Scatchard analysis of the PS5.M- hemin interaction indicated a 1:1 binding stoichiometry between the two (Travascio *et al.*, 1998). Hyperchromicity of the Soret band is usually seen as an indicator of the hydrophobicity of the hemin binding site (Slome-Schwok and Lehn, 1990). Travascio *et al.* (1998) hypothesized that this was true also for the PS5.M-hemin complex. *A priori*, however, it was difficult to conceptualize such a binding/active site made of DNA. One immediate question was: does the DNA aptamer supply an axial ligand to the hemin iron? Corwin *et al.* (1968) had shown that hemin's visible and near-visible electronic transitions were impacted also by stereoelectronic interactions between the iron's ligands and the porphyrin's π electrons. Thus, strong ligands cause the Soret band to be both more intense and to shift to longer wavelengths. PS2.M-hemin's visible spectrum (480–700 nm), relative to uncomplexed hemin, showed a more complex pattern of peaks, surprisingly reminiscent of Fe(III) hemoproteins (notably, the oxidized forms of myoglobin and hemoglobin, both of which show peroxidase activity). The iron moiety in these heme proteins is a six-coordinate, high-spin species (Adar, 1978). On that basis, Travascio *et al.* (1998) postulated that the iron(III) moiety within the DNAzyme-hemin complexes also existed in the high-spin state and had six-fold coordination, with one of the axial ligands being water.

Figure 9B shows the pH dependence of PS2.M-hemin-catalyzed peroxidation, relative to controls. Both the control and PS2.M-catalyzed reactions had sensitivity in the 6–5–10.0 pH range. For PS2.M-hemin, the bell-shaped pH dependence, peaking at pH 8.5, was much sharper than that of the background. At pH 8.0, however,

the rate acceleration by PS2.M-hemin was at its highest (~50:1); for this reason all further kinetic measurements by Travascio *et al.* (1998) were made at pH 8.0. One key finding was that both the catalyzed and background reactions were accelerated by the presence of nitrogenous buffers, such as ammonium or collidine (Travascio *et al.*, 1998, 2006). In the case of the DNAzyme-catalyzed reaction, the authors hypothesized that the nitrogenous buffer played the acid-base roles that distal residues within hemoprotein active sites typically play in activating hydrogen peroxide.

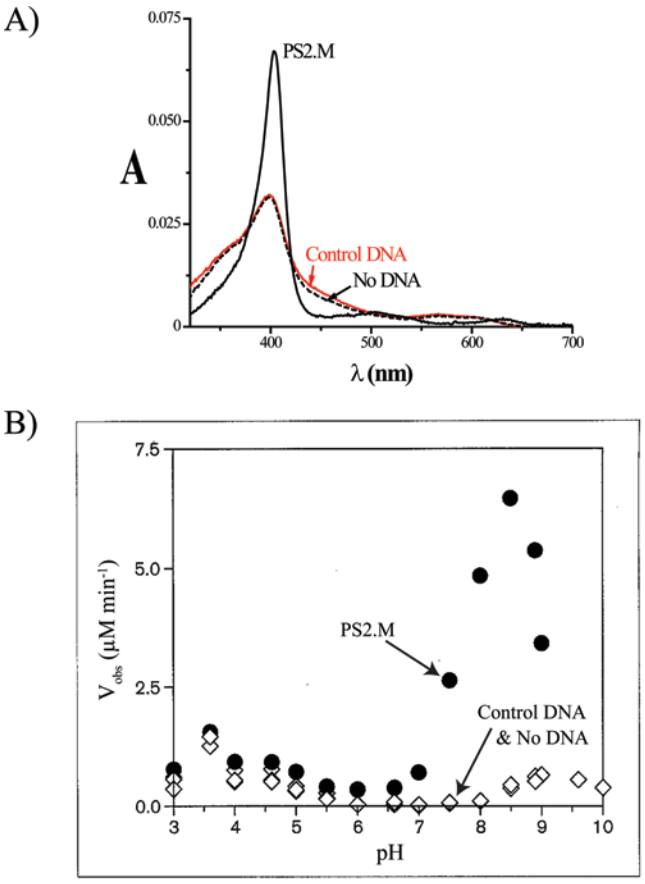

Figure 9. (A) The UV-visible absorption spectra of the PS2.M-hemin complex (black line); uncomplexed hemin in the absence of DNA (dotted black line) and mixed with a nonbinding control DNA oligonucleotide (red line). This figure has been adapted from Poon *et al.* (2011). (B) pH-dependence of the observed rates for PS2.M-hemin catalyzed (•) and uncomplexed hemin-catalyzed (◊) peroxidations of ABTS. This figure is adapted from Travascio *et al.* (1998).

