

## Promiscuous Catalysis by the *Tetrahymena* Group I Ribozyme

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Since the discovery of catalytic RNA, the possibility of an RNA world early in evolution has been widely discussed.<sup>1</sup> Indeed, a high degree of metabolic complexity in this RNA world has been suggested.<sup>2</sup> Although naturally occurring RNA enzymes are known to catalyze only two classes of reactions (P–O bond cleavage in phosphate diesters and aminoacyl transfer),<sup>3</sup> RNA enzymes catalyzing several other types of reactions, including C–C bond formation, have been discovered via *in vitro* selections.<sup>4</sup>

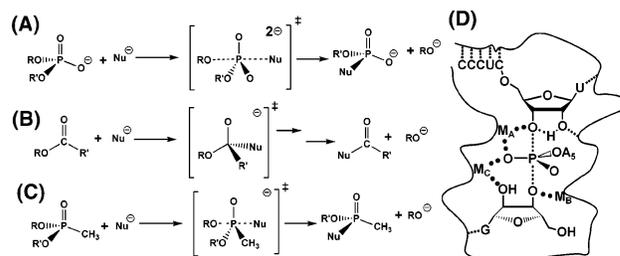
Metabolic complexity within an RNA world would have required facile routes for the evolution of new enzymatic activities. “Catalytic promiscuity” has been suggested to have played an important role in the evolution of new protein enzymes,<sup>5</sup> and it may have similarly been advantageous in an earlier RNA world. According to this view, products of duplicated genes would, in some instances, have a low level of activity for an alternate reaction, and this activity could afford a selective advantage either directly or following a limited number of mutations in the duplicated gene.<sup>5</sup>

There are a few known examples of promiscuous ribozymes,<sup>6</sup> but it is not known how common promiscuity is for RNA enzymes, and the mechanistic features important for RNA promiscuity have not been investigated. Herein we identify a new and rather efficient promiscuous activity of the L-21 *ScaI* ribozyme (E) derived from the Group I self-splicing intron from *Tetrahymena thermophila*, and we derive mechanistic insights via comparison to other reactions catalyzed by this ribozyme.

The *Tetrahymena* ribozyme, which normally catalyzes attack of guanosine (G) or water on a phosphodiester bond (CCCUCU<sub>P</sub>A<sub>5</sub> (S) + G<sub>OH</sub> (G) → CCCUCU<sub>OH</sub> (P) + G<sub>P</sub>A<sub>5</sub>),<sup>7</sup> also catalyzes attack on phosphate monoesters<sup>6a</sup> and hydrolysis of aminoacyl esters.<sup>6b</sup> However, the catalytic proficiency<sup>8</sup> toward aminoacyl esters is very low, less than 10-fold, and water is used instead of the normal G nucleophile.<sup>6b</sup> Although phosphoryl and aminoacyl transfers differ substantially, there are two simple differences that can be readily identified: (1) the geometry of the transition state and (2) the charge at the reaction center (Figure 1A, B).

To evaluate these contributions, and thereby learn more about catalysis by this ribozyme and the potential for catalytic promiscuity by RNA enzymes, we have utilized a phosphonate diester, S<sub>Me</sub> (Scheme 1). S<sub>Me</sub> lacks the negative charge of the natural phosphate diester substrate at the cleavage site (S<sub>ox</sub>, Scheme 1) but could undergo guanosine attack with the same trigonal bipyramidal transition-state geometry as the natural substrate (Figure 1A, C).<sup>9</sup> Thus, if charge were the most important feature for the ribozyme-catalyzed reaction, S<sub>Me</sub> would react with proficiency similar to that for acyl transfer. On the other hand, if geometry were the main factor controlling catalysis, S<sub>Me</sub> would be predicted to react with a rate acceleration similar to that for the normal phosphodiester substrate.

