Investigation of catalysis by bacterial RNase P via LNA and other modifications at the scissile phosphodiester

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ABSTRACT

We analyzed cleavage of precursor tRNAs with an LNA, 2′-OCH₃, 2′-H or 2′-F modification at the canonical (c₀) site by bacterial RNase P. We infer that the major function of the 2′-substituent at nt −1 during substrate ground state binding is to accept an H-bond. Cleavage of the LNA substrate at the c₀ site by Escherichia coli RNase P RNA demonstrated that the transition state for cleavage can in principle be achieved with a locked C3′-endo ribose and without the H-bond donor function of the 2′-substituent. LNA and 2′-OCH₃ suppressed processing at the major aberrant m₁ site; instead, the m₁ (nt +1/+2) site was utilized. For the LNA variant, parallel pathways leading to cleavage at the c₀ and m₁ sites had different pH profiles, with a higher Mg²⁺ requirement for c₀ versus m₁ cleavage. The strong catalytic defect for LNA and 2′-OCH₃ supports a model where the extra methylene (LNA) or methyl group (2′-OCH₃) causes a steric interference with a nearby bound catalytic Mg²⁺ during its recoordination on the way to the transition state for cleavage. The presence of the protein cofactor suppressed the ground state binding defects, but not the catalytic defects.

INTRODUCTION

Endonucleolytic 5′-end maturation of tRNA primary transcripts is catalyzed by the ribonucleoprotein enzyme Ribonuclease P (RNase P) in all three domains of life (Archaea, Bacteria and Eukarya) as well as in mitochondria and chloroplasts (1–4). An exception is the human mitochondrial tRNA 5′-end maturation enzyme that lacks an RNA subunit, with its three protein subunits recruited from related and unrelated biochemical pathways (5). Bacterial RNase P enzymes are composed of a catalytic RNA subunit (6), ~400 nucleotides (nt) in length, and a single small protein of typically 120 amino acids (7). The protein is essential in vivo, but in vitro its absence can be compensated by increased mono- and particularly di-valent cations (6). Studies with RNase P RNA ribozymes (P RNAs) from Escherichia coli and Bacillus subtilis have indicated a specific role for at least two metal ions in productive enzyme–substrate complex formation and cleavage chemistry (8–15). Since processing of precursor tRNAs (ptRNAs) by RNase P results in cleavage products with 3′-OH and 5′-phosphate termini, an inherent feature of RNase P catalysis must be to prevent the 2′-hydroxyl group at the cleavage site from attacking the scissile phosphodiester, which would otherwise result in 5′-OH and 2′,3′-cyclic phosphate ends. The 2′-hydroxyl group of the ribose at the cleavage site has indeed been identified as being crucial in the catalytic process. A 2′-deoxy or 2′-amino substitution at the canonical RNase P cleavage site (nt −1) affected substrate ground state binding, cleavage site recognition, binding of catalytically important Mg²⁺ and substantially reduced the cleavage rate by E. coli or B. subtilis P RNA (8,16–23). Initial evidence for the 2′-hydroxyl group at nt −1 acting as a ligand for a catalytic Mg²⁺ ion came from the observation that a 2′-deoxy modification at this location reduced the Hill coefficient for the Mg²⁺ dependence of substrate cleavage from 3 to 2 and increased the apparent Kₐ for Mg²⁺ binding 10³-fold (8). A 2′-amino modification at this position resulted in miscleavage at lower pH, consistent with the idea that protonation of the 2′-amino group at lower pH causes electrostatic repulsion of a metal at this location (20,21). Mn²⁺ rescued cleavage at the canonical c₀ site for ptRNA with a 2′-amino substitution at nt −1, which would be consistent with a direct metal ion coordination to the 2′-substituent at this location; however, a 2′-deoxy modification at the same site was rescued to a similar extent by Mn²⁺, thus questioning the possibility of a direct metal
modified ribose has a flexible conformation (Table 1) and the 2'-OCH₃ group can freely rotate around the C2'-O2' bond, whereas the position of the corresponding methylene is fixed in LNA. Other variants with 2'-fluoro (2'-F) or 2'-deoxy (2'-H) substitutions at nt −1 that affect the H–bonding pattern, the free energy of solvation and the electronegativity of the 2'-substituent were included as well. We then characterized the modified ptRNA substrates in reactions catalyzed by type A (E. coli) and type B (B. subtilis) RNase P (RNA) for (i) substrate ground state binding, (ii) cleavage site selection and (iii) cleavage kinetics. Our results indicate that the major function of the 2'-substituent at nt −1 during substrate ground state binding is to accept an H-bond. We demonstrate that cleavage at the canonical site by E. coli P RNA can occur in the presence of a locked C3'-endo ribose and without the H-bond donor function of the 2'-substituent, however, with very low efficiency. The strong catalytic defect in the presence of LNA and 2'-OCH₃ modifications at the c₀ site supports a model where the extra methylene (LNA) or methyl group (2'-OCH₃) causes a steric interference with a nearby bound catalytic Mg²⁺ during its recoordination on the way to the transition state for cleavage. LNA and 2'-OCH₃ at the c₀ site also suppressed processing at the major aberrant m₁ site, directing cleavage to the aberrant m₁ site (nt +1/+2). The protein cofactor, although suppressing the ground state binding defects, was unable to substantially relieve the severe catalytic defects. Differences between E. coli and B. subtilis enzymes included a stronger defect caused by 2'-H at nt −1 on the B. subtilis versus E. coli holoenzyme, and different cleavage site selection on the 2'-OCH₃ substrate.