Travascio *et al.* (1999) asked whether RNA-hemin complexes could also catalyze peroxidase reactions. Guanine-rich RNAs have also been shown to fold into G-quadruplexes (Cheong and Moore, 1992). The RNA version of PS2.M (rPS2.M) was tested for peroxidase activity, and found to be almost identically active as its DNA counterpart. By contrast, an unrelated G-quadruplex-forming DNA oligomer, OXY4 (representing sequence from a protozoan telomere), bound heme, but catalyzed ABTS peroxidation poorly (Travascio *et al.*, 1999). Overall, these results showed that (a) some RNAs and DNAs of identical sequence are able to fold to form comparable hemin-binding sites, and show comparable catalytic behavior; and (b) that complexing with a G-quadruplex does not in itself enhance the (low) intrinsic peroxidase activity of hemin. The notion was thus raised of distinct "active sites" within catalytically active aptamers, as opposed to mere binding sites for hemin on a G-quadruplex. In terms of what might distinguish an active site from a mere binding site, the issue of an axial ligand from DNA/RNA, suggested by the spectral features of the DNA/RNA-hemin complexes (above), was raised anew.

To probe the catalytic mechanism of DNA/RNA-hemin complexes, Travascio *et al.* (2006) carried out detailed kinetic analyses on both PS2.M-hemin and rPS2.M–hemin. First, control experiments involving peroxidation of hemin solubilized in detergent micelles (negatively charged, as well as uncharged micelles) showed little influence of the ambient charge on the efficiency of peroxidation. The authors therefore concluded that it could not be merely a negatively charged environment that

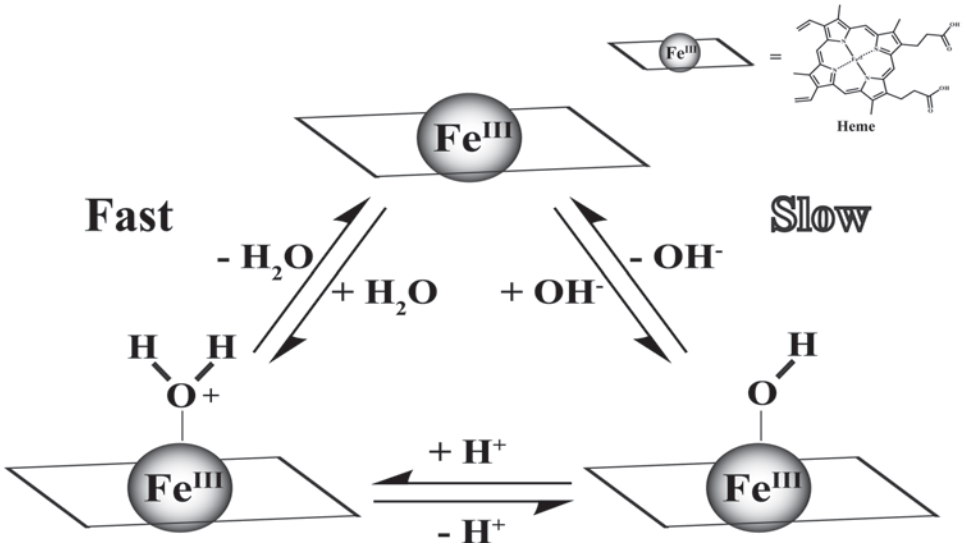

Figure 10. The alkaline transition of hemoproteins.

PS2.M and rPS2.M supplied to the complexed hemin. In summary, the polyanionic character of RNA and DNA was likely not a major contributor to the peroxidation catalysis.

Fe(III) hemoproteins are known to show an "alkaline transition" in their peroxidase activity, which arises from the ionization of a water molecule coordinated to the sixth (axial) position of the hemin iron (Antonini and Brunori, 1971). Figure 10 illustrates the concept, that as long as the sixth coordination position is occupied by water, it can exchange relatively easily with hydrogen peroxide, leading to the steps of peroxidase activity. However, deprotonation of this bound water to a hydroxide ion leads to a much slower exchange with hydrogen peroxide, and a concomitant drop in the hemin's peroxidase activity. Travascio *et al.* (2006) found that, detergent micelle-solubilized hemin showed transition pKa values of 3.4–4–0, depending on the detergent. Horseradish peroxidase shows a pKa of 10.9; while Fe(III)-hemoglobin, which has peroxidase activity, shows a pKa of 8.3. By way of comparison, PS2.M-hemin and rPS2.M-hemin show pKa values of 8.7 and 8.6, respectively (Travascio *et al.*, 2006). By this mechanistic criterion, as indeed by the criteria of the similarity of both their UV-visible spectra and peroxidation kinetics, the DNAzymes prove to be peroxidases that are at least as effective as the hemoprotein, Fe(III) hemoglobin.

