Porphyrin Metalation Catalyzed by a Small RNA Molecule

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Human ferrochelatase is a membrane-associated mitochondrial protein which catalyzes the insertion of Fe(II) into protoporphyrin IX, the final step in the biosynthesis of heme.[1] Deficiency in this enzyme is a dominant inherited condition resulting in erythropoietic protoporphyria, characterized by extreme sensitivity to light due to photoactivation of singlet oxygen by unmetalated porphyrin.[2] We report here the isolation and characterization of a 35-nucleotide (nt) RNA that catalyzes the related Cu(II) insertion into mesoporphyrin IX with a k_{cat}/K_m value of 2100 M^{-1} s^{-1}, close to the value for the metalation of mesoporphyrin with Fe(II) catalyzed by recombinant human ferrochelatase.[3]

The catalytic RNA was selected using the transition state analog approach that has been so successful in the arena of antibody catalysis[4] and has recently been demonstrated for RNA catalysis.[5] N-alkylated porphyrins are thought to be good structural analogs[6] of the distortion required of the porphyrin in the transition state of the metal insertion reaction, are very potent inhibitors of ferrochelatase (K_i = 9 nM),[7] and undergo uncatalyzed metalation up to 10^5-fold more readily[8] than the corresponding unalkylated porphyrins. Indeed, antibodies raised to *N*-methylmesoporphyrin were found to be efficient catalysts for the metalation of porphyrin with a variety of metal ions.[9]

A library containing approximately 10^{15} 86-nt oligonucleotides with 50 randomized positions, flanked by defined primer regions for reverse transcription (RT) and amplification by polymerase chain reaction (PCR), was generated.[10,11] *In vitro* PCR amplification of the double-stranded DNA template using the RT primer and a primer containing a class three T7 promoter sequence, followed by transcription with T7 polymerase, yielded the initial RNA library.[12] This library was then screened for its ability to bind *N*-methylmesoporphyrin (NMMP) in both the presence and absence of $Cu(OAc)_2$ (5 mM) under equilibrium binding conditions.[13,14] The biotin conjugate of NMMP (**1**)[15] was complexed with streptavidin-coated magnetic beads and incubated with a large excess of the RNA library (0.5−1 h) in 100 mM NaCl, 200 mM KCl, 5 mM $MgCl_2$, 20 mM Tris−acetate (pH 8.0) buffer containing 10% DMSO and 0.5% Triton X-100 to solubilize the hydrophobic porphyrin (buffer A). The beads were washed three times (0.5−5 min) to remove weakly-bound RNAs and then incubated in the presence of an excess of the transition analog **2** (1−1.5 h) to elute specifically bound RNAs (Table 1). The RNAs in solution were recovered by ethanol precipitation, reverse transcribed (AMV-RT), and amplified by PCR with Taq polymerase followed by runoff transcription to generate an enriched pool of RNA for a subsequent round of screening.[16,17] After 12 rounds of selection, 20 clones from each selection (+Cu, −Cu) were inserted into plasmid pUC118 and sequenced;[18] 18 unique sequences were identified from the +Cu selection, 14 from the −Cu selection. Individual sequences were generated by large-scale runoff transcription, purified by denaturing polyacrylamide gel electrophoresis and screened spectrophotometrically for their ability to catalyze the insertion of Cu(II) into mesoporphyrin IX.[19] Five sequences (two from +Cu, three from −Cu) displayed catalytic activity, and the most active of these, RNA+12.19, was further characterized.[20]

RNA+12.19 displays Michaelis−Menten kinetics with a V_{max} ($k_{cat,app}$) of 0.92 min^{-1} and K_m of 14 μM in the presence of 3 mM $Cu(OAc)_2$ and 0.5 μM RNA in buffer A (Figure 1). This corresponds to a turnover number ($k_{cat,app}$) of 0.92 min^{-1} and an observed acceleration of 460 over the background rate (k_{cat}/

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(12) Single stranded DNA (5′-ACGGCCCTTGCGGCCGCX$_{50}$-GGCGCGCCTTCGGGAGC-3′) was converted to double-stranded DNA with AMV reverse transcriptase, PCR-amplified (64-fold) on a large scale (200 mL), and purified by phenol/chloroform extraction and ethanol precipitation. One twenty-fifth of this DNA was used for the initial runoff transcription with T7 RNA polymerase to yield 700 μg of RNA for the first round of selection.

(13) Purchased from Porphyrin Products, Logan, UT, as a mixture of regioisomers.

(14) Our initial studies have been carried out with Cu(II) rather than Fe(II) to avoid complications from product inhibition and aerobic sensitivity. Mesoporphyrin IX is less photosensitive and less prone to aggregation than protoporphyrin IX.

