Toward an Efficient DNAzyme[†]

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ABSTRACT: A 33-nucleotide, guanine-rich DNA oligomer, PS5.ST1, has been reported to catalyze the metallation of mesoporphyrin IX (MPIX) by copper and zinc ions. In this paper we report a thorough investigation of the properties of this DNAzyme. We have established that a 24-nucleotide sequence (PS5.M), from within PS5.ST1, is both the minimal and most optimal catalytic unit. We have found that three related porphyrins are acceptable as substrates by this DNAzyme, of which protoporphyrin IX is preferred as a substrate over the expected substrate, MPIX. We have determined that it is unlikely that a strong, catalytically relevant binding site for copper ions exists in the DNAzyme and that high concentrations of copper destroy the active DNAzyme. This enzyme, whose folded structure likely contains guanine quartets, requires potassium ions for activity; we have shown that as little as 1 mM potassium is sufficient for its catalytic robustness, whereas as much as 0.5 M sodium still will not support catalysis. In determining the pH, temperature, and salt optima for the catalyzed reaction, we have found an unexpected stabilizing role for Tris buffer in both the catalyzed and background metallation reactions. As a consequence of various steps of optimization, we now have a vastly improved DNAzyme, one whose enzymatic parameters compare well both with those of natural ferrochelatases, as well as with those of artificially derived chelatases, composed of protein (a catalytic antibody) and RNA. The existence of this array of biocatalysts for porphyrin metallations allows one-to-one comparisons of the ways in which different biopolymers solve a given catalytic problem.

Transition state analogues (TSAs) (Haldane, 1930; Pauling, 1946) for chemical reactions have been successfully utilized for the generation of catalytic antibodies (Tramontano et al., 1986; Pollack et al., 1986), which are able to catalyze a large variety of such reactions [reviewed by Schultz and Lerner (1995)]. The use of TSAs has now been successfully extended, in conjunction with *in vitro* selection-amplification (SELEX) methods (Tuerk & Gold, 1990; Ellington & Szostak, 1990), toward the isolation of novel catalytic nucleic acids. Three successful cases have been reported recently: a catalytic RNA for the isomerization of a bridged biphenyl (Prudent et al., 1994) as well as DNA and RNA catalysts for porphyrin metallation (Li & Sen, 1996; Conn et al., 1996). In the first example, a catalytic RNA molecule for the isomerization of a bridged biphenyl was selected from a random RNA library by virtue of its binding to a TSA for the reaction (Prudent et al., 1994); the ribozyme was modestly catalytic, with a k_{cat}/k_{uncat} value of 90. The successful isolation of this particular ribozyme, however, confirmed that the transition state approach could be used to derive novel RNA catalysts.

In an earlier paper (Li & Sen, 1996), we demonstrated that a comparable methodology could be used to obtain catalytic DNAs as well. We reported a small DNA oligomer, PS5.ST1 (33 nucleotides), which could catalyze the metallation of mesoporphyrin IX (MPIX) by copper and zinc ions. Prior to this, three other catalytic DNAs had been reported (Breaker & Joyce, 1994, 1995; Cuenoud & Szostak, 1995), which carried out, respectively, cleavage of a RNA phosphodiester in the presence of lead (Breaker & Joyce, 1994) and of other cations (Breaker & Joyce, 1995) and a DNA ligase (Cuenoud & Szostak, 1995). However, these earlier DNAzymes had all been derived using "direct selection" techniques (Robertson & Joyce, 1990; Green et al., 1990; Bartel & Szostak, 1993), in which DNA molecules catalytic for a given reaction modify themselves as a consequence of their catalysis and are then purified out of a large pool of noncatalysts by virtue of their self-modification. By contrast, for our derivation of a catalytic DNA for porphyrin metallation (Li et al., 1996; Li & Sen, 1996), we carried out an *in vitro* selection (out of a random library) for DNA binders of *N*-methylmesoporphyrin (NMM), a distorted porphyrin that is a known transition state analogue for porphyrin metallation (Cochran & Schultz, 1990). Following 12 rounds of selection, NMM-binding DNA molecules were cloned, sequenced, and footprinted in the presence of bound NMM to determine the sequences of the binding sites (Li et al., 1996). A series of guanine-rich motifs (15−30 nucleotides long) were found to bind not only NMM, but also other porphyrins and metalloporphyrins [such as Fe(III)-protoporphyrin IX, or hemin], with lesser affinities. The NMM-binding site of one of the aptamers, synthesized as an autonomous oligomer (PS5.ST1), was found to be catalytic for the insertion of Cu^{2+} and Zn^{2+} into MPIX, with a k_{cat} of 13.7 h^{-1}, a K_M of 2.9 mM, and a catalytic ratio (k_{cat}/k_{uncat}) of ∼1400 for copper insertion into MPIX (Li & Sen, 1996). Interestingly, this k_{cat}/k_{uncat} value was comparable to that of a catalytic antibody derived by Cochran and Schultz (1990), using the same transition state analogue. The DNAzyme, however, was approximately 60-fold less efficient at binding and processing MPIX than the catalytic antibody (Cochran & Schultz, 1990), as well as a number of naturally occurring ferrochelatases [enzymes which insert Fe^{2+} ions into proto-

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	DNA Sequence	Relative activity
PS5.ST1	5'-TCGTG GGTCA TTGTG GGTGG GTGTG GCTGG TCC-3'	100
PS5.A	5'-TG GGTCA TTGTG GGTGG GTGTG GCTGG TCC-3'	44
PS5.B	5'-TCA TTGTG GGTGG GTGTG GCTGG TCC-3'	4
PS5.C	5'- TTGTG GGTGG GTGTG GCTGG TCC-3'	15
PS5.E	5'- TG GGTGG GTGTG GCTGG T-3'	4
PS5.F	5'-TCGTG GGTCA TTGTG GGTGG GTGTG GCTGG T-3'	78
PS5.G	5'-TCGTG GGTCA TTGTG GGTGG GTGTG GCT-3'	117
PS5.H	5'-TCGTG GGTCA TTGTG GGTGG GTGT-3'	4
PS5.J	5'-TGG GTGTG GCT-3'	0
PS5.K	5'-GAAGA TCGTG GGTCA TTGTG GGTGG GTGTG GCT-3'	106
PS5.L	5'-GTGTC GAAGATCGTG GGTCA TTGTG GGTGG GTGTG GCT-3'	111
PS5.M	5'-GTG GGTCA TTGTG GGTGG GTGTG G-3'	137
OXY4	5'-TTTTG GGGTT TTGGG GTTTT GGGGT TTTGG GG-3'	0
REP2	5'-AATAC GACTC ACTAT AGGAA GAGAT GGTTT TTCCA TCTCT	0
BLD	5'-AATAC GACTC ACTAT AGGAA GAGAT GG-3'	0

FIGURE 1: Sequences of DNA molecules used to determine the optimal catalytic sequence within the aptamer PS5. REP2 and BLD were control oligomers, not derived from the aptamer selections (Li et al., 1996); OXY4 was a guanine-rich telomeric sequence (Williamson et al., 1989). The relative activity was defined as follows: Relative activity = 100 $(v - v_{bld})/(v_{PS5.ST1} - v_{bld})$, in which $v_{PS5.ST1}$, v_{bld}, and v are the initial rates for PS5.ST1, BLD (reference DNA), and the other oligomers above, respectively.

porphyrin IX (PPIX) in the final step of the heme biosynthetic pathway (Lavallee, 1988)].

In our earlier study (Li & Sen, 1996), we found that the DNAzyme PS5.ST1 had several novel features. These included modest but definite inhibitions of catalytic activity with increasing concentrations of magnesium in the assay buffer and a requirement for potassium ions (which could not be substituted for by sodium ions). This last and the highly guanine-rich sequence (Figure 1) of PS5.ST1, suggested that the folded form of the DNAzyme probably contained guanine quartets, which are stabilized preferentially by potassium ions (Sen & Gilbert, 1990; Williamson et al., 1989). The observed methylation protection of certain guanines in the TSA-binding site of the parent aptamer PS5 (Li et al., 1996) was also consistent with this conjecture.