The disparity between the alkaline transition pK_a values of uncomplexed hemin (<4.0) and PS2.M-hemin (8.7) provides insight into the DNAzymes' superior catalysis at or near neutral pH. In uncomplexed hemin, the bound water is well deprotonated at pH 7.0, and the resultant hydroxide complex exchanges poorly with H_2O_2; whereas in the DNAzymes, the bound water is not deprotonated, with a concomitant fast exchange with H_2O_2 and peroxidase activity. What structural or other features of the PS2.M-hemin active site enable this suppression of deprotonation? That remains to be fully elucidated; however, the data of Travascio *et al.* (2006) provide degrees of insight into how the DNAzymes may provide a chemical environment for accelerated peroxidation. These include: possible nucleobase coordination to the 5th axial position of the hemin; provision of a polar environment in the vicinity of the hemin iron; and, favorable hydrophobic contacts for the periphery of the porphyrin ring. As described above, nitrogenous buffers enhance peroxidation rates, likely participating in acid-base chemistry for the generation of Compound I.

PS2.M-hemin was also studied by Travascio *et al.* (2001) and by Witting *et al.* (2001) using EPR spectroscopy. At low temperatures the complex showed both a low-field g ~6 and a high-field g ~2 signal, which are characteristic of high-spin ferric heme with axial symmetry (i.e. the spectra are comparable to those of the six-coordinate heme iron in acidic Fe(III)-myoglobin–Ikeda-Saito *et al.*, 1992; Bogumil *et al.*, 1995). These data are indeed consistent with the presence of *two* axial ligands on the iron of PS2.M-hemin, with one of these ligands being water. Low-temperature EPR, along with parallel spin-trapping experiments following treatment of PS2.M-hemin with H_2O_2, indicated the formation of a carbon-centered radical, presumably on the PS2.M DNA oligonucleotide. Follow-up probing of the chemical integrity of PS2.M found oxidative damage to specific guanine within the DNA. Cumulatively, these findings led Travascio *et al.* (2001) and Witting *et al.* (2001) to propose that specific guanines within PS2.M contribute to the enhanced peroxidase activity of PS2.M-hemin and related DNAzymes–perhaps by supplying the 5th axial ligand to the hemin?

Generality of hemin- RNA/DNA complexes with peroxidase activity, and some unique properties

In 2007, Rojas *et al.* reported a deep exploration of the potential of PS2.M-hemin to catalyze the peroxidation of a variety of substrates in enantio-specific and/or regio-specific manner. They also examined the relative effectiveness of oxidants other than H_2O_2 for PS2.M-hemin activation. The authors' stated goal was to establish the extent to which this DNAzyme constituted a specific catalyst, and how its DNA matrix, beyond majorly activating the bound hemin, contributed to its ability to utilize specific substrates and oxidants and generate unique oxidation products. First, the authors confirmed that PS2.M-hemin was indeed a far more effective oxidation catalyst than uncomplexed hemin. They found that depending on the substrate, PS2.M contributed up to ~70-fold rate acceleration over the activity of naked hemin. PS2.M-hemin was found to oxidize a broad range of substrates; a remarkable finding was that with certain phenolic substrates, including L- and D-tyrosine, N-acetyl-L-tyrosine, and hydroxycinnamic acid, PS2.M-hemin showed rates superior to horseradish peroxidase (Rojas *et al.*, 2007).

These authors reported that in addition to hydrogen peroxide, bulkier oxidants such as t-butyl hydroperoxide and cumene hydroperoxide were also effective at activating PS2.M-hemin. A key finding of these authors was that although PS2.M-hemin showed some regioselectivity with regard to the substrates that it oxidized, it showed almost no enantioselectivity. These are points of striking departure beween the hemin-utilizing DNAzymes/ribozymes and protein peroxidases such as HRP. The absence of enantioselectivity in the nucleic acid-hemin complexes suggests relatively "open" active sites for them (Figure 11A); however, whether more sophisticated active sites can be built around the hemin, remains a priority for future research.