(15) The NMMP−biotin conjugate was prepared by carbodiimide-mediated (4 mol equiv of EDC, 3 mol equiv of NHS, catalytic DMAP) coupling of *N*-methylmesoporphyrin (50 μmol) and biotin-X-cadaverine (2 mol equiv, Molecular Probes, Eugene, OR) in DMF. Biotinylated NMMP was recovered by column chromatography on neutral alumina (5−20% EtOH/$CHCl_3$).

(16) EDTA (5 mM) was included in the precipitation to chelate Cu^{2+} which was found to inhibit Taq polymerase. Reverse transcription was performed in 40 μL volume with 20 mM Tris (pH 8.9), 50 mM KCl, 0.1% Triton X-100, 1.25 mM dNTPs, 5 mM $MgCl_2$, 5 μM primer (GCTC-CCGAAGGCGCGCC), 20 u rRNasin, and 2.5 u AMV RT at 50 °C for 30 min. Amplification by PCR of half of the RT reaction was carried out in the same buffer with 0.25 mM dNTPs, 2 mM $MgCl_2$, and 1 μM primers (RT primer and GATAATACGATCACTATACCACGGCCCTTGCGG-CCGC) for 8−15 cycles of 94 °C (30 s), 54 °C (30 s), and 74 °C (45 s). RNA was reannealed before selection by heating to 72 °C for 5 min in the absence of copper and then slowly cooling to room temperature before addition of copper.

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(18) DNA amplified by PCR was elongated with primers to introduce 5′-*Eco*RI and 3′-*Hin*dIII restriction sites. After restriction digestion, the library was ligated into pUC118, transformed into *Escherichia coli* MC1061 by electroporation, and streaked onto agar plates. Plasmid DNA was isolated from overnight cultures using a Promega Wizard miniprep kit and sequenced using the M13 reverse primer with Sequenase 2.0. DNA suitable for runoff transcription was prepared by PCR of transformed colonies from an overnight culture (10 μL), followed by extraction with phenol/chloroform.

(19) Kinetic samples (99 μL) were prepared in buffer A containing 0.5 μM RNA. There is some indication that RNA+12.19 aggregates, so all kinetics were performed at this low concentration. Before addition of mesoporphyrin (as a 10× DMSO solution) and $Cu(OAc)_2$, the RNA was reannealed by heating to 80 °C for 45 s and cooled to room temperature. Following addition of mesoporphyrin and a minimum 15-min equilibration period, the reaction was initiated by addition of 100 mM $Cu(OAc)_2$ (2.97 μL). The formation of mesoporphinatocuprate was monitored at 559 nm (ϵ_{559} = 20.9 mM^{-1}), and the initial rate of reaction over 5 min was calculated by least-squares linear regression. The reaction shows a nonlinear dependence on the copper concentration from 1 to 10 mM. The maximum rate is observed at 3 mM. The complexity of porphyrin metalation rates resulting from multiple copper ion species has been previously reported: Funahashi, S.; Yamaguchi, Y.; Tanaka, M. *Bull. Chem. Soc. Jpn.* **1984**, *57*, 204−208.

(20) RNA pools from previous rounds were assayed for activity and found to be inactive.

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Table 1. Summary of Selection

round	[RNA] (μM)	[biotin−NMMP]	[NMMP] (μM)
1−6	30	1 μM	50
7	30	500 nM	100
8	30	250 nM	100
9	30	125 nM	100
10	30	60 nM	100
11	30	30 nM	100
12	30	15 nM	100

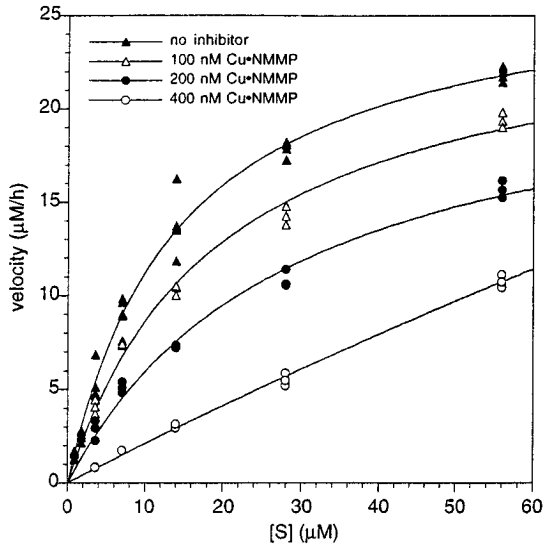
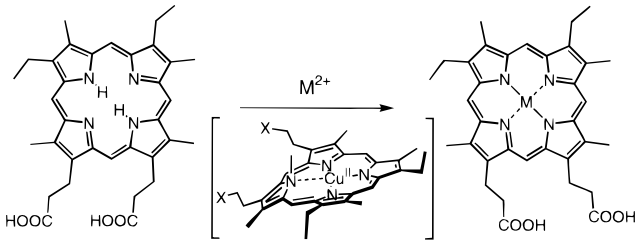


Figure 1. Dependence of reaction rate on mesoporphyrin IX and *N*-methylmesoporphyrin IX Cu(II) concentration.