MATERIALS AND METHODS
RNA synthesis, 5'-phosphorylation of RNA, 5'-end-labeling of RNA and assembly of ptRNA variants

Chemical and enzymatic RNA synthesis, purification, 5'-phosphorylation and 5'-³²P-end-labeling of RNA, as well as the assembly of ptRNA variants with single-site modifications were performed exactly as described (20,30).

Preparation of recombinant RNase P proteins
E. coli and B. subtilis RNase P proteins carrying an N-terminal His-tag were overexpressed and purified as described (31).

Reconstitution of RNase P holoenzymes
(i) For cleavage experiments, a solution containing 133 mM E. coli or B. subtilis P RNA, 107 mM KCl, 53 mM MES, pH 6.0 and 10.7 mM MgCl₂ was incubated for 5 min at 55°C and 50 min at 37°C. To 15 µl of this solution, 1 µl of P protein solution (freshly thawed and diluted) was added to adjust the solution to 0.5 µM B. subtilis or 1.2 µM E. coli P protein and to 125 nM P RNA, 100 mM KCl, 50 mM MES and 10 mM MgCl₂, pH 6.0. This mixture was incubated for another 5 min at 37°C;
16 μl thereof were then combined with 4 μl ptRNA solution (preincubated in the same buffer for 5 min at 55°C and 25 min at 37°C) to start the processing reaction.

(ii) For binding experiments, a solution containing 1.18 μM *E. coli* or *B. subtilis* P RNA, 235 mM NH₄OAc, 59 mM Tris–acetate, pH 7.1, 17.7 mM CaCl₂ and 0.059 (w/v) Nonidet P40 was incubated for 1 h at 37°C. To 8.5 μl of this mixture, 1.5 μl of P protein solution was added to adjust the solution to 5 μM *B. subtilis* or 12 μM *E. coli* P protein (freshly thawed and diluted), 1 μM P RNA, 200 mM NH₄OAc, 50 mM Tris–acetate, pH 7.1, 15 mM CaCl₂ and 0.05 (w/v) Nonidet P40. This holoenzyme stock solution was incubated for 15 min at 37°C and then used to prepare serial dilutions in the same buffer. The diluted solutions were incubated for another 5 min at 37°C. 10 μl of individual holoenzyme solutions were combined with an equal volume of 5'-32P-labeled ptRNA solution (<1 nM ptRNA, 80 000 Cerenkov cpm), which had been preincubated for 30 min at 37°C in the same buffer, followed by a final incubation step for 5 min at 37°C before conduction of the spin column assay (see below). For cleavage and binding experiments, the final RNase P holoenzyme concentration was considered equal to the final P RNA concentration. This represents an upper estimate of the enzyme concentration, since 100% ribonucleoprotein complex formation may not have been reached.

Nuclease P1 hydrolysis

A solution of 5'-32P-end-labeled 24-mer RNA (10⁴ Cerenkov cpn/μl), 0.1 μg/μl carrier RNA, 40 mM NH₄OAc, 0.4 mM ZnSO₄, pH 5.3 and 0.01 ng/μl nuclease P1 was incubated for 20 s at 70°C in a heating block. After incubation the reaction tube was placed on ice, the RNA was concentrated and desalted by ethanol precipitation and analyzed by denaturing PAGE.

Kinetics

The ptRNA variants were processed under single turnover conditions at saturating enzyme concentrations (5 μM *E. coli* or *B. subtilis* P RNA or 100 nM of reconstituted *E. coli* or *B. subtilis* RNase P holoenzyme), 1 M NH₄OAc (for RNA-alone reactions) or 0.1 M KCl (for holoenzyme reactions), and at Mg²⁺ concentrations and pH conditions as specified. The pH of 7.0 was chosen to provide conditions where substantial cleavage of all variants occurred, even at low Mg²⁺ concentrations; pH values of 5.5 or 6.0 allowed us to analyze cleavage at rate-limiting chemistry for all substrates (9). All processing reactions were performed at 37°C, P RNA solutions were preincubated for 1 h at 37°C and ptRNA solutions for 5 min at 55°C and 25 min at 37°C before mixing the two. For holoenzyme kinetics, an additional enzyme reconstitution procedure was included (see above). Aliquots withdrawn at different time points from enzyme–substrate mixtures were subjected to ethanol precipitation in the presence of 20 μg of glycogen before analysis by 20% PAGE/8 M urea. Data analysis and calculation of single turnover rate constants of cleavage (*k*_obs) was performed as described (32). For the Mg²⁺ titration experiments in Figure 4E, data were analyzed by non-linear regression analysis using the Hill equation

\[ k_{obs} = \frac{k_{obs, max}}{[Mg^{2+}]^n} \]

ptRNA binding by spin column assays

Spin column assays for the determination of equilibrium dissociation constants (*K*_D) of enzyme–substrate complexes were performed as described (10). Binding of ptRNAs to P RNA was analyzed in a buffer containing 50 mM MES, 1 M NH₄OAc, CaCl₂ (15 mM) or SrCl₂ (5 or 80 mM), 0.1 % (w/v) SDS, 0.05 % (w/v) Nonidet P40, pH 6.0. Binding of ptRNAs to holoenzymes was analyzed in a buffer containing 50 mM Tris-acetate, 0.2 M NH₄OAc, 15 mM CaCl₂, 0.05 % (w/v) Nonidet P40, pH 7.1.