In this paper, we report a thorough investigation of the properties of this DNAzyme. We wished to establish the most optimal conditions for catalysis by the DNAzyme by systematically examining the roles of the individual components within the binding/assay buffer, as well such parameters as pH and temperature. Another important goal was to determine the elements within the DNA sequence of PS5.ST1 that gave the most optimal catalysis. In addition to the above, we carried out a number of experiments to test the range of porphyrin substrates utilizable by PS5.ST1; to investigate the existence of a catalytically relevant copper-binding site within the DNAzyme; and, finally, to make appropriate comparisons, where these were possible, of the catalytic properties of protein, DNA, and RNA catalysts for porphyrin metallation.

MATERIALS AND METHODS

Materials. Porphyrins were purchased from Porphyrin Products (Logan, UT) and used without further purification, except that in the sodium dependent catalysis experiments mesoporphyrin IX was washed extensively with double-distilled water to remove possible metal ion contaminants. High concentration stock solutions of the various porphyrins were made by directly dissolving the porphyrins in the appropriate buffer solution in which the enzyme activity was going to be assayed. These stocks were stored in the dark at 5 °C. All porphyrin concentrations were quantitated by standard spectroscopic methods (Smith, 1975; Gunter et al., 1989). Under these conditions, the MPIX stock solution was found to be stable for weeks without decomposition or precipitation; stocks of PPIX, however, needed to be made up fresh daily, for these showed a significant decomposition with prolonged storage (assayed by HPLC, data not shown) (Dinello & Chang, 1978).

Purification and Treatment of DNA Oligomers. DNA oligomers were synthesized at the University Core DNA Services at the University of Calgary. Synthesized DNA sequences for DNAzyme assays were purified as follows: the oligomers were size-purified in preparative polyacrylamide gels; the gel slices containing the DNA were eluted with TE buffer (10 mM Tris, pH 7.4, 0.1 mM EDTA) overnight. The eluants were then passed through Spice C18 (Rainin) columns, and the retained DNA was washed with water and eluted with 30% acetonitrile. DNAs purified in this way were lyophilized and dissolved in 10 mM Tris acetate, pH 7.4, and stored at −20 °C. It was important to avoid all salt solutions during the C18 purification procedure because metal ions (Mg^{2+}, Na^+, K^+, and possibly, others) had significant effects on the catalytic rates (see Results and Discussion). For the metallation assays, the DNAzyme oligomer was denatured in 10 mM Tris acetate buffer at 90 °C for 5 min and allowed to cool slowly (over 1 h) to room temperature.

General Assay Protocols. For the HPLC analysis of all metallation reactions involving MPIX, Ni(II)-MPIX was used as an internal standard.

For accuracy in the making up of the final reaction mixtures, each of MPIX, copper, and Triton were assembled from 2× to 20× stock solutions made up in the appropriate buffer solutions. The order of addition of the different reaction components was particularly important. DNA was preincubated with MPIX at the assigned reaction temperature, for 10−20 min, and then combined with appropriate volumes of the same solution containing the copper acetate (all stock buffers were brought to the reaction temperature first). For analysis, aliquots of the reaction mixtures were withdrawn at given times, quenched with 200 mM Tris, pH 9.0, and 15 mM EDTA, mixed with a fixed volume of buffer containing the internal standard, and injected into the HPLC column. Initial rates were calculated from the linear portions (at less that 10% product formation; at least five time points were taken for this portion, and no end-point corrections were made) of product concentration versus time plots. The initial rate measurements were duplicated in most cases (with <15% variation) and average values were taken.

Once quenched, as above, the reaction mixtures were very stable [with the ratio of Cu(II)-MPIX:MPIX remaining virtually unchanged for at least one week after quenching, at 25 °C storage]. The reactions were analyzed by HPLC (a Waters 600E Multisolvent System with a Waters 991 photodiode array detector), at 25 °C. The column used was a μBondapak C18 10 μm, and the mobile phase was 85% methanol and 15% 1 M ammonium acetate, pH 5.2.

Protocols for Individual Experiments: Substrate Usage . The metallation reactions were carried out in SB buffer [100 mM Tris, pH 7.1 (note: all pH values in this report were for the final solution, and measured at 20 °C), 200 mM NaOAc, 25 mM KOAc, 10 mM $Mg(OAc)_2$, 5% DMSO, 0.5% Triton X-100]. PS5.ST1 was at 5 μM, and the Cu^{2+} and porphyrin ([S]) concentrations were as indicated in Table 2. The reactions were allowed to proceed at 25 °C. HPLC

conditions used to separate and analyze the different porphyrins and their product metalloporphyrins are summarized in Table 5.

Sequence Optimization. The reaction was carried out at 25 °C in 225KB buffer (100 mM Tris, pH 7.1, 225 mM KOAc, 5% DMSO, and 0.5% Triton X-100), with 33 μM MPIX and 5 μM DNA. All the DNA oligomers had been gel-purified and recovered by ethanol precipitation.

pH Dependences. Reactions were carried out under conditions similar to those described for the sequence optimization experiments, except that the buffers were as follows: For buffers at pH ≥7.0, 100 mM Tris was used; for pH 5.5−7.0, 100 mM MES was used; for pH 4.0−5.6, 50 mM potassium acetate buffer was used (the MES and acetate buffers also contained 100 mM Tris acetate). Each buffer also contained 50 mM potassium acetate, 5% DMSO, and 0.5% Triton X-100. Individual buffers were made up as 2× stocks, and their pH was adjusted by titrating with 10% acetic acid. The MES buffers (containing Tris acetate) appeared to buffer their specified pH stably.

Temperature Dependence. The reactions were performed in a thermal cycler (utilized as a constant temperature bath). The buffer used was the MES-Tris buffer 50KB (100 mM MES, pH 6.2, 50 mM KOAc, 100 mM Tris acetate, 5% DMSO, and 0.5% Triton X-100), with DNA at 5 μM, MPIX at 33 μM, and $Cu(OAc)_2$ at 1 mM. Metallation initial rates were measured at 0, 5, 15, 20, 25, 35, 40, 45, 55, and 65 °C.

DNA Concentration Dependence and $Cu(OAc)_2$ Concentration Dependence. These were both carried out at 15 °C, under the same conditions as described for the temperature dependence experiments, except that the DNA concentrations and Cu(II) concentrations were varied, respectively. For the copper ion concentration assays, the DNA was incubated first with MPIX, for 10−20 min, followed by addition of the copper acetate to start the reaction. Reactions in which the copper was added first gave unpredictable and irregular results.

The DNA preincubation experiments in the presence of copper were carried out as follows. The DNAzyme samples, in 50KB buffer, had copper acetate added to them to final concentrations of 2, 10, and 20 mM, respectively. After an appointed period of preincubation, aliquots were withdrawn and mixed with an equal volume of 66 μM MPIX (2× MPIX) in the same buffer and the metallation reactions allowed to proceed for 20, 3, and 1 min, respectively. The analysis of product and substrate was carried out by HPLC, as usual.

To monitor for precipitation of the DNA following the copper preincubations, ^{32}P-labeled PS5.M was used, and the samples were spun in a microfuge at 13 000 rpm. The counts remaining in solution were monitored by scintillation counting.

DMSO and Triton X-100 Concentration Dependences. The experimental conditions were as described for the temperature dependence experiments, at 15 °C, except that the DMSO and, separately, Triton X-100 concentrations of the buffer were varied. DMSO concentrations (v/v%) of 0, 0.8, 2.5, 5, 10, and 20% were assayed, as were Triton X-100 concentrations of 0.08, 0.25, 0.5, 1.0, and 2.5% (w/v).