Scheme 1. Metalation of Mesoporphyrin and Transition-State Analog *N*-Methylmesoporphyrin



1, X = $CONH(CH_2)_5NHCO(CH_2)_5NH$-biotin,
2, X = COOH

k_{uncat}).[21] The specificity constant of RNA+12.19 for mesoporphyrin IX (k_{cat}/K_m) is 1100 M^{-1} s^{-1}. Rate profiles in the presence of the preformed (*N*-methylmesoporphinato)copper complex[22] **2** allow calculation of an apparent K_i of 120 nM[23] (although it is likely that there is variation in the dissociation constants for each regioisomer[24]). The value of the ratio K_m/

(21) The first-order kinetic rate constant for the reaction of mesoporphyrin IX and $Cu(OAc)_2$ (3 mM) in these reaction conditions is 0.12 h^{-1}.

(22) Aqueous $Cu(OAc)_2$ (100 mM) and *N*-methylmesoporphyrin (5 mM in DMSO) were mixed in equimolar amounts and allowed to stand in the dark for 18 h and then diluted to 1.0 mM. The red-brown NMMP turns green upon Cu(II) chelation.

(23) The rate data at each substrate concentration were plotted as a function of inhibitor concentration and the resultant curves fit to a modified Michaelis−Menten equation where [S] was expressed as a rational-number multiple of K_m. The average K_i of the five curves was taken.

(24) It is known that the four *N*-methylmesoporphyrin regioisomers inhibit ferrochelatase to different degrees: De Matteis, F.; Gibbs, A. H.; Harvey, C. *Biochem. J.* **1985**, *226*, 537.

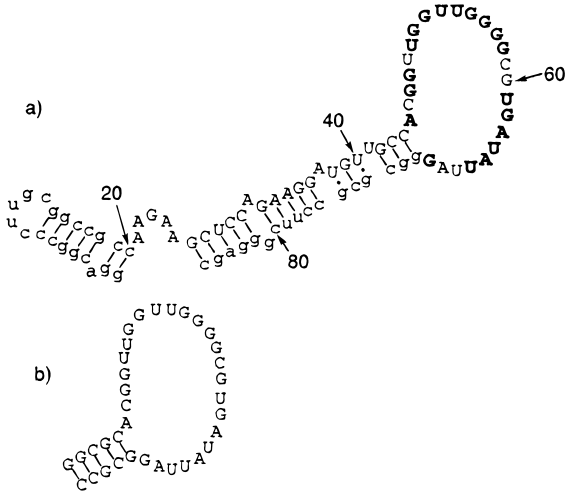


Figure 2. (a) Predicted secondary structure of RNA+12.19. Primer regions are lowercase, and conserved residues are bold. A short stem (3 bp) predicted in the conserved loop was not accurately reproduced in folding predictions for the other two ribozymes and has been omitted. (b) Sequence and predicted secondary structure of 35-nt catalytic RNA.

K_i (117) varies by a factor of approximately 4 from that calculated by k_{cat}/k_{uncat}. This discrepancy may be due to our use of a mixture of four different pyrrole N-Me regioisomers, a nonchemical partial rate-limiting step, or other factors.

The thermal denaturation profile of RNA+12.19 shows a sharp transition at 80 °C, indicating a stable, cooperatively-folded structure. The predicted secondary structure[25] of this RNA indicates a loop structure of 25 nucleotides at the end of a long stem (Figure 2a). This same sequence motif was also found in two of the catalysts isolated from the −Cu selection. These two RNAs (−12.04, −12.15) were found to have similar activity as RNA+12.19 (89% and 110%, respectively). Among these three oligonucleotides, 19 of the 25 nucleotides in the loop are completely conserved. Synthesis of the loop region as a 35-nt RNA (Figure 2b) results in a ribozyme with almost an identical K_m (16 μM), but with an apparent k_{cat} (2.0 min^{-1}) more than twice as great as that of RNA+12.19. This is most likely due to reduced misfolding during renaturation in the assay buffer. For this small RNA, the specificity constant (k_{cat}/K_m) is 2100 M^{-1} s^{-1} which rivals the values calculated for recombinant human ferrochelatase with iron and mesoporphyrin (1290 and 8210 M^{-1} s^{-1}).[3]

This work demonstrates the ability of RNA to catalyze a reaction closely related to that required for the biosynthesis of the essential cofactor, heme. The small size of the catalytic sequence motif found here should facilitate structural studies. Moreover, mutants of this RNA may be selected to display other porphyrin-based activities: ferrochelatase activity, reversible oxygen binding with RNA-bound heme, peroxidase activity, or complementation of ferrochelatase-deficient (heme H^-) auxotrophs which could ultimately lead to *in vivo* complementation of ferrochelatase deficiency using gene transfer techniques. We will report on these developments in due course.

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