RESULTS

The bacterial ptRNA Gly variants with single modifications at nt −1 were constructed by ligation in the D loop after annealing of a synthetic 24-mer to a 5'-truncated tRNA transcript (Figure 1A). The different 2'-ribose modifications at nt −1 are illustrated in the boxes on the left. Several physico-chemical properties of the 2'-substituents investigated here are summarized in Table 1. The locked C3′-endo conformation of an LNA residue is shown in comparison to that of regular nucleotides in Figure 1B. To put our data into a broader perspective, we analyzed the substrates in reactions catalyzed by *E. coli* and *B. subtilis* RNase P (RNA), the main model systems for the two structurally distinct classes of bacterial P RNAs, type A (for 'ancestral') and type B (for 'Bacillus') (33–37).

Effect of modifications at nt −1 on substrate binding by *E. coli* P RNA

Two sets of variants of ptRNA Gly were investigated. The first set carried ribothymidines (rT) at nt −1 and −2 and a 2'-OH, 2'-OCH₃ or LNA modification at nt −1 [referred to as 2'-OH (T-1), 2'-OCH₃ (T-1) and LNA (T-1)] or LNA at nt −2 [LNA (T-2)]. The ribothymidines at positions −1 and −2 were introduced for reasons of comparability with variants carrying LNA substitutions at nt −1 or −2, because LNA analogs for uridine were not available. The second set of substrates carried a C at nt −1 and U at nt −2, and a 2'-OH, 2'-fluoro (2'-F) or 2'-deoxy (2'-H) substituent at position −1 [referred to as 2'-OH (C-1), 2'-F (C-1) and 2'-H (C-1)]. We determined equilibrium dissociation constants (*K*_D) for binding of ptRNA Gly variants to *E. coli* P RNA at two Sr²⁺ (5 and 80 mM) and one Ca²⁺ (15 mM) concentration (Table 2). Both metal ions support substrate binding to *E. coli* P RNA with similar efficiency as Mg²⁺, but are inactive (Sr²⁺) or very inefficient (Ca²⁺) as catalytic cofactors ([30] and references therein). The ptRNA with rT residues at −2 and −1, variant 2'-OH (T-1), bound to *E. coli* P RNA with the same affinity (within 2-fold) as the variant with U at −2 and C at −1,
variant 2'-OH (C-1) (Table 2), indicating that the extra methyl groups on the bases as well as the pyrimidine identity at nt −1 had no or very marginal effects on substrate binding in the ground state. This essentially pertains to the LNA (T-2) substrate as well, which differed from the control substrate 2'-OH (T-1) only by a 3–4-fold increase in $K_d$ at the lowest divalent metal ion concentration (5 mM Sr$^{2+}$). Thus, an LNA modification at nucleotide −2 had little effect on substrate binding by *E. coli* P RNA.

At all three tested metal ion conditions, binding defects were seen for the 2'-F (C-1) (2–4-fold), LNA (T-1) (2–13-fold), 2'-H (C-1) (9–60-fold) and 2'-OCH$_3$ (T-1) substrate (30–50-fold). Remarkably, the binding defect for LNA (T-1) was essentially suppressed (within 3-fold) at 15 mM Ca$^{2+}$ or 80 mM Sr$^{2+}$ (Table 2). For comparison, the 2'-H (C-1) ptRNA was still bound with 9–11-fold reduced affinity under the same conditions, thus even exceeding the defect of a 2'-F substitution (4-fold effect on $K_d$). In summary, the LNA substitution at position −1 had only a very minor impact on ptRNA ground state binding, despite its bulky extra methylene group and a sugar pucker arrested in C3'-endo.

In contrast, the structurally related 2'-OCH$_3$ modification caused a >30-fold affinity decrease at all tested metal ion concentrations. The 2'-deoxy substitution caused the largest affinity decrease, 60-fold at 5 mM Sr$^{2+}$, which could be mitigated to 10-fold at the higher divalent metal ion concentrations. From the comparison of an LNA versus 2'-H and 2'-F modification at nt −1 we infer that the presence of a 2'-substituent at nt −1 with good H-bond acceptor capacity, such as the 2'-oxygen, is a major determinant for metal-dependent formation of E–S ground state complexes. Such information could not be extracted from the results obtained with the related 2'-OCH$_3$ modification, because the steric effect dominates for 2'-OCH$_3$.