The findings of Rojas *et al.* raised some fundamental questions: (a) do all folds and topologies of DNA and RNA G-quadruplex bind hemin comparably well? And, (b) are all such complexes competent at catalyzing one-electron oxidations? In recent years, a number of groups have examined these questions. As described above, RNA and, particularly, DNA G-quadruplex folds are highly polymorphic–being capable of adopting wholly parallel,

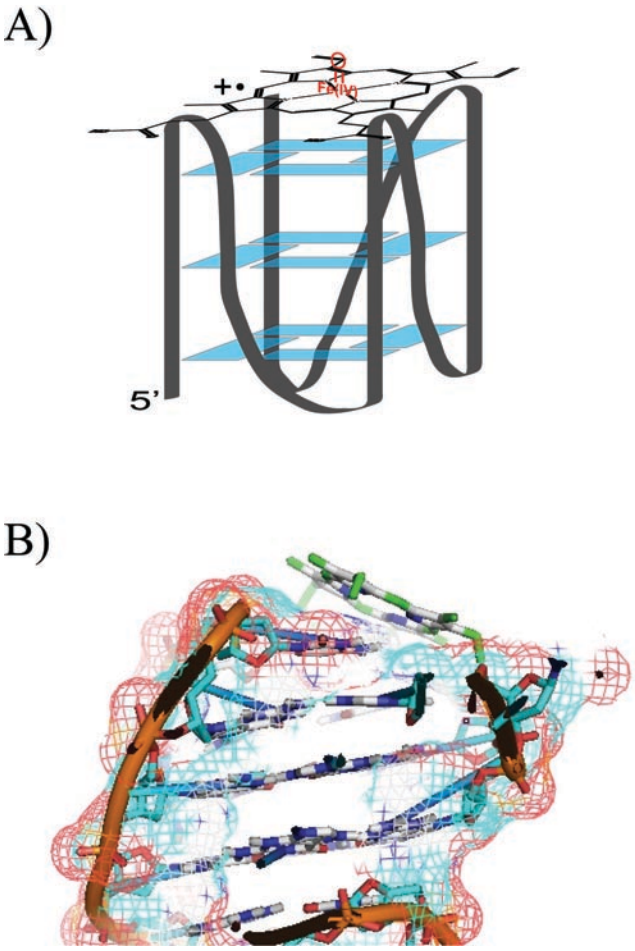

Figure 11. (A) A schematic diagram showing the stacking of hemin (shown here as Compound I) upon the terminal guanine-quartet of a DNA or RNA G-quadruplex. (B) Docked location of hemin upon the G-quadruplex formed by the Bcl-2 DNA oligomer (adapted from Poon *et al.*, 2011).

partially parallel, as well as antiparallel strand orientations. Cheng *et al.* (2009) carried out a broad investigation of the hemin-binding, as well as peroxidation properties of a variety of not only aptamers, but genomic DNA and RNA sequences that are postulated to form G-quadruplexes *in vivo* (reviewed by Lipps and Rhodes, 2009). They reported that DNAs and RNAs that formed parallel-stranded G-quadruplexes were most optimal, both for heme binding and peroxidase activity. In a series of papers, Kong and colleagues have explored in some detail the structural characteristics (topology; loops) that a G-quadruplex requires in order to bind heme and catalyze peroxidation (Kong *et al.*, 2009a,b, 2010). These authors elaborated that both intramolecular and multi-stranded quadruplexes were effective, as long as their strand orientations were all-parallel. It is likely therefore, that PS2.M also forms a wholly parallel quadruplex. Such had indeed been anticipated by Majhi and Shafer (2006).

Thirstrup and Baird (2010) took the interesting step of covalently coupling hemin, by way of a DNA linker, to PS2.M, and found that many of the salt and buffer requirements of the non-covalent PS2.M-hemin complex were obviated for this conjugate. More recently, Nakayama *et al.* (2011) have reported that the covalent linkage of hemin and DNA enables even antiparallel G-quadruplexes to function effectively as DNAzymes. In terms of optimizing the activity of G-quadruplex-hemin complexes to obtain the fastest peroxidase activity possible, Li *et al.* (2009a) reported a DNA G-quadruplex, AGRO100, which they claimed, had the highest hemin-binding affinity and fastest peroxidase activity of all hemin-binding aptamers tested to date.

Unfortunately, no high-resolution NMR or crystal structure has been reported to date for a DNA/RNA-hemin complex. High-resolution structures do exist, however, for certain DNA G-quadruplexes (such as the Bcl-2 G-quadruplex–Dai *et al.*, 2006), that have separately been shown to bind hemin and catalyze oxidative reactions (Poon *et al.*, 2011). Poon *et al.* carried out flexible docking of hemin onto the NMR structure of the Bcl-2 G-quadruplex. In all of the lowest energy structures, the heme stacked upon a loop cytosine, C6, which in turn was stacked upon the G-quartets of the quadruplex (Figure 11B). One feature of particular interest was that the exocyclic amine functionality of the C6 base lay close to and approximately axial to the heme iron (separated by 2.74 Å; whereas, in heme enzymes, the separation between the iron atom and the axial ligand is in the 2.1–2.4 Å range–Hersleth *et al.*, 2006). With the undoubted appearance of high-resolution structures of G-quadruplex-heme complexes in the near future, it will be interesting to see if an axial coordination of the heme iron by a nucleobase is both a correct and a universal feature of peroxidase DNAzymes and ribozymes.