Buffer Effects. These were tested with 2.5 μM DNA, 33 μM MPIX, 50 mM KOAc, 0.25% Triton X-100, and 1% DMSO held constant. The buffers used were made up as described in the pH dependence section.

Sodium and Potassium Dependences. They were carried out at 25 °C, in 50 mM Tris, pH 7.3, 1% DMSO, and 0.25% Triton, with variations in the concentrations of either sodium (at 0, 5, 10, 25, 50, 100, 225, and 500 mM) or potassium (at 0.1, 1, 10, 25, 50, 100, and 200 mM).

k_{cat} and K_M Measurements for MPIX and PPIX. These were carried out at 15 °C, in 40KB buffer (see text), with DNA at 1.5 μM and $Cu(OAc)_2$ at 1.0 mM. The initial rates were determined for both enzymatic and background reactions at 10, 15, 20, 30, 45, 70, and 100 μM MPIX and, separately, at 3.5, 5.0, 7.0, 10.0, 14.0, and 20.0 μM PPIX, respectively. The k_{cat} and K_M values were calculated using the GraFit 3.0 program.

RESULTS AND DISCUSSION

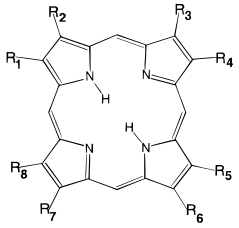
Substrate Range of PS5.ST1. Although PS5.ST1 had been derived to have the porphyrin MPIX as its expected substrate (on the basis of the fact that PS5 had been selected for binding to methylated MPIX, i.e., NMM), our previous study (Li & Sen, 1996) had indicated that the porphyrin PPIX was possibly an even better substrate for PS5.ST1 than MPIX. We therefore examined six different porphyrins to see if they were acceptable as substrates by PS5.ST1, for copper insertions. Table 1 summarizes their names and structures. Experiments were carried out in SB buffer, at 25 °C, in the presence of either 5 μM PS5.ST1 or 5 μM noncatalytic (Li & Sen, 1996) oligomer, REP2 (see Figure 1).

Table 2 shows the results for both the enzymatic and nonenzymatic reactions. The acceptable substrates for PS5.ST1 are MPIX, PPIX, and DPIX, whereas the other porphyrins are not. The three substrate porphyrins are closely related to one another in structure; however, HPIX (which differs from MPIX in the replacement of one secondary hydrogen atom from each of the two ethyl groups, at positions 2 and 4, by a hydroxyl group) is not a substrate. Thus, the acceptable functionalities at positions 2 and 4 of the porphyrin ring may be vinyl or ethyl or hydrogen (as in DPIX); however, the presence of the hyrdoxyl groups in HPIX abolishes catalysis. The experiments above do not reveal whether HPIX simply does not bind to PS5.ST1, or whether, having bound, it is not amenable to the structural distortion necessary for catalysis. Conceivably, the hydroxyl groups could interfere with binding into the active site by being sterically larger than the hydrogen atoms they replace or by introducing polar entities into what might be a hydrophobic region of the active site. Future experiments with other porphyrins, and mutagenesis of PS5.ST1, should clarify these findings.

The finding that CPIII and UPIII (Table 1) were not substrates could be rationalized in terms of their divergent structures, the high degree of steric clash that their extra carboxylic groups might encounter in the active site, and electrostatic repulsion between the highly negatively charged DNA and the carboxylates.

In terms of substrate usage, PS5.ST1 shows both similarities and differences with natural ferrochelatases (from various sources) in that both appear to act on MPIX, PPIX, and DPIX (Taketani & Tokunaga, 1981, 1982; Dailey & Smith, 1984; Lavallee, 1988) but not on coproporphyrin III (Taketani & Tokunaga, 1981, 1982). However, the ferrochelatases also

Table 1: Porphyrins Tested as Substrates[a]



porphyrin	R1	R2	R3	R4	R5	R6	R7	R8
mesoporphyrin IX (MPIX)	Me	Et	Me	Et	Me	P^H	P^H	Me
protoporphyrin IX (PPIX)	Me	V	Me	V	Me	P^H	P^H	Me
deuteroporphyrin IX (DPIX)	Me	H	Me	H	Me	P^H	P^H	Me
hematoporphyrin IX (HPIX)	Me	CH(OH)Me	Me	CH(OH)Me	Me	P^H	P^H	Me
coproporphyrin III (CPIII)	Me	P^H	Me	P^H	Me	P^H	P^H	Me
uroporphyrin III (UPIII)	A^H	P^H	A^H	P^H	A^H	P^H	P^H	A^H

[a] Names, abbreviations, and side-chains (R1−R8). Side-chain abbreviations: Me = methyl; Et = ethyl; V = vinyl; P^H = CH_2CH_2COOH; A^H = CH_2COOH. Side chains R6 and R7 = P^H for all porphyrins.

Table 2: Rate Parameters for DNAzyme-Catalyzed and Background (Noncatalyzed) Metallations of Different Porphyrins

porphyrin	[S], μM	[Cu(II)], mM	V_{total} μM/h	V_{uncat} μM/h	V_{total}/V_{uncat}
MPIX	100	1.0	3.71	0.82	4.5
PPIX	100	1.0	10.55	1.06	9.9
DPIX	100	1.0	20.25	5.51	3.7
HPIX	100	1.0	95	95	1
CPIII	50	0.2	600	600	1
UPIII	50	0.2	1200	1200	1

use hematoporphyrin as a substrate, at the same level as protoporphyrin (Taketani & Tokunaga, 1982; Dailey & Smith, 1984; Lavallee, 1988). And, by contrast to both the DNAzyme and the ferrochelatases, the catalytic antibody for porphyrin metallation (Cochran & Schultz, 1990) accepts only MPIX as substrate. No data on substrate usage have been reported for a recently described catalytic RNA for porphyrin metallation (Conn et al., 1996). Although the high substrate specificity of the catalytic antibody was consistent with the properties of many protein active sites and binding sites in general, recent papers have also reported highly specific bindings of small organic ligands by RNA aptamers (Famulok & Szostak, 1992; Jenison et al., 1994; Geiger et al., 1996). The nature of interactions between active sites and substrates in the different DNA, RNA, and protein catalysts for metallation remains, therefore, an important area for investigation in the future.

Optimally Catalytic Fragment of Aptamer PS5. The 5′ and 3′ boundaries of the PS5.ST1 fragment of the 112-nucleotide, and NMM-binding, aptamer PS5 (Li et al., 1996; Li & Sen, 1996) had been chosen initially on the basis of footprinting studies carried out on PS5 [in the presence of bound NMM and hemin (Li et al., 1996)]. Those data had indicated that the first G-motif (underlined, below) within PS5.ST1 (5′-TC**GTG GGTCA TTGTG GGTGG GTGTG G***CTGG TCC*-3′), was not footprinted (whereas the guanines in the sequence not underlined did footprint). However, the 5′ motif, being directly adjacent to the footprinted region within PS5, had been included in the design of PS5.ST1. To determine whether the motif was required after all, and to pinpoint the most optimal catalytic sequence within PS5, a number of oligomers centered around the footprinted region were synthesized (Figure 1) and tested for their ability to catalyze metallation. The results are summarized in Figure 1. Even the deletion of TCG from the 5′ end (as in PS5.A) resulted in a 50% loss in activity, whereas the deletion of the entire 5′-most G-motif (as in PS5.B) essentially abolished catalytic activity. By contrast, the 3′-most G-motif (italicized above), which footprinted in the presence of NMM in PS5 (Li et al., 1996), appeared not to be important for catalysis. The optimal (and minimal) catalytic sequence therefore corresponded to the 24-nucleotide, very guanine-rich (14 guanines out of 24 bases) oligomer PS5.M (shown in bold type above), and this was the sequence chosen for the further studies described below.