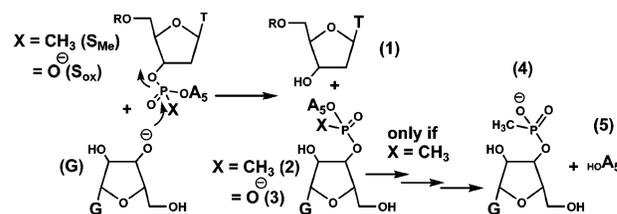
The transition state of the phosphodiester reaction involves base-pairing, metal ion coordination, and hydrogen bonds (Figure 1D) and proceeds with a catalytic proficiency of 10<sup>20</sup>.<sup>10</sup> If S<sub>Me</sub> were to react with guanosine as normal substrates do (e.g., S<sub>ox</sub>), the pathway



**Figure 1.** Reactions considered herein. Solution transition states for nucleophilic attack on (A) phosphate diesters, (B) aminoacyl esters (full bonding to the nucleophile is shown for simplicity), and (C) phosphonate diesters. (D) Transition state interactions in the *Tetrahymena* ribozyme-catalyzed phosphoryl transfer derived from biochemical data;<sup>7</sup> charges and partial charges have been omitted for simplicity.

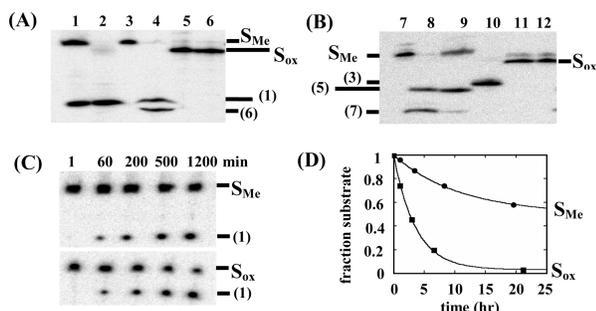
### Scheme 1

R = m(CCC)d(TC); m = 2'-methoxyribose; d = 2'-deoxyribose



depicted in Scheme 1 would be followed. The intermediate product (2) is unstable in buffer solutions<sup>11</sup> and predicted to decompose to (4) and (5) (see Scheme S2 for details). Further, the two diastereoisomers of S<sub>Me</sub> are predicted to react at much different rates, as thio-substitution experiments have revealed a large deleterious effect for substitution of the pro-S<sub>P</sub> oxygen atom with sulfur. This effect presumably arises because the S<sub>P</sub> sulfur cannot be accommodated between M<sub>A</sub> and M<sub>C</sub> (Figure 1D),<sup>12</sup> so that the corresponding methyl group in S<sub>Me</sub> (R<sub>P</sub> isomer) would similarly be expected to slow the reaction.

S<sub>Me</sub> was supplied commercially and its identity confirmed by gel migration, mass spectroscopy, HPLC, and reaction product analysis (see below and Supporting Information). As expected, due to its lower charge, 5'-radiolabeled S<sub>Me</sub> migrated more slowly in gel electrophoresis than S<sub>ox</sub> (Figure 2A, lanes 3 and 5). When 5'-radiolabeled S<sub>Me</sub> and S<sub>ox</sub> were incubated with E and G, the labeled 5'-products had the same gel migration (Figure 2A, lanes 1 and 2), suggesting formation of the same product (1), as predicted in Scheme 1. Conversely, the reactions of 3'-radiolabeled S<sub>Me</sub> and S<sub>ox</sub> led to different products, with gel mobilities consistent with compounds (5) and (3), respectively, (Figure 2B, lanes 9 and 10), again in agreement with Scheme 1. No reaction was observed in the absence of ribozyme (not shown). Further, only about 50% of S<sub>Me</sub> was converted to products, whereas S<sub>ox</sub> gave full conversion (Figure 2C, D), suggesting that only one S<sub>Me</sub> diastereoisomer reacts, again in agreement with prediction. These and other observations (see below) indicate that S<sub>Me</sub> is a substrate for the *Tetrahymena* ribozyme and that the reaction depicted in Scheme 1 is followed.



**Figure 2.** Characterization of starting materials and products (see text and Supporting Information for reaction details). **(A)** Gel migration of reactions with 5'-radiolabeled substrates. Lane 1:  $S_{Me}$ , ribozyme reaction. Lane 2:  $S_{ox}$ , ribozyme reaction. Lane 3:  $S_{Me}$ . Lane 4:  $S_{Me} + 0.1$  M NaOH. Lane 5:  $S_{ox}$ . Lane 6:  $S_{ox} + 0.1$  M NaOH. **(B)** Gel migration of reactions with 3'-radiolabeled substrates. Lane 7:  $S_{Me}$ . Lane 8:  $S_{Me} + 0.1$  M NaOH. Lane 9:  $S_{Me}$ , ribozyme reaction. Lane 10:  $S_{ox}$ , ribozyme reaction. Lane 11:  $S_{ox}$ . Lane 12:  $S_{ox} + 0.1$  M NaOH. Compounds (6) and (7) correspond to the second products of alkaline hydrolysis of  $S_{Me}$  (see Supporting Information). **(C)** Typical autoradiograms of the reactions of  $S_{Me}$  (top) or  $S_{ox}$  (bottom) in 450 nM E, 2 mM G, 10 mM  $Mg^{2+}$ , pH 8.3. **(D)** Fraction of unreacted substrate plotted versus time and fit to a single-exponential decay.