Catalysis of 2-electron oxidations

Using as a starting point DNA/RNA-hemin complexes that catalyzed peroxidase (1-electron oxidation) reactions, Poon *et al.* (2011) investigated whether other known reactions of hemoproteins, such as 2-electron oxidations, could also be catalyzed by these complexes. Natural peroxidases such as HRP oxidize a variety of substrates and appear not to require substrate binding close to their activated heme; mere collisions of the substrates with the edges of activated hemes within these peroxidases enables electron transfer between the heme and substrate (Ator *et al.*, 1987). DNA/RNA-hemin complexes, too, catalyze the peroxidation of a wide variety of structurally distinct substrates (Rojas *et al.*, 2007). Therefore, by analogy, it is also likely that DNAzymes and ribozymes carry out 1-electron oxidations by enabling collisions of the substrate with their activated heme moieties.

Catalysis of 2-electron, "oxygen transfer" reactions, however, might prove intrinsically more challenging for these DNAzymes and ribozymes. Figure 12A schematizes two alternative mechanisms by which protein monooxygenases and peroxygenases are thought to transfer oxygen to substrate: by a direct, one-step, oxygen transfer; or, a two-step, "oxygen rebound" mechanism involving two successive 1-electron oxidations. Both of these mechanisms, however, in order to transfer the ferryl oxygen atom to the substrate,