Two points of interest are raised by the above results: (a) that the NMM-footprinted region within PS5 and the catalytic sequence, overlap but do not coincide and (b) that the relatively low catalytic activity of the parent sequence, PS5, compared to those of the fragment PS5.ST1 (and PS5.M), may be connected with somewhat different folding patterns for the two. Regarding point a, it is conceivable that the guanines identified by methylation protection in PS5 (Li et al., 1996) participate purely in the DNA folding (i.e., that the footprint reflects intra-DNA interactions, rather than DNA−NMM interactions). If so, some residues that did not footprint could yet be involved in NMM binding, and therefore required for catalysis. As for point b, it may be that PS5 on the one hand, and the PS5.M and PS5.ST1 sequences on the other, fold similarly but not identically, leading to differences in catalytic activity, or else that the noncatalytic elements of PS5 interfere with the diffusion of substrate molecules into the active site. Detailed studies to resolve these possibilities are underway.

Optimal pH for Catalysis. The effectiveness of catalysis by PS5.M in the pH range of 4−8 was examined. Figure 2 shows the velocities for the PS5.M-catalyzed and the uncatalyzed (in the presence of the control oligomer BLD; Figure 1) reactions. For our original experiments carried out around neutral pH (in SB buffer), Tris had been the buffer used. However, preliminary measurements carried out at non-neutral pHs, using other buffering agents (such as MES), showed sharp, and buffer-specific, changes in the background reaction rates. We found that the inclusion of a certain concentration of Tris (as Tris acetate) in the MES-buffered acidic solutions appeared to counter this effect (discussed

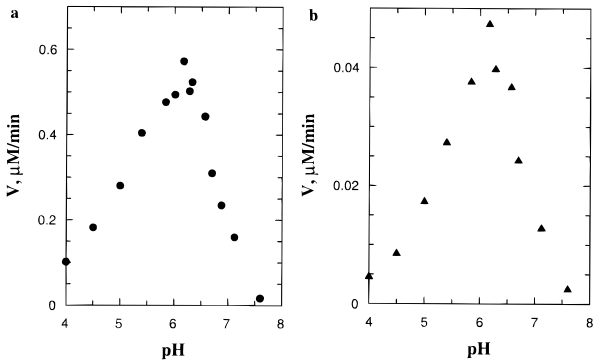


FIGURE 2: pH profiles of the DNAzyme-catalyzed and background (noncatalyzed) metallations. (a) v_{cat} plotted against pH for the PS5.M-catalyzed reaction (●), and (b) v_{uncat} plotted against pH for the uncatalyzed reaction, in the presence of the control oligomer BLD (▲). The experimental protocols are described in Materials and Methods.

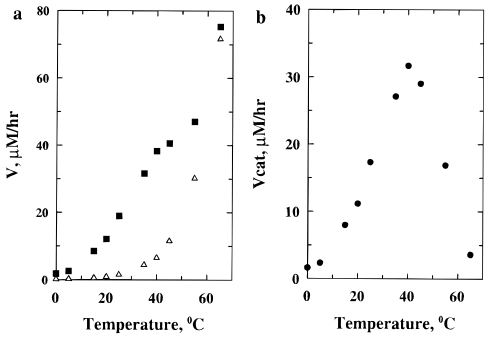


FIGURE 3: Temperature profiles of the DNAzyme-catalyzed and background (non-catalyzed) metallations. (a) v_{total} (■), in the presence of 5 μM PS5.M; and v_{uncat} (△), in the presence of 5 μM BLD) versus temperatures. (b) v_{cat} (●; $v_{cat} = v_{total} - v_{uncat}$) vs temperature. The experimental conditions are outlined in Materials and Methods.

in detail in a later section on buffer-specific effects). Therefore, for pH values > 7.0, 100 mM Tris was used for buffering, whereas below pH 7.0, 100 mM MES (between pH 5.5 and 7.0) or potassium acetate (between pH 4.0 and 5.5) was used (in each case, containing 100 mM Tris acetate, in addition to the other metallation reaction components such as potassium acetate, DMSO, and Triton X-100).

Figure 2 shows that both the PS5.M-catalyzed and the background rates peaked at around pH 6.2. The DNA-mediated catalysis mechanism appears to retain the pH dependence of the uncatalyzed reaction. The bell-shaped profiles for both the catalyzed and background rates could reflect a number of events, including the increase in rates from pH 7.6 to 6.2 correlating with progressively lower concentrations of the unprotonated Tris base [Tris base is reported to chelate weakly with Cu^{2+} ions (Hall et al., 1971; Sun & Martell, 1969)]. In addition, at the basic end of the spectrum, the copper complexes would become resistant to losing water (necessary for metallation) by virtue of becoming hydroxy complexes [the first pK_a of a Cu^{2+}-bound water is ~6−7 (Burgess, 1978)]; this, too, might be reflected in the overall metallation rate. On the other side of the curve, the drop in rate from pH 6.2 to 4.2 may reflect the protonation of a pyrollic nitrogen atom of the porphyrin (Smith, 1975). The slope of log(V) vs pH is only ~0.3, far smaller than 1.0, and this suggests that no proton transfer takes place in the transition state.

Interestingly, a pH of approximately 8.0 was found to be optimal for catalysis by the ferrochelatases [utilizing as substrates MPIX and Zn^{2+} (Li et al., 1987), MPIX and Co^{2+} (Roberts, 1987), as well as MPIX and/or PPIX with Fe^{2+} (Taketani & Tokunaga, 1981; Labbe & Hubbard, 1960; Goldin & Little, 1969; Porra & Jones, 1963; Krueger et al., 1956)]. However, none of these studies reported the pH profiles for the *background*, uncatalyzed reactions, leaving it uncertain whether the pH had a direct influence on the structure and activity of these enzymes. Separate studies on the nonenzymatic formation of mesoheme [Fe(II)-MPIX] reported pH optima of 9.5 (Kassner & Walchak, 1973) and 8.8 (Taketani & Tokunaga, 1984), respectively, under similar experimental conditions as used for the ferrochelatase studies. Thus, it was conceivable that the reported pH optima of 8.0 for the ferrochelatases did reflect attributes of the enzymes themselves.

On the basis of our own finding of a pH optimum of 6.2 for metallation (under our experimental conditions), this pH was used for all subsequent optimization experiments.

Catalyzed Rate vs Temperature. We investigated the rates of metallation of MPIX by copper at different temperatures, in the presence of PS5.M, or of the control oligomer BLD. Figure 3a plots rate as a function of temperature for both the total (background plus catalyzed) reaction and for the background reaction itself. Figure 3b shows the net catalyzed rate, v_{cat} [obtained by subtracting the background rate, v_{uncat}, measured in the presence of BLD from the total rate, v_{total} (equal to $v_{cat} + v_{uncat}$), measured in the presence of PS5.M]. The background rate increases exponentially with temperature (Figure 3a), whereas the net catalyzed rate (Figure 3b) shows an approximately bell-shaped profile, with a maximum at 40 °C. The bell-shaped catalyzed rate profile may be modeled as a balance of two opposed tendencies: the natural temperature dependent increase of the catalyzed rate, combined with a progressive instability, and tendency to denaturation, of the folded PS5.M. In addition, G-quadruplex structures [such as PS5.ST1, Li and Sen (1996)] are a highly polymorphic class of structures, which are able to interconvert with changes in conditions. A preliminary melting curve of PS5.M (data not shown) appears to be consistent with this notion. We are currently carrying out experiments to address these structure-related questions. Interestingly, the ratio v_{cat}/v_{uncat} reaches a maximum at 15 °C. For our remaining experiments in this paper, we picked 15 °C as the reaction temperature (at which temperature it was possible to measure accurately a number of very fast catalyzed reactions, described in later sections).