$S_{Me}$  and  $S_{ox}$  gave similar ribozyme-catalyzed reaction rates (Figure 2D and Table S1). Measurement of presteady-state reaction parameters revealed rate constants within 2-fold for the two substrates under standard reaction conditions (G nucleophile, 10 mM  $Mg^{2+}$ , pH 5.5–8.5; Table S1 and Figure S1). However, as  $S_{Me}$  is intrinsically about 14 orders of magnitude more reactive than  $S_{ox}$ ,<sup>10,13</sup> the enzymatic rate enhancement is far smaller for  $S_{Me}$  than for  $S_{ox}$  (see below).

We further characterized the reaction of  $S_{Me}$  by using different nucleophiles. Prior results have shown that 3'-dG, 2'-dG, ATP, and 2-aminopurine are, at best, poor substitutes for G, whereas 2'-aminoguanosine ( $G_{NH_2}$ )<sup>7,14</sup> and UCG<sup>15</sup> are highly reactive. Accordingly, we found that  $S_{Me}$  reacted only with UCG and  $G_{NH_2}$ . Whereas G reacted with the same rate constant for both substrates, UCG has a 5-fold preference for reaction with  $S_{ox}$ , and  $G_{NH_2}$  reacts about 4-fold faster with  $S_{Me}$  (Table S1). Further, in the absence of guanosine,  $S_{Me}$  reacts in the ribozyme-catalyzed, guanosine-independent reaction (Table S1) ~20-fold faster than  $S_{ox}$ . Finally, the G reaction with 10 mM  $Mn^{2+}$  and 10 mM  $Mg^{2+}$  was 4-fold faster for  $S_{Me}$  than for  $S_{ox}$ . Overall, these results suggest that the reaction of  $S_{Me}$  is highly similar to that for  $S_{ox}$ , but with minor differences in peripheral transition-state interactions. These differences presumably arise from the electrostatic differences between the substrates.

Our results show that the *Tetrahymena* ribozyme is able to catalyze guanosine attack on neutral phosphonate diesters, with a rate enhancement of a million fold.<sup>13</sup> This reaction is much more proficient than acyl transfer to water (catalytic proficiency <10, see above),<sup>6b</sup> suggesting the importance of transition-state geometry. Interestingly, the guanosine binding site of the ribozyme has been shown to act as a separable module.<sup>16</sup> Further, a catalytic role of metal ions has been suggested in in vitro-evolved ribozymes that catalyze aminoacyl transfer.<sup>17</sup> Thus, one can imagine evolutionary scenarios in which RNA domains were swapped and/or reoriented to overcome geometrical and charge problems and create new ribozymes, akin to the mixing of protein domains that has aided in the evolution of new enzymes.<sup>18</sup>

Nevertheless, the reaction of  $S_{Me}$  is about 14 orders of magnitude less proficient than the cognate reaction of a phosphate diester anion. This reduced catalytic proficiency suggests the importance of negative charge at the reactive center of the ribozyme, despite the overall polyanionic nature of the RNA catalyst. RNA apparently uses its negative charge to create metal ion binding sites and local positive potentials. This view is in agreement with the transition state depicted in Figure 1D, in which an oxygen atom with a partial negative charge is caged between two metal ions.

It will be of interest to further explore the catalytic potential and promiscuity of natural and in vitro-evolved ribozymes and to better understand the features of RNA molecules that endow them with their catalytic power and potential.

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**Supporting Information Available:** Experimental procedures, Schemes S1 and S2, Table S1, and Figure S1. This material is available free of charge via the Internet at <http://pubs.acs.org>. See any current masthead page for ordering information and Web access instructions.

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