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^{2+} do not affect binding of P_H and P_{OH}

Analog	Mn^{2+}	K_d (nM)[a]	
		pH 5.0	pH 8.0
P_{OH}	-	3.9 ± 1.0	2.0 ± 0.52
P_{OH}	+	2.7 ± 0.70	2.3 ± 0.74
P_H	-	64 ± 16	18 ± 3.6
P_H	+	36 ± 2.8	39 ± 6.1

[a] 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^{2+} (10 mM Mg^{2+}, 2 mM G_{OH}, 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 10^8 M^{-1} min^{-1}, as has been observed (*2, 3*).

Table S2. pH and Mn^{2+} do not affect the substrate association rate constant for P_{SH}

Analog	Mn^{2+}	k_{on} (M^{-1} min^{-1})[a]	
		pH 5.2	pH 7.2
P_{SH}	-	0.8×10^8	0.6×10^8
P_{SH}	+	ND	0.7×10^8

[a] 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^{2+} (10 mM Mg^{2+}, 30 °C). ND, not determined.

Supplementary Methods

Synthesis of P_H and P_SA. P_H and P_SA ($CCCUCU_{3'S}A$), an RNA substrate bearing a 3′-S phosphorothiolate linkage, were prepared by published procedures (*5, 6*).

Generating P_{SH}. To generate P_{SH} from P_SA, the 3′-S phosphorothiolate linkage was cleaved with $AgNO_3$, as previously described (*7*). 1 nmol of P_SA was treated with 30 nmol of aqueous $AgNO_3$ 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_{SH} was collected. This 3′-(2-pyridyldithio) derivative of P_{SH} was 5′-labeled with ^{32}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_{18} column (Waters) according to the manufacturer's protocol and dried using a speed-vac. Immediately prior to initiating any reaction with P_{SH}, the 3′-(2-pyridyldithio) derivative of P_{SH} 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_{SH}. 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_2,

and redox-active metal ions such as Mn^{2+} and Co^{2+} (*8*). When working with the 3′-SH group of P_{SH} in the presence of 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 Mn^{2+}. In control reactions with P_{OH}, these reagents did not change the kinetic and thermodynamic parameters of the *Tetrahymena* ribozyme reaction (data not shown). In reactions with Mn^{2+}, the integrity of P_{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}P-labeled P_{SH} ([$^*P_X$] < 1 nM), 10 mM $MgCl_2$, 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 µL aliquots were removed from the 10 µL reaction at specified times and quenched in 4 µL of stop solution (1.5 M DTT, 50 mM NaMES, pH 6.5, 0.1 mM EDTA). Starting material ($^*P_{SH}$) and product ($^*P_{S\text{-}ICH_2C(O)NH_2}$) 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 Mn^{2+} had no effect on this solution pK_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 $MgCl_2$ 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^{2+} 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 ≥3$t_{1/2}$ except for very slow reactions. Good first-order fits to the data, with endpoints of ≥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′-^{32}P-labeled *P_X ([*P_X] <1 nM) and with ribozyme saturating with respect to P (500 nM E, K_d ≤ 64 nM, Table SI) and with saturating guanosine (2 mM, K_d ≤ 1 mM, (*1*)). Following addition of *P_X to initiate the miscleavage reaction, five 1 µL aliquots of the reaction mixture were removed from the 10 µL reaction at specified times and quenched in 7 µL 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′-^{32}P-labeled P_X

([$^*P_X$] < 1 nM) was incubated with saturating ribozyme (300 nM) for 5 min and then diluted 10-fold with a chase solution containing 5 μM 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 μL aliquots from the 20 μL reaction were withdrawn at six time points, and each was immediately added to 2 μL 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 $MgCl_2$). 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•$^*P_X$) 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 (≤ 15%) and slow (≥ 85%) phase. The slow phase reflects P_{SH} dissociation since: a) in control experiments where 5′-^{32}P-labeled P_{SH} was loaded onto a 20% denaturing polyacrylamide gel, P_{SH} is the major band (≥85%); the remaining bands (≤15%) correspond to sulfur-oxidized products and b) k_{off} from the slow phase decreases with increasing [Mn^{2+}] while k_{off} from the fast phase is Mn^{2+}-independent (~ 5 min^{-1}).

Measuring association rate constants (k_{on}): P_{SH} association was measured at several ribozyme concentrations (2 – 20 nM) at 30 ^{o}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′-^{32}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•*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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