Catalyzed Rate as a Function of Enzyme Concentration. An important test for the catalytic behavior of any putative enzyme is that at constant substrate concentrations (and with all other parameters remaining unchanged), the rate of the catalyzed reaction is directly proportional to the concentration of the enzyme. Figure 4 shows the catalyzed (total minus background) initial rates for different concentrations of PS5.M, measured at two different, and fixed, MPIX concentrations: 33 and 100 μM. Linear relationships between rates and DNAzyme concentrations were obtained in both cases, demonstrating unequivocally the enzymatic aspect of PS5.M.

In carrying out these experiments, we observed anomalous results if the DNA had been purified in a certain way. Thus, at the higher DNA concentrations in particular, residual Mg^{2+}

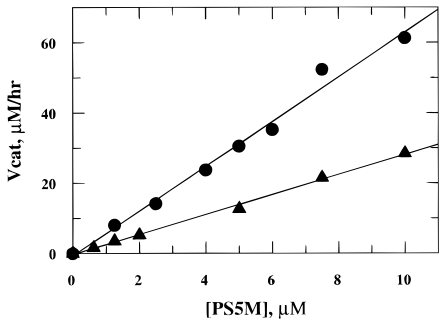


FIGURE 4: v_{cat} (catalyzed initial rates) as a function of PS5.M concentration. The v_{cat} values were obtained at fixed MPIX concentrations of 33 μM (▲) and 100 μM (●). The reaction conditions are detailed in Materials and Methods.

ions from ethanol precipitations were found to have an inhibitory effect on the catalysis. Elimination of the ethanol precipitation steps entirely (and the purification of the synthetic DNA oligomers on a desalting G-50 column, followed by water elution and vacuum lyophilization) led to the data reported above.

Is There a Binding Site for Copper at the Active Site of PS5.M? An important question that had remained unresolved in our previous report (Li & Sen, 1996) concerned the existence of a catalytically relevant binding site for a copper ion at the DNAzyme's active site. The dependence of the rate of reaction, at constant MPIX concentration, on copper concentration up to 2.5 mM had given a straight line plot, with no evident sign of saturation. This appeared to indicate that there was no strong, catalytically relevant copper-binding site in the DNAzyme (as with the catalytic antibody, but unlike the ferrochelatases). However, the possibility that a copper-binding site with a K_M value larger than 2.5 mM existed could not rigorously be ruled out.

We therefore carried out metallation experiments in 50KB buffer (100 mM MES, pH 6.2, 100 mM Tris acetate, 50 mM potassium acetate, 5% DMSO, and 0.5% Triton X-100) and varied the copper ion concentration from 0 up to 20 mM. Initial rates for the metallation of MPIX (itself at a fixed concentration of 33 μM) were then measured. During the initial rate measurements it was found that for copper concentrations up to 10 mM, the formation of Cu-MPIX was reliably linear with time usually below 10% conversion levels of substrate (for both the enzymatic and the nonenzymatic reactions). With copper above 10 mM, linear initial rates could not be measured at all, for the formation curves leveled off even before 5% of the substrate had been converted to product.

Figure 5a shows the plots of the initial rates for both the PS5.M-catalyzed and the background (in the presence of the oligomer BLD) reactions; the rate of the catalyzed reaction was linear with copper concentration up to 5 mM copper, beyond which there appeared signs of a gradual saturation of rate, indicating that there might be a catalytically relevant but weak copper-binding site in PS5.M. However, copper ions have been shown, in the absence of other salts, to be potent denaturants of double-stranded DNA (Eichhorn & Shin, 1968), and the "saturation kinetics" seen in Figure 5a would also be consistent with the destruction of the active enzyme at high copper concentrations.

To distinguish between the two possibilities, we carried out experiments in which we preincubated the folded DNAzyme in buffer containing copper ions at 2, 10, and 20

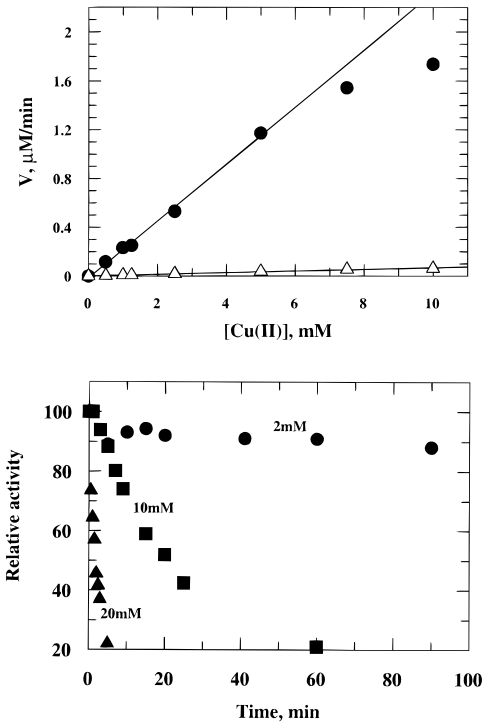


FIGURE 5: (a) Metallation rates as functions of $Cu(OAc)_2$ concentrations. The metallations were carried out with a fixed MPIX concentration of 33 μM, and 5 μM of either PS5.M (●) or BLD (△). Other conditions are detailed in Materials and Methods. (b) The enzymatic activity of PS5.M after preincubation with copper ions. The preincubations were carried out with $Cu(OAc)_2$ at 2 mM (●), 10 mM (■), and 20 mM (▲), respectively, for the times indicated. Metallation reactions were carried out with PS5.M at 5 μM and MPIX at 33 μM. The detailed protocols are given in Materials and Methods.

mM, with each of these preincubations being carried out for a number of different time points. Following each preincubation, one volume of the copper- and PS5.M-containing solutions (in reaction buffer) were mixed with 1 volume of reaction buffer containing 66 μM MPIX (to give final MPIX concentrations of 33 μM). The measured initial rates of the various preincubated samples, as a function of the time of preincubation, are shown in Figure 5b. Enzyme samples preincubated in 2 mM copper gave approximately the same initial rate, regardless of the length of the preincubation; however, in the 10 mM preincubations, the enzyme activity (as indicated by initial rates of the catalyzed reaction) dropped to 50% after as little as 20 min of preincubation. With the 20 mM copper samples, the initial rates dropped to 50% after only 2 min of preincubation.

These data demonstrated that the higher concentrations of copper clearly had a destructive effect on the active form of the DNAzyme. It was therefore likely that the "saturation" of rate seen in Figure 5a did not reflect a real saturation of a binding site for copper in the DNAzyme, but was, rather, the consequence of a time dependent inactivation of DNAzyme in the high-concentration copper samples. Clearly, the criterion of saturation kinetics could not be used in this case to test for a copper-binding site. Other experiments are currently being designed to detect whether such a copper-binding site does in fact exist.

To test whether the DNA was in fact precipitated in these samples, the samples (containing ^{32}P-labeled PS5.M) were centrifuged in a microfuge for extended periods. However,

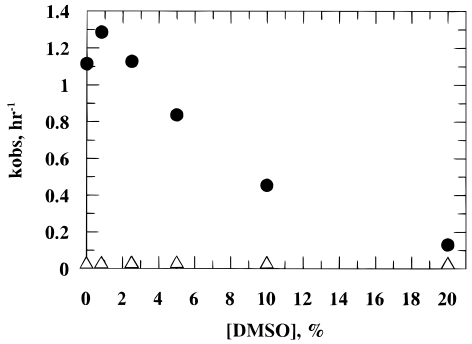


FIGURE 6: Observed rate constants for metallation, k_{obs}, plotted as a function of dimethyl sulfoxide (DMSO) concentration. k_{obs} for metallation reactions was obtained at various DMSO concentrations, in the presence of 2.5 μM PS5.M (●), or BLD (△). The detailed reaction conditions are outlined in Materials and Methods.

no radioactive pellet was detected. Therefore, the suspected DNA−copper aggregates, if indeed formed, were not sufficiently insoluble to precipitate. Another possibility is that, instead of forming DNA−copper aggregates, copper may destroy the *catalytically active* folded form of the DNA folding by competing for essential hydrogen-bonding sites in the DNA.