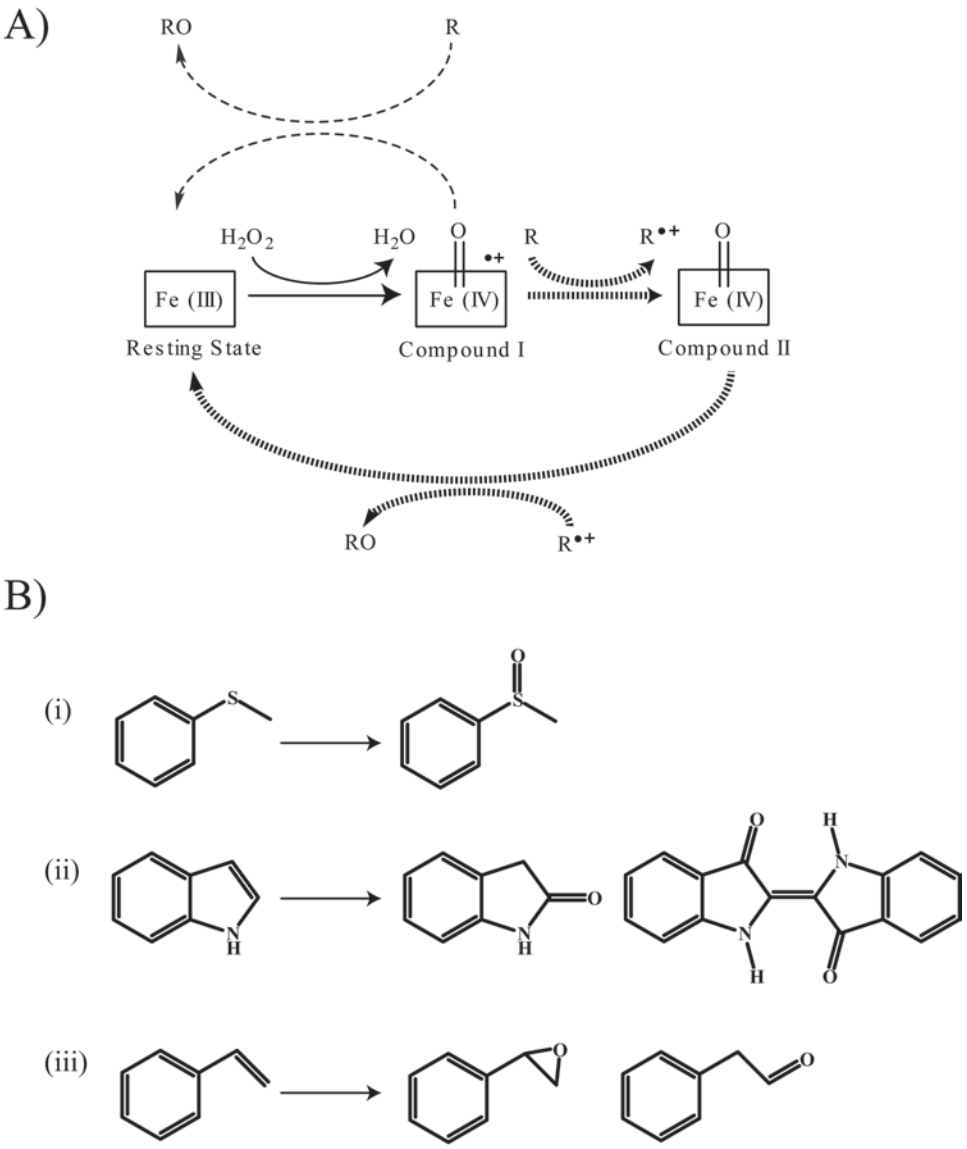

Figure 12. (A) The peroxygenase (oxygen transfer; 2-electron oxidation) catalytic cycle. (B) Substrates for the peroxygenase activity of PS2.M-hemin: (i) thioanisole to thioanisole sulfoxide; (ii) indole to 2-oxyndole and indigo; (iii) styrene to styrene oxide and phenylacetaldehyde.

necessarily require the binding (or at least prolonged localization) of the substrate close to the activated heme. Given the significant evidence for "open" or "exposed" active sites within DNA/RNA-hemin complexes (Figure 11), it was not clear *a priori* that DNA/RNA-hemin complexes would be competent to catalyze oxygen transfer reactions.

Poon *et al.* (2011) investigated whether three classic oxygen transfer reactions could be catalyzed by a variety of DNA/RNA-hemin complexes (including PS2.M-hemin and rPS2.M-hemin). These reactions are summarized in Figure 12B, and include (i) oxidation of a heteroatom, in this case, sulfur (thioanisole to its sulfoxide); (ii) oxidation of an electron-rich alkene (indole) and (iii) a less electron-rich alkene (styrene) to various products. Poon *et al.* (2011) found that all three substrates were readily oxidized to the expected products, with kinetics in each case comparable to those of protein hemoenzymes. Use of ^{18}O-labeled H_2O_2 revealed that the oxygen atom transferred to form both thioanisole sulfoxide and styrene oxide originated in the added H_2O_2 (rather than in dissolved oxygen, for instance). Hammett analysis of the kinetics of thioanisole sulfoxide formation, however, could not distinguish cleanly between the one-step and two-step mechanisms for this catalysis. One distinctive feature of these 2-electron oxidations by PS2.M-hemin, compared to those catalyzed by a variety of hemoproteins, was PS2.M-hemin's lack of enantioselectivity (as judged by the production of racemic thioanisole sulfoxide). Most protein hemoenzymes, by contrast, show a significant enantioselectivity (reviewed in van Rantwijk and Sheldon, 2000). PS2.M-hemin's lack of enantioselectivity, however, is consistent with the earlier findings of Rojas *et al.* (2007). In both instances, it likely reflects the relative openness of the PS2.M-hemin active site (specifically, a lack of structure on the hemin's distal side, where the substrate is expected to encounter the ferryl oxygen to be transferred). Given that PS2.M-hemin and

