

**Thermodynamic Evidence for Negative Charge Stabilization by a Catalytic  
Metal Ion within an RNA Active Site**

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**SUPPORTING INFORMATION**

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**Table S1.** pH and Mn<sup>2+</sup> do not affect binding of P<sub>H</sub> and P<sub>OH</sub>

Analog	Mn <sup>2+</sup>	K <sub>d</sub> (nM) <sup>a</sup>	
		pH 5.0	pH 8.0
P <sub>OH</sub>	-	3.9 ± 1.0	2.0 ± 0.52
P <sub>OH</sub>	+	2.7 ± 0.70	2.3 ± 0.74
P <sub>H</sub>	-	64 ± 16	18 ± 3.6
P <sub>H</sub>	+	36 ± 2.8	39 ± 6.1

<sup>a</sup> Dissociation constants were measured by monitoring the rate of product miscleavage as a function of ribozyme concentration at pH 5.0 (MES) and pH 8.0 (EPPS), in the absence or presence of 3 mM Mn<sup>2+</sup> (10 mM Mg<sup>2+</sup>, 2 mM G<sub>OH</sub>, 50 °C), as previously described (1). Dissociation constants were independently verified by measuring dissociation rate constants through a gel mobility shift assay using pulse-chase methods (data not shown) and assuming an association rate constant of 10<sup>8</sup> M<sup>-1</sup> min<sup>-1</sup>, as has been observed (2, 3).

**Table S2.** pH and Mn<sup>2+</sup> do not affect the substrate association rate constant for P<sub>SH</sub>

Analog	Mn <sup>2+</sup>	k <sub>on</sub> (M <sup>-1</sup> min <sup>-1</sup> ) <sup>a</sup>	
		pH 5.2	pH 7.2
P <sub>SH</sub>	-	0.8 x 10 <sup>8</sup>	0.6 x 10 <sup>8</sup>
P <sub>SH</sub>	+	ND	0.7 x 10 <sup>8</sup>

<sup>a</sup> Association rate constants were measured by a gel mobility shift assay using pulse-chase methods (4), as described in supplementary methods. Measurements were made at pH 5.2 (MES) and pH 7.2 (MOPS) in the absence or presence of 1 mM Mn<sup>2+</sup> (10 mM Mg<sup>2+</sup>, 30 °C). ND, not determined.

## Supplementary Methods

**Synthesis of P<sub>H</sub> and P<sub>S</sub>A.** P<sub>H</sub> and P<sub>S</sub>A (CCCUCU<sub>3</sub>S<sub>3</sub>A), an RNA substrate bearing a 3'-S phosphorothiolate linkage, were prepared by published procedures (5, 6).

**Generating P<sub>SH</sub>.** To generate P<sub>SH</sub> from P<sub>S</sub>A, the 3'-S phosphorothiolate linkage was cleaved with AgNO<sub>3</sub>, as previously described (7). 1 nmol of P<sub>S</sub>A was treated with 30 nmol of aqueous AgNO<sub>3</sub> in a final volume of 43 μL. After 30 min at room temperature in the dark, the mixture was treated with methanolic 2,2'-dipyridine disulfide (0.1 M, 2 μL) and 0.5 M Tris-HCl (pH 8.0, 5 μL), resulting in the formation of a suspension. After 1 h at room temperature, the suspension was centrifuged for 5 min and the supernatant containing the 3'-(2-pyridyldithio) derivative of P<sub>SH</sub> was collected. This 3'-(2-pyridyldithio) derivative of P<sub>SH</sub> was 5'-labeled with <sup>32</sup>P as described in the main text except that DTT was omitted. Following purification on a 20% native polyacrylamide gel (90 mM Tris, 90 mM boric acid) the products were visualized by autoradiography, excised from the gel, and eluted at 4 °C overnight in water. The eluant was subsequently desalted using a Sep-Pak C<sub>18</sub> column (Waters) according to the manufacturer's protocol and dried using a speed-vac. Immediately prior to initiating any reaction with P<sub>SH</sub>, the 3'-(2-pyridyldithio) derivative of P<sub>SH</sub> was pretreated with 1 mM aqueous DTT for 5 min at room temperature to reduce the disulfide bond.