As an interesting note, we observed that the prior addition of the substrate MPIX to the DNAzyme (before the addition of copper) substantially preserved the DNAzyme from denaturation (data not shown). It was therefore a good practice for the study of the metallation reactions (particularly at high copper concentrations) to incubate the DNAzyme first with MPIX for 10−20 min to allow the formation of DNA−porphyrin complexes, prior to initiating the reaction by introducing copper.

Experiments were also carried out to determine whether the inactivation by copper was reversible, whether catalytic activity could be recovered by decreasing the copper ion concentration. Our results (data not shown) indicated that the inactivation was irreversible. Future experiments will focus on establishing the mode of copper-mediated inactivation of the DNAzyme, i.e., whether it is the result of DNA denaturation or of a copper-mediated free radical destruction of the DNA or both.

Effects of Dimethyl Sulfoxide and of Detergent on Catalysis. All the buffers described thus far for the assaying of catalysis contained 5% dimethyl sulfoxide (DMSO) and 0.5% of the nonionic detergent, Triton X-100. The reason for their inclusion was the presumption that they would help increase the overall solubility of the sparingly soluble substrate, MPIX. In addition, Triton X-100 and other nonionic detergents have been widely used in kinetic studies of metallation reactions (Cochran & Schultz, 1990; Taketani & Tokunaga, 1981, 1984; Li et al., 1987; Roberts, 1987; Labbe & Hubbard, 1960; Goldin & Little, 1969; Porra & Jones, 1963; Krueger et al., 1956; Kassner & Walchak, 1973), mainly because they were found to reduce the background metallation rate and helped prevent the aggregation of the dissolved hydrophobic porphyrins (Li et al., 1987). In the course of these studies, we observed that the presence of the 5% DMSO could be dispensed with as long as the porphyrin stocks were made directly in a Tris acetate buffer.

We therefore investigated the effect of the 5% DMSO, and the presence of DMSO in general, upon catalysis by PS5.M. Figure 6 shows the k_{obs} values for metallation at various DMSO concentrations, for both the DNA-catalyzed and the background reactions carried out in 50KB buffer, pH 6.2. Even though DMSO had little effect on the background reaction, its impact on the catalytic activity of PS5.M appeared profound. At 20% DMSO, the catalysis was only ∼10% as effective as found at 1% DMSO, which, rather surprisingly, gave the highest level of catalysis. The progressive denaturation of folded nucleic acid structures in the presence of increasing concentrations of ethanol and other organic solvents has been exhaustively documented. The decrease of the DNAzyme's activity at higher DMSO concentrations might be due to this, or due to changes in solvation of the DNA, the porphyrin, and the copper ions. It is interesting to note here, however, that even at 20% DMSO, the catalysis by PS5.M is not totally abolished; it is therefore conceivable, that with a greater stabilization of the DNAzyme's folded structure (by changes in sequence or by other means), catalysis of metallation may be observable at quite high concentations of DMSO and other organic solvents.

We also tested the effect of increasing concentrations of Triton X-100 on catalysis (data not shown). Increasing detergent concentrations above 0.25% appeared to reduce both the catalyzed and background rates exponentially. Below 0.25% Triton, however, the background reaction appeared to be unaffected; therefore, k_{obs}(catalyzed)/k_{obs}(uncatalyzed) was at a theoretical maximum with the detergent completely removed. However, the complete removal of detergent was not practical, owing to its requirement for substrate solubilization. Therefore, 0.25% Triton, as well as 1% DMSO, were retained in buffers for our further investigations.

Buffer Effects on Catalysis. In the section on the pH optimization of catalysis (above), we discussed the importance of Tris to porphyrin metallation, as carried out under our experimental conditions. Tris, which buffers effectively betwen pH 7 and 9, has also been used widely for the study of ferrochelatase enzymes. In our own rate vs pH measurements (above), we found that the presence of >50 mM Tris acetate in otherwise MES-buffered acidic solutions preserved the "regular", or monotonic, behavior of the measured initial rates for both catalyzed and background metallations of MPIX with copper. Figure 7 illustrates the behavior of initial rates in a solution buffered by 100 mM MES, pH 6.2, as compared to one buffered by 100 mM MES, pH 6.2, but containing in addition, 50 mM Tris acetate (the effective buffering capacity of MES lies between pH 5.5 and 6.8). Figure 7a shows the PS5.M-catalyzed rates in both buffers (which both also contained potassium acetate and the other buffer components) as functions of time, and Figure 7b shows the background rates. These figures show that in the MES-only buffer, the background rate was much slower and curved off earlier than that in the MES buffer containing Tris acetate, whereas the catalyzed reaction in the MES-only buffer experienced an early burst up to 10% conversion of the substrate to product, and then slowed dramatically, to the point where the metallation reaction had virtually stopped. In MES buffers containing Tris acetate at any level above 50 mM, by contrast, both the enzymatic and nonenzymatic reactions proceeded smoothly with time and appeared to go on to completion.

Tris base has the ability to chelate Cu^{2+} in aqueous solution with modest equilibrium constants (with log K values of ∼3−4) (Hall et al., 1971; Sun & Martell, 1969). It is therefore

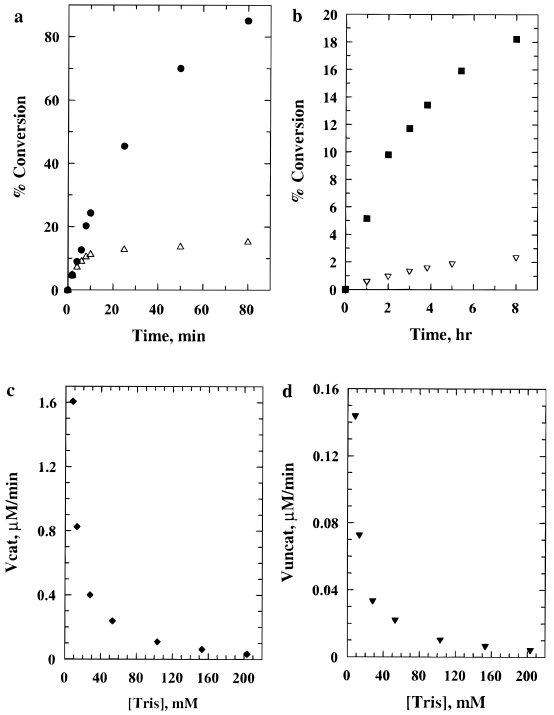


FIGURE 7: Percentage conversions of substrate to product, measured in different buffer solutions, and plotted as functions of time. (a) Time dependence plots for PS5.M-catalyzed metallations in 100 mM MES, pH 6.2, + 50 mM Tris acetate (●); and in 100 mM MES, pH 6.2 (△). (b) Analogous time dependence plots for the background (noncatalyzed) metallations in the MES-Tris buffer (■) and in the MES-only buffer (▽). (c) v_{cat} (◆) and (d) v_{uncat} (▼) of metallations carried out in Tris-only buffers (pH 7.3), as functions of Tris concentration. Detailed reaction conditions are given in Materials and Methods.

likely that in the presence of Tris, the Cu^{2+} ion exists substantially as Cu^{2+}−Tris complexes. Because of the relatively modest complexing constant, such complexes would still dissociate relatively easily to give hydrated copper ions, which in turn could be chelated by the porphyrins [the formation constants for the porphyrin metallation reactions are, by contrast, very high, for example, log $K_f \geq 25$ for HPIX with $Zn(OAc)_2$, at 25 °C (Smith, 1975)]. It has been reported that MES, too, forms coordination complexes with copper(II) ions, but with a smaller formation constant (log $K = 1.39$) (Balikugeri, 1989). Therefore, in MES-only buffers, the hydrated copper ions may begin to chelate instead with the *carboxylate* groups of the porphyrin, to form aggregated complexes (we observed a discernable color change of the dissolved porphyrin in these situations). Assuming that the formation of such porphyrin−copper aggregates was not instantaneous, we would still observe experimentally the initial burst of the catalyzed reaction, seen in Figure 7a, during the first few minutes of the reaction. The presence of relatively high concentrations of Tris in the buffer, on the other hand, and the formation of Tris base−Cu^{2+} complexes, might preclude the formation of such porphyrin−copper aggregates.