**Effect of modifications at nt -1 on substrate binding by *B. subtilis* P RNA**

*B. subtilis* P RNA bound the control all-ribose substrates at 15 mM Ca$^{2+}$ with 19–26-fold lower affinity than *E. coli* P RNA (Table 2), consistent with previous findings (37). Again, whereas the LNA (T-1) substrate showed little perturbation of substrate binding relative to the 2'-OH (T-1) substrate (2.6-fold increase in $K_d$), the defect was most pronounced for the substrate with 2'-OCH$_3$ at −1 (11-fold increase in $K_d$). Here, the 2'-H substitution reduced affinity only 2-fold and a 2'-F substitution was bound with roughly equal affinity as the 2'-OH (C-1) control substrate. These results are qualitatively similar to those obtained with *E. coli* P RNA.

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**Table 1. Some physico-chemical properties of ribose moieties with different 2'-substituents**

<table>
<thead>
<tr>
<th>2'-substituent</th>
<th>H-bond donor</th>
<th>H-bond acceptor</th>
<th>Flexible ribose conformation</th>
<th>Preferentially adopted sugar pucker$^a$</th>
<th>Relative solubility$^b$</th>
<th>Electronegativity of 2'-substituent</th>
<th>Sterical impediments</th>
</tr>
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<tbody>
<tr>
<td>2'-OH</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>C3'-endo</td>
<td>Highest</td>
<td>High</td>
<td>No</td>
</tr>
<tr>
<td>LNA</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Locked C3'-endo</td>
<td>Intermediate</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td>2'-OCH$_3$</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>C3'-endo</td>
<td>Intermediate</td>
<td>High</td>
<td>Yes</td>
</tr>
<tr>
<td>2'-H</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>C2'-endo</td>
<td>Lowest</td>
<td>Low</td>
<td>No</td>
</tr>
<tr>
<td>2'-F</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>C3'-endo</td>
<td>Low</td>
<td>Highest</td>
<td>No</td>
</tr>
</tbody>
</table>

$^a$Information for LNA taken from Petersen et al. (52) for conformational equilibria of ribose and 2'-substituted riboses (2'-OH, 2'-H and 2'-OCH$_3$), see Gusichlauer and Jankowski (53).

$^b$According to Cramer and Truhlar (54).
Table 2. Substrate ground state binding to \textit{E. coli} and \textit{B. subtilis} P RNAs and holoenzymes

<table>
<thead>
<tr>
<th>[Me(^{2+})]</th>
<th>ptRNA</th>
<th>\textit{E. coli}</th>
<th>\textit{B. subtilis}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\text{P RNA}</td>
<td>\text{Holoenzyme}</td>
<td>\text{P RNA}</td>
</tr>
<tr>
<td></td>
<td>(K_d) (nM)</td>
<td>(K_{d,rel})</td>
<td>(K_d) (nM)</td>
</tr>
<tr>
<td>5 mM Sr(^{2+})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'-OH (T-1)</td>
<td>86 ± 22</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>LNA (T-1)</td>
<td>1143 ± 232</td>
<td>13.3</td>
<td>–</td>
</tr>
<tr>
<td>2'-OCH(_3) (T-1)</td>
<td>4168 ± 733</td>
<td>48.5</td>
<td>–</td>
</tr>
<tr>
<td>LNA (T-2)</td>
<td>295 ± 70</td>
<td>3.4</td>
<td>–</td>
</tr>
<tr>
<td>2'-OH (C-1)</td>
<td>48 ± 20</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>2'-F (C-1)</td>
<td>103 ± 49</td>
<td>2.1</td>
<td>–</td>
</tr>
<tr>
<td>2'-H (C-1)</td>
<td>2887 ± 874</td>
<td>60.1</td>
<td>–</td>
</tr>
<tr>
<td>15 mM Ca(^{2+})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'-OH (T-1)</td>
<td>6.1 ± 2.3</td>
<td>1.0</td>
<td>1.1 ± 0.11</td>
</tr>
<tr>
<td>LNA (T-1)</td>
<td>15.5 ± 3.7</td>
<td>2.5</td>
<td>1.1 ± 0.34</td>
</tr>
<tr>
<td>2'-OCH(_3) (T-1)</td>
<td>191.4 ± 4</td>
<td>31.4</td>
<td>3.8 ± 0.64</td>
</tr>
<tr>
<td>LNA (T-2)</td>
<td>6.7 ± 3</td>
<td>1.1</td>
<td>–</td>
</tr>
<tr>
<td>2'-OH (C-1)</td>
<td>7.6 ± 1.3</td>
<td>1.0</td>
<td>0.92 ± 0.21</td>
</tr>
<tr>
<td>2'-F (C-1)</td>
<td>27.1 ± 3.7</td>
<td>3.6</td>
<td>1.24 ± 0.35</td>
</tr>
<tr>
<td>2'-H (C-1)</td>
<td>82.5 ± 11.3</td>
<td>10.9</td>
<td>1.9 ± 0.77</td>
</tr>
<tr>
<td>80 mM Sr(^{2+})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'-OH (T-1)</td>
<td>5.9 ± 2.9</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>LNA (T-1)</td>
<td>11.5 ± 4.2</td>
<td>1.9</td>
<td>–</td>
</tr>
<tr>
<td>2'-OCH(_3) (T-1)</td>
<td>211 ± 61</td>
<td>35.8</td>
<td>–</td>
</tr>
<tr>
<td>LNA (T-2)</td>
<td>7.4 ± 2.4</td>
<td>1.25</td>
<td>–</td>
</tr>
<tr>
<td>2'-OH (C-1)</td>
<td>7.4 ± 2</td>
<td>1.0</td>
<td>–</td>
</tr>
<tr>
<td>2'-F (C-1)</td>
<td>29.9 ± 8.3</td>
<td>4.0</td>
<td>–</td>
</tr>
<tr>
<td>2'-H (C-1)</td>
<td>67.9 ± 16.4</td>
<td>9.2</td>
<td>–</td>
</tr>
</tbody>
</table>