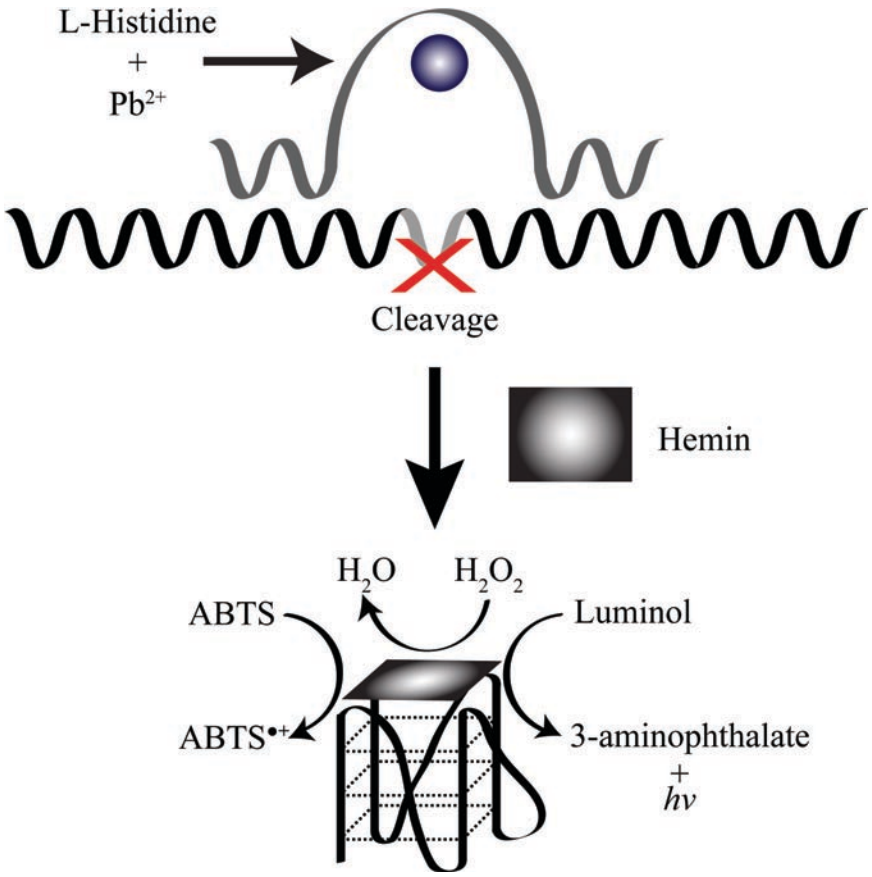

Figure 13. A practical application of the peroxidase activity of DNA-hemin complexes. Shown is a DNAzyme cascade for the amplified detection of lead ions (or the amino acid, L-histidine). Within this cascade, the hemin-utilizing DNAzyme is the final component. In its first stage, an allosteric, lead/L-histidine-responsive DNAzyme cleaves a substrate strand. The released, cleaved products can then fold to form a G-quadruplex, bind hemin, and generate photons as output by way of the catalyzed peroxidation of luminol. This figure is adapted from Elbaz *et al.* (2008).

other hemin-containing ribozymes/DNAzymes catalyze both one- and two-electron oxidations efficiently, and appear to have relatively open and accessible active sites, they somewhat resemble the microperoxidases. Indeed, lessons from the study of the microperoxidases may prove useful for the structural, as well as mechanistic dissection of hemin-containing ribozymes and DNAzymes.

Practical applications

In the past decade, the efficient peroxidase activity of PS2.M-hemin and of hemin complexed with other DNA/RNA G-quadruplexes, have found a versatile practical utility. This review does not attempt to provide a comprehensive account of the range of applications reported for the so-called "peroxidase-mimicking DNAzyme" (excellent reviews on the subject have been written by Willner *et al.*, 2008; and Liu *et al.*, 2009). However, a brief summary of the major applications must include chemical sensing using colorimetry (Kong *et al.*, 2008; Li *et al.*, 2008; Deng *et al.*, 2008; Nakayama and Sintim, 2009); chemical and biological sensing using fluorescence (Nakayama and Sintim, 2010; Qiu *et al.*, 2011); sensing using electrochemistry (Zhang *et al.*, 2010; Yuan *et al.*, 2011) and, immunoblotting (Li *et al.*, 2009b). Both Li *et al.* (2011) and Willner and colleagues (Pavlov *et al.*, 2004; Sharon *et al.*, 2010; Freeman *et al.*, 2010) have used DNA aptamer-hemin complexes to monitor and quantitate the activity of the cancer-related telomerase enzyme. Other applications include bioelectronics (Willner *et al.*, 2007); the construction of a molecular machine (Weizmann *et al.*, 2008); and, use as an electrocatalyst (Pelossoff *et al.*, 2010). Figure 13 illustrates a paradigmatic application reported by Elbaz *et al.* (2008). Here, a DNAzyme cascade (within which a hemin-utilizing DNAzyme is the final component) is used for the amplified detection of lead ions, or of the amino acid, L-histidine.

A completely different kind of utility, and one that is classically associated with horseradish peroxidase, is immunohistochemistry. Recently, Thirstrup and Baird (2010) compared the relative tissue immuno-staining properties of PS2.M-hemin and HRP. Rather than using a non-covalent complex between PS2.M and hemin, these authors covalently linked the porphyrin and DNA, and then used the conjugate for successful staining for the PSA antigen in human prostate tissue sections. Figure 14 shows representative slides from these staining experiments.

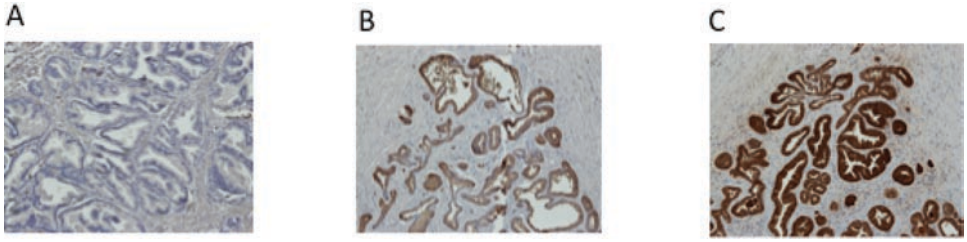

Figure 14. An immunohistochemical application of the peroxidase activity of DNA-hemin complexes. Shown are human prostate tissue sections stained with an anti-prostate specific antigen (PSA) antibody, which have then been developed with peroxidases and diaminobenzidine. (A) No peroxidase control; (B) PS2.M-hemin as peroxidase; and (C) Horseradish peroxidase. These data are shown with the permission of Dr. Geoffrey Baird.