Of course, the chelation of copper ions by Tris base might be expected to slow down the overall metallation reaction. We examined the effect on metallation rate of raising Tris concentrations in purely Tris buffers (pH 7.3) containing the usual potassium acetate and DMSO and Triton X-100. Figure 7, panels c and d, shows data for the PS5.M-catalyzed and background reactions, respectively; both rates decrease exponentially with increases in Tris concentration. Interestingly, replotting the rates as reciprocal functions of the anticipated concentration of free Tris *base* in these buffers (V vs 1/[Tris base]) shows straight line (data not shown), suggesting that the chelation of copper by Tris base was in fact a factor in the kinetics of metallation.

By contrast, increasing MES concentrations in MES-buffered solutions at pH 6.2 (all solutions also containing Tris acetate, at 50 mM) did not significantly change the metallation rates.

In summary, our data suggested that Tris played two roles in the porphyrin metallation reactions (in both the catalyzed and background versions). First, it chelated copper ions and reduced the formation of presumptive "unproductive" copper−porphyrin aggregates, and, second, it reduced the overall metallation rates.

Contrasting Effects of Sodium and Potassium Ions on Catalysis. In our previous paper (Li & Sen, 1996), we reported data that sodium and potassium ions had contrasting roles in the catalysis by PS5.ST1: K^+ was absolutely necessary for catalysis whereas Na^+, even up to 225 mM concentrations, was ineffective in supporting catalysis. We also found that in the absence of sodium and magnesium, 25−50 mM K^+ was optimal for catalysis. This striking difference in the requirements for potassium and sodium, the heavily guanine-rich sequence of the DNAzyme, as well as methylation protection evidence from the parent aptamer, PS5 (Li et al., 1996), had cumulatively suggested that the folded and active form of the DNAzyme might contain guanine quartets [which are significantly better stabilized by potassium ions than by sodium ions (Sen & Gilbert, 1990; Williamson et al., 1989; Sundquist & Klug, 1989)].

Na^+ ions, however, do support the formation and stabilization of G quartets, except that significantly higher concentrations are needed than K^+ ions required for a comparable task (Sen & Gilbert, 1990). The free energy *difference* for quadruplex formation by $d(G_3T_4G_3)_2$ in 100 mM NaCl and in 100 mM KCl has been reported to be −4.2 kcal/mol at 25 °C (Scaria et al., 1992), corresponding to an equilibrium constant ratio in excess of 10^3. Besides, there is considerable evidence for the occasional formation of *different* G quadruplex structures by the same oligomer in potassium solutions vs sodium solutions (Sen & Gilbert, 1990; Venczel & Sen, 1993). In measuring catalytic rates of PS5.M across a large sodium concentration range (0, 5, 10, 25, 50, 100, 225, and 500 mM), we found catalysis to be nowhere higher than 10−15% above the background rate (data not shown). This appeared to support our original conjecture (Li & Sen, 1996) that the folded structure of the DNAzyme contained probably two or fewer complete G quartets (which could exist stably only in the presence of potassium ions). The other possibilty is, of course, that in sodium-only solutions PS5.M folds to form a folded structure different from the catalytically active one.

We wished to test whether the optimal K^+ concentration required for catalysis had changed following the various steps taken above for the optimization of catalysis. In addition, we wished to establish how little potassium was needed to support a significant catalysis. Reactions were therefore carried out in 50 mM Tris acetate, pH 7.3, 1% DMSO, 0.25% Triton, and varying concentrations of potassium acetate. Figure 8 shows the PS5.M-catalyzed and background rates as functions of potassium concentrations up to 200 mM. The

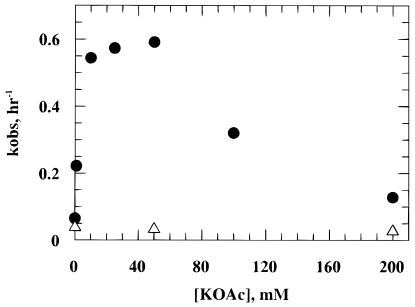


FIGURE 8: Metallation rates plotted against potassium ion concentrations. (a) k_{obs} for the catalyzed (in the presence of 2.5 μM PS5.M, ●); and, background (in the presence of 2.5 μM BLD, △) reactions.

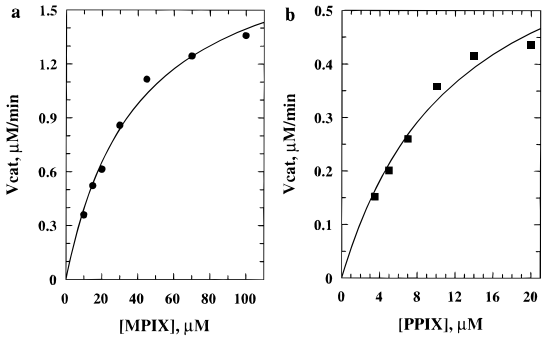


FIGURE 9: Initial rates of metallation plotted against substrate porphyrin (MPIX and PPIX) concentrations. (a) PS5.M-catalyzed rates (v_{cat}, equal to $v_{total} - v_{uncat.}$) for Cu-MPIX formation, plotted against [MPIX], in the presence of 1.5 μM PS5.M (●). (b) Analogous plot for the formation of Cu-PPIX (■).

DNAzyme appeared to function well even at very low (~10 mM) potassium concentrations, and the optimal activity extended at least to 50 mM K^+. At the lower end, in as little as 0.1 mM K^+, the enzyme activity was 70% above background; at 1 mM K^+, it had increased to 480% and at 10 mM K^+, up to 1400%. At higher than 50 mM K^+, the catalytic activity began to decrease, but more slowly than the pace of its rise at lower potassium concentrations.

Optimized Enzymatic Parameters for the Utilization of MPIX and PPIX as Substrates. The above process of optimization of the various parameters connected with the catalytic reaction dramatically changed the observed $v_{cat+uncat}/v_{uncat}$ ratio from 4.5 in SB buffer (100 mM Tris, pH 7.1, 200 mM sodium acetate, 25 mM potassium acetate, 10 mM magnesium acetate, 0.5% Triton X-100, 5% DMSO, at 25 °C) (Li & Sen, 1996) to a ratio of ~100 in 40KB buffer (100 mM MES, pH pH 6.2, 40 mM potassium acetate, 50 mM Tris acetate, 0.25% Triton X-100, 1% DMSO, at 15 °C). It could therefore be expected that the kinetic parameters, k_{cat} and K_M, for the DNAzyme would also have changed. Experiments were therefore carried out to determine k_{cat} and K_M for the utilization of both MPIX and PPIX by PS5.M, in 40KB buffer, at 15 °C (with 1.5 μM PS5.M and 1 mM copper acetate).

Figure 9, panels a and b, shows the catalyzed velocities of copper insertion into MPIX and PPIX, respectively, as functions of the concentrations of those porphyrins. These data were analyzed with the program GraFit 3.0, and the kinetic parameters obtained are shown in Table 3.