The analysis of ptRNA binding was performed using the spin column assay (10). The dissociation constant \((K_d)\) was measured in the presence of 50 mM MES/NaOH, pH 6.0, 1 M NH\(_4\)OAc (P RNA) and Ca\(^{2+}\) (15 mM) or Sr\(^{2+}\) (5 or 80 mM) as the divalent metal ion; ptRNA binding to the holoenzyme was assayed at 50 mM Tris/CH\(_3\)COOH, 0.2 M NH\(_4\)OAc, 15 mM Ca\(^{2+}\), pH 7.1. Relative \(K_d\) values \((K_{d,rel})\): individual \(K_d\) divided by \(K_d\) for the corresponding all-ribose ptRNA. \(K_d\) values for the 2'-F (C-1) and 2'-H (C-1) substrates at 15 mM Ca\(^{2+}\) are consistent (within 2-fold) with \(K_d\) values determined in a previous study under identical conditions (20).

P RNA, except for the generally weaker substrate affinity of \textit{B. subtilis} P RNA and its relatively low apparent sensitivity to the 2'-H substitution at C-1.

**Effect of modifications at nt –1 on substrate binding by RNase P holoenzymes**

We further investigated binding of the modified substrates to \textit{E. coli} and \textit{B. subtilis} RNase P holoenzymes at 15 mM Ca\(^{2+}\) but in the presence of a 5-fold lower monovalent salt concentration (0.2 M) than in RNA-alone assays. The presence of the protein cofactor increased and equalized substrate affinity \((K_d)\) values between 1 and 13 nM; Table 2) and abolished the substrate affinity differences seen for \textit{E. coli} relative to \textit{B. subtilis} P RNA. The 2'-H (C-1) ptRNA was exceptional, as it was bound with 6-fold lower affinity than its control substrate by the \textit{B. subtilis} holoenzyme, thus even exceeding the defect of the 2'-OCH\(_3\) (T-1) substrate (3.5-fold for \textit{E. coli}, 4.1-fold for \textit{B. subtilis} RNase P). Thus, a pronounced defect of 2'-H, not evident in the \textit{B. subtilis} P RNA-alone affinity assay, emerged in the holoenzyme context. LNA at nt –1 affected ground state binding to the holoenzymes insignificantly.

**Cleavage site selection**

\textit{Escherichia coli} P RNA is known to form alternative E–S complexes in response to structural changes in the enzyme and/or substrate RNA, which lead to aberrant cleavage usually one nucleotide upstream (m–1 site, between –2 and –1) of the canonical cleavage site (c–0 site, between –1 and +1) (see e.g. 14,27). More rarely, aberrant cleavage at the m–1 site (between nt +1 and +2) has also been observed in reactions catalyzed by \textit{E. coli} and \textit{B. subtilis} P RNA (Figure 2A) (8,19,22).

Except for the LNA (T-1) and 2'-OCH\(_3\) (T-1) substrates, all ptRNA variants listed in Table 2 were processed exclusively at the c–0 site by \textit{E. coli} P RNA (data not shown). The cleavage sites for ptRNA with LNA or 2'-OCH\(_3\) at –1 were analyzed by co-electrophoresis of nuclease P1 hydrolysis ladders (P1 like RNase P generates 5'-phosphate termini) along with the products derived from \textit{E. coli} P RNA cleavage. This revealed cleavage of the LNA (T-1) ptRNA at the c–0 and m–1 sites (Figure 2B and C, lanes 2 and 9, respectively), whereas the 2'-OCH\(_3\) (T-1) ptRNA was cleaved exclusively at the m+1 site (Figure 2B and C, lanes 4 and 2, respectively). The 7-nt 5'-cleavage product with the 3'-terminal LNA sugar generated by cleavage at the c–0 site (Figure 2B, lane 2) migrated somewhat faster than the corresponding all-ribose counterpart (Figure 2B, lane 6). Changes in the mobility of short oligonucleotides with 3'-terminal ribose modifications in high-resolution gels have been documented before (20,22).