**Maintaining the Integrity of the –SH Group of P<sub>SH</sub>.** Previous work using 2'-SH cytidine in the hammerhead ribozyme reaction showed that the –SH group is susceptible to oxidation in the combined presence of 1 mM DTT (or TCEP), O<sub>2</sub>,

and redox-active metal ions such as  $\text{Mn}^{2+}$  and  $\text{Co}^{2+}$  (8). When working with the 3'-SH group of  $\text{P}_{\text{SH}}$  in the presence of  $\text{Mn}^{2+}$ , we encountered similar effects. We were able to avoid -SH oxidation under aerobic conditions by including 200 mM DTT and 1 mM ascorbic acid in all reactions with  $\text{Mn}^{2+}$ . In control reactions with  $\text{P}_{\text{OH}}$ , these reagents did not change the kinetic and thermodynamic parameters of the *Tetrahymena* ribozyme reaction (data not shown). In reactions with  $\text{Mn}^{2+}$ , the integrity of  $\text{P}_{\text{SH}}$  was verified by demonstrating that the -SH group is susceptible to alkylation by iodoacetamide and by observing that the bands corresponding to the oxidized products (<15%) did not accumulate over the reaction time course as observed on 20% denaturing polyacrylamide gels (90 mM Tris, 90 mM boric acid, 0.05 mM EDTA, and 7 M urea) (data not shown).

**Iodoacetamide Modification Assay.** Reactions were performed at 50 °C with trace amounts of 5'- $^{32}\text{P}$ -labeled  $\text{P}_{\text{SH}}$  ( $[\text{*P}_X] < 1 \text{ nM}$ ), 10 mM  $\text{MgCl}_2$ , 50 mM buffer (see Methods), 1 mM sodium ascorbate, 0.1 mM EDTA, and 5 mM iodoacetamide). Following addition of iodoacetamide to initiate the reaction, five 1  $\mu\text{L}$  aliquots were removed from the 10  $\mu\text{L}$  reaction at specified times and quenched in 4  $\mu\text{L}$  of stop solution (1.5 M DTT, 50 mM NaMES, pH 6.5, 0.1 mM EDTA). Starting material ( $\text{*P}_{\text{SH}}$ ) and product ( $\text{*P}_{\text{S-ICH}_2\text{C(O)NH}_2}$ ) were separated by electrophoresis on 20% denaturing polyacrylamide gels, and their ratio at each time point was quantitated as described in Methods. Control reactions showed that 1 mM  $\text{Mn}^{2+}$  had no effect on this solution  $\text{p}K_{\text{a}}$  (data not shown).

**General Kinetics.** Ribozyme reactions were single-turnover, with ribozyme (E) in excess of 5'-labeled oligonucleotide substrate. Prior to initiating the reaction,

ribozyme was preincubated with 10 mM MgCl<sub>2</sub> and 10 mM NaMOPS (pH 6.8) at 50 °C for 15 minutes to renature the ribozyme, diluted 10-fold in the appropriate buffer, and adjusted to the desired Mn<sup>2+</sup> concentration at 50 °C. For all reactions, starting material and product were separated by electrophoresis on 10 or 20% polyacrylamide gels, and their ratio at each time point was quantitated with a Typhoon Phosphorimager (GE Healthcare). Reactions were typically followed for  $\geq 3t_{1/2}$  except for very slow reactions. Good first-order fits to the data, with endpoints of  $\geq 90\%$  were typically obtained (Sigma Plot, Systat software). Slow reactions were followed for up to 3 hours and endpoints of 95% were assumed to obtain the observed rate constants.