With MPIX as substrate, the optimized conditions gave rise to a moderate, ~6-fold increase in k_{cat}, but a dramatic, 70-fold decrease in the K_M compared to those derived in SB buffer (Li & Sen, 1996). Overall, these parameters combined

Table 3: Kinetic Parameters for Metallation of MPIX and PPIX Prior to Optimization (in SB Buffer) and Following Optimization (in 40 KB Buffer)

substrate	k_{cat}, min^{-1}	K_M, μM	k_{cat}/K_M, M^{-1} min^{-1}	k_{cat}/k_{uncat}
MPIX (in SB)	0.23 ± 0.06	2900 ± 900	79	1400
MPIX (in 40 KB)	1.30 ± 0.07	39.7 ± 4.9	3.25×10^4	1200
PPIX (in 40 KB)	0.49 ± 0.06	12.3 ± 2.9	3.97×10^4	3700

to give a large, 400-fold increase in the DNAzyme's catalytic efficiency, k_{cat}/K_M. The major change in K_M implied that the optimization process had worked significantly to improve the DNAzyme's affinity for its substrate, MPIX, presumably by eliminating or reducing detrimental conditions found in our initial selection buffer (SB buffer). On the other hand, the k_{cat} increase was probably not due to an increase in the robustness of the DNAzyme itself, for the background rate constant, k_{uncat}, also improved under the optimized conditions to almost the same extent (up ~7-fold, from 0.0096 to 0.065 h^{-1}). Thus, the catalytic "gain", k_{cat}/k_{uncat}, remained essentially unchanged through the optimization process.

Table 3 shows further that the K_M value for PPIX as substrate was superior to that for MPIX, the "intended" substrate for PS5.M. The k_{cat}/k_{uncat} for PPIX, too, was 3-fold larger than that for MPIX, confirming our preliminary results (Li & Sen, 1996) that PPIX was in fact a superior substrate for this DNAzyme.

Ferrochelatases and the "Artificial" Biocatalysts for Porphyrin Metallation. Ferrochelatases are the protein enzymes that catalyze the insertion of Fe^{2+} ions into protoporphyrin IX (PPIX) in the final step of heme biosynthesis (Lavallee, 1988). A number of studies have established that the *N*-methylporphyrins were a class of potent and reversible inhibitors of the enzymes (De Matteis et al., 1980; Tephly et al., 1979; Ortiz de Montellano et al., 1981; Dailey & Fleming, 1983), probably because these methylated porphyrins were close structural representations of the transition state for porphyrin metallation (Cochran & Schultz, 1990). This notion was successfully made use of by Cochran and Schultz (1990), who immunized mice with NMM, and successfully derived antibodies catalytic for porphyrin metallation. A similiar technology for isolating DNA and RNA sequences that bound specifically to NMM led eventually to the discovery of catalytic DNA and RNA molecules for this reaction (Li & Sen, 1996; Conn et al., 1996).

On the basis of the fact that the catalytic antibody, catalytic DNA, and catalytic RNA are all derived using the same transition state analogue (NMM), it may be reasonable to make an initial assumption that they all catalyze porphyrin metallation via similar mechanisms (which might in turn mirror those of the ferrochelatases). Table 4 summarizes the key kinetic parameters for all of these enzymes. As such, a detailed comparison of the individual properties of these catalysts is only of limited utility, for the experimental conditions [such as particulars of pH, temperature, substrate metal ion (Cu^{2+}, Zn^{2+}, or Fe^{2+}) concentrations, as well as the concentrations of individual buffer components] were different in each case. The k_{cat} of the ferrochelatase enzyme appears to be higher than that of the artificial enzymes, but under the particular assay conditions used for the ferrochelatase (50 μM Zn^{2+} or 100 μM Fe^{2+}), the enzyme molecules were already at saturation (*vide* K_M values, below) in terms of their binding capacity for the substrate metal ions, whereas

Table 4: Comparison of Key Kinetic Parameters of Ferrochelatases, Catalytic Antibody, DNA, and RNA

enzyme type	k_{cat} min^{-1}	K_M for porphyrins, μM	K_M for metal ions, μM	k_{cat}/K_M, M^{-1} min^{-1}	k_{cat}/k_{uncat}
ferrochelatase[a]	0.96 (Fe^{2+})[e]	12.5 (MPIX and PPIX)	11.8 (Zn^{2+})	76 800[e]	N/A
	7.2 (Zn^{2+})[e]		6.7 (Fe^{2+})	576 000[e]	
antibody[b]	0.14 (Cu^{2+})[e]	50 (MPIX)	no binding site	2900[e]	1700
	1.3 (Zn^{2+})[e]			26 800[e]	2600
DNA[c]	1.3 (Cu^{2+}, MPIX)	40 (MPIX)	no or weak binding site	32 500	1200
	0.49 (Cu^{2+}, PPIX)	12.3 (PPIX)		39 700	3700
RNA[d]	2.0 (Cu^{2+})	16 (MPIX)	N/A	125 000[e]	460

[a] Experiment conditions: 100 mM Tris-HCl, pH 8.0, 0.1% Tween 20, 25 μM zinc acetate or 100 μM iron citrate 37 °C (Okuda et al., 1994). [b] Experiment conditions: 90 mM Tris acetate, pH 8.0, 0.5% (w/v) Triton X-100, 5% (v/v) DMSO, 1 mM copper acetate or zinc acetate, 26 °C (Cochran and Schultz, 1990). [c] Experiment conditions: 50 mM Tris acetate, 100 mM MES, pH 6.2, 0.25% (w/v) Triton X-100, 1% (v/v) DMSO, 40 mM potassium acetate, 1 mM copper acetate, 15 °C. [d] Experiment conditions: 20 mM Tris acetate, 100 mM sodium chloride, 200 mM potassium chloride, 0.5% Triton X-100, 10% DMSO, 3 mM copper acetate, 25 °C (Conn et al., 1996). [e] The values shown were calculated using the data in the cited references.

Table 5: HPLC Conditions for the Analysis of Metallation by Different Porphyrins

porphyrin	flow rate, mL/min	retention time for substrate, min	retention time for product, min	wavelength for monitoring, nm
MPIX	2.0	6.2	15.6	390
PPIX	2.0	7.1	16.5	400
DPIX	1.5	4.7	10.0	390
HPIX	1.0	4.1	5.8	390
CPIII	1.0	3.6	4.7	390
UPIII	1.5	3.1	5.2	400

the artificial enzymes (at least the antibody, and the DNAzyme) do not appear to have strong substrate metal ion binding sites (for the DNAzyme, the K_M value would have to be higher than the 10 mM copper ion concentration tested for data shown above).

Unlike the artificial enzymes, ferrochelatases have very high affinities for their metal ion substrates (11.8 μM for Zn^{2+}; 6.7 μM for Fe^{2+}) (Okuda et al., 1994), and this is the most striking difference between the ferrochelatases, on the one hand, and the catalytic antibody and DNA, on the other. The lack of such binding sites in the artificial catalysts probably stems from the fact that no specific driving force (comparable to the use of the transition state, NMM, for creating a binding site for the substrate porphyrin) was built into the selection procedures to induce metal-ion binding sites in the catalytic antibody and DNA and RNA.

The two recently reported nucleic acid (DNA and RNA) catalysts for porphyrin metallation (Li & Sen, 1996; Conn et al., 1996) share a number of features: they were both selected out of their respective random libraries for binding to the same transition state analogue, NMM; both were cloned after 12 cycles of binding, elution, and amplification; now, as we report in this paper, their catalytic capabilities appear to be very similar. Most interesting, however, is the almost identical size of their optimally functioning units (24 nucleotides for the DNAzyme PS5.M; 25 nucleotides in the conserved RNA loop) and the significant homology that appears to exist between the two G- and T (or U)-rich sequences. The G richness of the catalytic RNA and the fact that it was selected, as well as assayed, in a buffer containing a high concentration of potassium (Conn et al., 1996), suggest that, as in the case of PS5.M, there may be guanine quartets in its folded structure. The overall similarity of the two enzymes raises the interesting question: would a RNA version of our DNA sequence, or vice versa, also have catalytic properties? These, and other questions, are being actively investigated in our laboratory.

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