The \textit{E. coli} RNase P holoenzyme cleaved the LNA (T-1) and 2'-OCH\(_3\) (T-1) ptRNAs with very low efficiency relative to the unmodified substrate (Figure 2C, lanes 5...
and 12 versus 4 and 11). Under the applied holoenzyme assay conditions (buffer B), essentially no cleavage of the LNA (T-1) and 2'-OCH₃ (T-1) substrates was seen in the absence of the protein cofactor (Figure 2C, lanes 7 and 14). Thus, the protein exerted its known effect, i.e. improvement of activity under low salt conditions, but a severe catalytic defect caused by LNA (T-1) and 2'-OCH₃ at nt -1 remained despite the presence of the protein. Interestingly, the protein switched the cleavage site for the 2'-OCH₃ (T-1) ptRNA such that cleavage occurred predominantly at the m₁ site and not at the m₃₁ site as in the RNA-alone reaction (Figure 2C, lanes 2 versus 5). As in the RNA-alone reaction, the E. coli holoenzyme cleaved the LNA (T-1) substrate with low efficiency at the c₀ and m +₁ sites, and overexposure of gels even revealed some cleavage at the m₁ site (Figure 2C, lane 12). Processing inhibition by LNA and 2'-OCH₃ at -1 also led to appearance of an aberrant cleavage product in reactions catalyzed by the E. coli holoenzyme (marked by asterisks in Figure 2C, lanes 5 and 12). This aberrant cleavage site, attributable to a minor alternative conformation of ptRNA Gly, was tentatively assigned (data not shown) to the anticodon arm. Indeed, cleavage of such weakly populated aberrant tRNA conformers was observed before by Kikuchi and coworkers and termed hyperprocessing (38). The underlying rearrangement of the tRNA structure apparently results from base pairing between the 3₀-strand of the acceptor stem and nucleotides of the variable loop and/or the 3₀-strand of the anticodon stem to form a hairpin-like structure (38–40).

In the reaction catalyzed by B. subtilis P RNA and holoenzyme, we saw very weak cleavage at the c₀ site for 2'-OCH₃ (T-1) ptRNA and at the m₃₁ and c₀ sites for LNA (T-1) ptRNA. Examples are shown in Figure 3.
for the *B. subtilis* RNA-alone reaction (Figure 3A, lanes 6–15) and the *B. subtilis* holoenzyme (Figure 3B, lanes 15–18). In conclusion, all four tested activities (*E. coli* and *B. subtilis* P RNAs and holoenzymes) cleaved the LNA (T-1) substrate with very low efficiency relative to unmodified ptRNA, and utilized the aberrant m₁ site in addition to the c₀ site. However, the 2’-OCH₃ (T-1) ptRNA was cleaved with very low efficiency at the c₀ site by *B. subtilis* P RNA and holoenzyme, whereas *E. coli* P RNA selected exclusively the m₁ site and the 

**Rate constants of cleavage by *E. coli* P RNA**

All modifications introduced at nt −1 significantly affected the cleavage reaction catalyzed by *E. coli* P RNA. The rate constants for c₀ cleavage under single turnover conditions (E >> S) and rate-limiting chemistry (120 mM Mg²⁺, pH 5.5) decreased in the order 2’-OH (T-1) > LNA (T-2) > 2’-OH (C-1) > 2’-F (C-1) > 2’-H (C-1) >> LNA (T-1), and for the 2’-OCH₃ (T-1) ptRNA no cleavage at all was detected at the c₀ site (Table 3). The same hierarchy was seen at 20 mM Mg²⁺, pH 5.5, but at this lower Mg²⁺ concentration cleavage of the LNA (T-1) and 2’-OCH₃ (T-1) substrates was at the detection limit and thus not further analyzed. Rate differences were mitigated at pH 7 and 120 mM Mg²⁺, which was expected because at this higher pH value cleavage rates are generally faster, but another step than cleavage chemistry begins to limit the turnover of ptRNA substrates with 2’-OH at nt −1 (20). At lower Mg²⁺ concentrations (20 mM), the defects owing to ribose modifications at nt −1 were exacerbated, consistent with the documented Mg²⁺ binding defect caused by the absence of the 2’-hydroxy group at this location (8,20). Low efficiency cleavage of ptRNA 2’-OCH₃ (T-1) at the m₁ site was comparable at 20 and 120 mM Mg²⁺ (pH 7), although the endpoint of the reaction was lower at 20 mM Mg²⁺ (0.3 versus 0.55; Table 3).

**Rate constants of cleavage by *B. subtilis* P RNA**

In single turnover reactions (5 μM P RNA) at 120 mM Mg²⁺ and rate-limiting chemistry (pH 5.5) (10), the hierarchy of effects caused by the different ribose modifications equaled that observed for *E. coli* P RNA (cf. Tables 3 and 4), but the decreases in rate constant were more pronounced for the 2’-F (C-1) and particularly the 2’-H (C-1) ptRNA analyzed under the same conditions. Furthermore, the endpoint for c₀ cleavage of the LNA (T-1) substrate by *B. subtilis* P RNA was ~2.5% (Table 4), compared with 65% (fitted endpoint) and 42% (highest substrate conversion measured experimentally; Table 3) for the *E. coli* P RNA reaction. Another peculiarity was seen for *B. subtilis* P RNA-catalyzed cleavage of 2’-OCH₃ (T-1) ptRNA at the c₀ site and LNA (T-1) ptRNA at the m₁ site. Here, cleavage was already at saturation at the first time point (1 h; Table 4 and Figure 3A, lanes 6–15), with very low endpoints (1–4%; Table 4). A possible explanation is that weakly populated E−S conformers gave rise to these cleavages.