**Miscleavage Reactions.** Miscleavage reactions were carried out with trace amounts of 5'-<sup>32</sup>P-labeled \*P<sub>X</sub> ([\*P<sub>X</sub>] <1 nM) and with ribozyme saturating with respect to P (500 nM E,  $K_d \leq 64$  nM, Table SI) and with saturating guanosine (2 mM,  $K_d \leq 1$  mM, (1)). Following addition of \*P<sub>X</sub> to initiate the miscleavage reaction, five 1  $\mu$ L aliquots of the reaction mixture were removed from the 10  $\mu$ L reaction at specified times and quenched in 7  $\mu$ L of stop solution [8 M Urea, 50 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, 0.5x TB (45 mM Tris, 45 mM boric acid)]. Starting material and product were separated by electrophoresis on 20% denaturing polyacrylamide gels, and their ratio at each time point was quantitated as described above.

**Pulse-Chase Experiments.** Dissociation rate constants ( $k_{off}$ ) for the product analogs were measured through a gel mobility shift assay using pulse-chase methods as reported previously (9). In a typical experiment, 5'-<sup>32</sup>P-labeled P<sub>X</sub>

( $[^*P_X] < 1 \text{ nM}$ ) was incubated with saturating ribozyme (300 nM) for 5 min and then diluted 10-fold with a chase solution containing 5  $\mu\text{M}$  unlabeled  $P_{OH}$  in the appropriate buffer at the same temperature. Guanosine was excluded from the reaction mixture to avoid miscleavage of P. Following addition of chase, five 3.5  $\mu\text{L}$  aliquots from the 20  $\mu\text{L}$  reaction were withdrawn at six time points, and each was immediately added to 2  $\mu\text{L}$  of loading buffer containing 60% glycerol and 0.5% xylene cyanol and carefully loaded onto a running (150 V) 10% native polyacrylamide gel (90 mM Tris, 90 mM boric acid, 0.05 mM EDTA, and 10 mM  $\text{MgCl}_2$ ). For reactions with  $P_{SH}$ , native gels were cast with 10 mM DTT and allowed to polymerize overnight to avoid smearing of the bands as reported previously (10). Electrophoresis buffer contained the same components as the gel. The enzyme-bound ( $E \cdot ^*P_X$ ) and unbound ( $P_X$ ) species were separated by electrophoresis and their ratios at each time point were quantitated as described above. In control experiments, addition of chase to ribozyme prior to adding  $^*P_X$  blocked binding of  $^*P_X$ , demonstrating that the chase was effective (data not shown). For pulse-chase experiments with  $P_{SH}$ , plots of fraction bound vs. time were biphasic, with a fast ( $\leq 15\%$ ) and slow ( $\geq 85\%$ ) phase. The slow phase reflects  $P_{SH}$  dissociation since: a) in control experiments where 5'- $^{32}\text{P}$ -labeled  $P_{SH}$  was loaded onto a 20% denaturing polyacrylamide gel,  $P_{SH}$  is the major band ( $\geq 85\%$ ); the remaining bands ( $\leq 15\%$ ) correspond to sulfur-oxidized products and b)  $k_{off}$  from the slow phase decreases with increasing  $[\text{Mn}^{2+}]$  while  $k_{off}$  from the fast phase is  $\text{Mn}^{2+}$ -independent ( $\sim 5 \text{ min}^{-1}$ ).

**Measuring association rate constants ( $k_{on}$ ):**  $P_{SH}$  association was measured at several ribozyme concentrations (2 – 20 nM) at 30 °C. The lower temperature reduces the dissociation rate constant, preventing rapid dissociation of  $P_{SH}$  upon substrate binding. As  $k_{on}$  reflects simple duplex formation, this value is temperature-independent, as has been observed (11, 12). In a typical experiment, 5'-<sup>32</sup>P-labeled  $P_{SH}$  ( $[^*P_X] < 1$  nM) was added to ribozyme and aliquots were withdrawn at six time points and carefully loaded onto a running (150 V) 10% native polyacrylamide gel, as described above. The enzyme-bound ( $E \cdot ^*P_X$ ) and unbound ( $P_X$ ) species were separated by electrophoresis and their ratios at each time point were quantitated to obtain an observed association rate constant. The second order association rate constant,  $k_{on}$ , was measured as the slope of observed association rate versus ribozyme concentration.

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