Implications for biology, and future directions

The formation of RNA and DNA G-quadruplexes *in vivo* is a subject of significant current research interest: for instance, chromosomal telomeres within ciliate macronuclei have been shown convincingly to form G-quadruplexes (Paeschke *et al.*, 2008); a DNA G-quadruplex has been implicated in the pilin antigenic variation in *Neisseria gonorrheae* (Cahoon and Seiffert, 2009); and, a diversity of mammalian DNA and RNA sequences, ranging from oncogene promoters to chromosomal telomeres, have also been postulated to form G-quadruplexes *in vivo* (reviewed in Lipps and Rhodes, 2009). On the basis of the results reviewed in this paper, we believe that researchers who are concerned with intracellular G-quadruplexes should take cognizance of the potential for oxidative catalysis by such G-quadruplexes *in vivo*. Atamna *et al.* (2009) state that in the cell, amino acids, peptides, and proteins are also believed to transiently bind the newly synthesized heme. The pool of this transiently bound heme is referred to as "regulatory heme". Given the evidence here of folded RNAs and DNAs that strongly bind heme, the likely pool of intracellular sequestering agents for regulatory heme must now include guanine-rich RNAs and DNAs. Regarding the potentially deleterious effect of heme activation *in vivo*, a particularly interesting observation has been reported recently, that in Alzheimer's disease patients, the toxic agent of the disease, amyloid-β peptide, both sequesters and binds heme (Atamna and Boyle, 2006). This binding leads not only to a functional heme deficiency, but generates a surprisingly strong and potentially toxic intracellular peroxidase activity (Atamna and Boyle, 2006). Given this, it is possible to imagine that certain disease states accumulate an overabundance of guanine-rich transcripts in the cell. Such RNAs may then, like the amyloid-β peptide, serve to sequester heme away from its optimal utilization in the cell, as well as catalyze 1- and 2-electron oxidative reactions detrimental of the cell.

Like other metabolic cofactors that contribute to the enlarged chemical functionality repertoire of enzymes, heme is most likely an ancient compound, a likely player in the postulated RNA world (Yarus, 2002). The body of evidence summarized above–namely, that DNA/RNA-heme complexes efficiently recapitulate at least two of the known catalytic functions of contemporary hemoproteins, lends credence to heme's link to the RNA world. It also raises an obvious question: what *other* properties of hemoproteins, catalytic or otherwise, could be the property of DNA/RNA-heme complexes?

Various synthetic, cationic porphyrins are known to bind to different DNA secondary structures, including to G-quadruplexes; these porphyrins have been found typically to stabilize G-quadruplexes to which they bind (reviewed by Neidle and Read, 2000–2001). Though direct evidence is not yet available, it is plausible that the binding of heme also stabilizes the RNA and DNA quadruplexes, as well as other secondary structural motifs. Recently, much attention has focused on riboswitches, metabolite-binding RNA motifs within mRNAs that serve as feedback modules for the control of different metabolic pathways, including biosynthetic pathways for DNA and RNA bases and enzymatic cofactors (reviewed by Tucker and Breaker, 2005). It will be interesting to see if bioinformatic analyses on the enzymes involved in heme biosynthetic pathways in different organisms uncover riboswitches in these pathways, whose activity is controlled either by the binding of heme itself or by the metabolic precursors of heme.

The relatively simple RNA and DNA quadruplex folds that have been explored to date for their heme-binding and concomitant oxidative properties, likely generate "open" active sites, such as shown in Figure 11. However, creation of more complex RNA and DNA folds (with in-built heme-binding sites) could allow DNA/RNA-heme complexes to approach the quality of substrate usage and reaction specificity that are characteristic of many protein hemoenzymes. Cytochrome P450 enzymes have been regarded for many years as promising "green" catalysts for the synthesis of valuable chemicals on an industrial scale (van Rantwijk and Sheldon, 2000). It is not inconceivable that DNA/RNA-heme complexes in the near future may find the same utility, especially given excellent price and chemical stability advantages they may enjoy over many recombinant proteins.

As summarized briefly above, the practical, *in vitro* utility of the catalytic properties of heme-utilizing ribozymes and DNAzymes has already found numerous and broad ranging outlets. No doubt, more applications will be described in the years to come. To date, most of these applications make use of the peroxidase activity of

these enzymes. It will be interesting to see if the ability of RNA/DNA-heme complexes to also catalyze oxygen transfer reactions will find a comparably fertile ground in terms of application.

Acknowledgments

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Declaration of interest

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