**Rate constants of cleavage by RNase P holoenzymes**

Rates in the RNase P holoenzyme reactions were measured under single turnover conditions at pH 6.0 and 4 mM Mg²⁺, or at 10 mM Mg²⁺ for the most inhibitory LNA and 2’-OCH₃ modifications. Effects were similar in reactions catalyzed by the *E. coli* and *B. subtilis* holoenzymes, with two major exceptions: (i) similar to the *B. subtilis* RNA-alone reaction, the defect caused by the 2’-H (C-1) ptRNA was much higher in the *B. subtilis* holoenzyme than in the *E. coli* holoenzyme; (ii) *B. subtilis* holoenzymes cleaved 2’-F (C-1) ptRNA with lower efficiency than their *E. coli* counterparts. This is consistent with a previous observation (10) that the *B. subtilis* holoenzyme was more sensitive to ribose modifications than the *E. coli* holoenzyme.
Table 3. Observed ($k_{obs}$) and relative ($k_{rel}$) rate constants for cleavage of modified ptRNAs by *E. coli* P RNA

<table>
<thead>
<tr>
<th>ptRNA</th>
<th>pH 5.5</th>
<th></th>
<th>pH 7</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 mM Mg$^{2+}$</td>
<td>120 mM Mg$^{2+}$</td>
<td>20 mM Mg$^{2+}$</td>
<td>120 mM Mg$^{2+}$</td>
</tr>
<tr>
<td>2'-OH (T-1)</td>
<td>$k_{obs}$ (min$^{-1}$)</td>
<td>$k_{rel}$</td>
<td>$k_{obs}$ (min$^{-1}$)</td>
<td>$k_{rel}$</td>
</tr>
<tr>
<td>LNA (T-1)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.d.</td>
</tr>
<tr>
<td>c$_0$ site</td>
<td>0.9 ± 0.25</td>
<td>1.0</td>
<td>3.2 ± 1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>LNA (T-1)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>(1 ± 0.5) x 10$^{-3}$</td>
<td>1.0</td>
</tr>
<tr>
<td>m$_{+1}$ site</td>
<td>0.0018</td>
<td>1.7</td>
<td>3.23 ± 0.16</td>
<td>1.4</td>
</tr>
<tr>
<td>2'-OCH$_3$ (T-1)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>1.2 ± 0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>c$_0$ site</td>
<td>0.52 ± 0.02</td>
<td>1.7</td>
<td>0.34 ± 0.06</td>
<td>1.4</td>
</tr>
<tr>
<td>LNA (T-2)</td>
<td>1.2 ± 0.1</td>
<td>0.8</td>
<td>5.2 ± 0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>2'-OCH$_3$ (T-1)</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0.05 ± 0.07</td>
<td>6.4</td>
</tr>
<tr>
<td>m$_{+1}$ site</td>
<td>2.3 (± 0.9) x 10$^{-2}$</td>
<td>39</td>
<td>1.3 ± 0.08</td>
<td>6.7</td>
</tr>
<tr>
<td>2'-H (C-1)</td>
<td>7 (± 3) x 10$^{-4}$</td>
<td>1286</td>
<td>(4.0 ± 0.7) x 10$^{-2}$</td>
<td>80</td>
</tr>
</tbody>
</table>

n.a.: not analyzed; n.d.: cleavage not detectable. Cleavage experiments were performed under single turnover conditions (5 µM *E. coli* P RNA, <1 nM ptRNA, 1 M NH$_4$OAc and the indicated pH and Mg$^{2+}$ concentration, essentially as described (30); for low efficiency cleavage of the LNA (T-1) and 2'-OCH$_3$ (T-1) substrates, average values for the endpoint ($L = \text{limit}$) of the reaction determined by curve fitting and average values for the highest substrate turnover measured experimentally are given; $k_{rel}$ is the ratio of the respective $k_{obs}$ value to that of the 2'-OH (T-1) ptRNA. For the LNA (T-1) and 2'-OCH$_3$ (T-1) substrates, cleavage is differentiated for the c$_0$ and m$_{+1}$ sites; all other substrates were cleaved exclusively at the c$_0$ site.

Table 4. Observed ($k_{obs}$) and relative ($k_{rel}$) rate constants for cleavage of modified ptRNAs by *B. subtilis* P RNA and *E. coli* and *B. subtilis* RNase P holoenzymes

<table>
<thead>
<tr>
<th>ptRNA</th>
<th><em>E. coli</em> holoenzyme</th>
<th><em>B. subtilis</em> P RNA</th>
<th><em>B. subtilis</em> holoenzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 6</td>
<td>pH 5.5</td>
<td>pH 6</td>
</tr>
<tr>
<td></td>
<td>4 or 10 mM Mg$^{2+}$</td>
<td>120 mM Mg$^{2+}$</td>
<td>4 or 10 mM Mg$^{2+}$</td>
</tr>
<tr>
<td>2'-OH (T-1)</td>
<td>$k_{obs}$ (min$^{-1}$)</td>
<td>$k_{rel}$</td>
<td>$k_{obs}$ (min$^{-1}$)</td>
</tr>
<tr>
<td>LNA (T-1)</td>
<td>2.5 ± 0.5</td>
<td>1.0</td>
<td>2.6 ± 0.4</td>
</tr>
<tr>
<td>c$_0$ site</td>
<td>Extent: 0.03 at 1 h;</td>
<td></td>
<td>(3 ± 1) x 10$^{-3}$</td>
</tr>
<tr>
<td></td>
<td>≤ 0.2 at 20 h$^a$</td>
<td>L = 0.027/0.027</td>
<td>L = 0.027</td>
</tr>
<tr>
<td>LNA (T-1)</td>
<td>2.5 ± 0.2</td>
<td>1.0</td>
<td>5.8 ± 0.1</td>
</tr>
<tr>
<td>m$_{+1}$ site</td>
<td>Extent: 0.03 at 1 h;</td>
<td></td>
<td>(6.0 ± 0.5) x 10$^{-2}$</td>
</tr>
<tr>
<td></td>
<td>≤ 0.7 at 20 h$^a$</td>
<td>L = 0.19/0.34</td>
<td>L = 0.10 ± 0.12</td>
</tr>
<tr>
<td>2'-OCH$_3$ (T-1)</td>
<td>0.39 ± 0.06</td>
<td>1.0</td>
<td>1.57 ± 0.04</td>
</tr>
<tr>
<td>c$_0$ site</td>
<td>(6.0 ± 0.1) x 10$^{-3}$</td>
<td>417</td>
<td>(1 ± 0.2) x 10$^{-3}$</td>
</tr>
<tr>
<td>m$_{+1}$ site</td>
<td>L = 0.19/0.34</td>
<td></td>
<td>(L = -0.08)</td>
</tr>
</tbody>
</table>

n.d.: Cleavage not detectable. Cleavage experiments were performed under the following conditions: 5 µM *B. subtilis* P RNA, <1 nM ptRNA, 1 M NH$_4$OAc, 50 mM MES, pH 5.5 and Mg$^{2+}$ as specified above each column or 100 nM holoenzyme, <1 nM ptRNA, 100 mM KCl, 50 mM MES, pH 6 and 4 or 10 mM Mg$^{2+}$ (*E. coli*/*B. subtilis* holoenzymes). Holoenzyme reconstitution and kinetic experiments were performed as described under Materials and Methods. Cleavage is differentiated for the c$_0$ and m$_{+1}$ site in the case of the 2'-OCH$_3$ (T-1) substrate, and for the c$_0$ and m$_{+1}$ site in the case of ptRNA LNA (T-1); all other substrates were cleaved exclusively at the c$_0$ site. For the 2'-OCH$_3$ (T-1) and LNA (T-1) substrates, cleavage occurred to very low extents [in most cases with saturation already reached at the first data point (1 h)]; thus, only the extent of cleavage instead of cleavage rate constants is given here.

$^a$Determined at 10 mM Mg$^{2+}$.

$^b$Determined from the linear range of substrate turnover (no endpoint from curve fitting/ highest turnover experimentally measured).
than *E. coli* holoenzyme reaction; (ii) weak cleavage of the 2'-OCH₃ substrate at the c₀ site occurred with *B. subtilis* but not with *E. coli* RNase P, as mentioned before. As observed for *B. subtilis* P RNA (see above), the extent of cleavage for the LNA (T-1) and 2'-OCH₃ (T-1) substrates by both holoenzymes was extremely low (Table 4).

**Mg²⁺ dependence of LNA (T-1) ptRNA cleavage by *E. coli* P RNA at sites c₀ and m₊₁**

The LNA (T-1) ptRNA was simultaneously processed at the c₀ and m₊₁ site by *E. coli* P RNA (Figure 2B and C). Simultaneous processing at different sites can be attributed to parallel reaction pathways (14,19,22,23,41) as illustrated in Scheme 1. Here we investigated the Mg²⁺ dependence of c₀ and m₊₁ cleavage, with the following outcome: First, at low [Mg²⁺], the fraction of substrate cleaved at the c₀ site (Fₓ) was substantially lower at early versus late time points (Figure 4A); with increasing [Mg²⁺], these differences largely disappeared (Figure 4B–D). This indicates that the two (EoS) complexes reacted with different single exponential decay rate constants (kₒbs) at low [Mg²⁺] and similar rate constants at high [Mg²⁺]. Second, Fₓ values at experimental endpoints (last measured time point) and theoretical endpoints (derived from the single exponential decay curve fit) were lower at 20 mM Mg²⁺ (Fₓ < 0.7) than at e.g. 120 mM Mg²⁺ (Fₓ > 0.8). Third, plotting kₒbs as a function of [Mg²⁺] (Figure 4E) and allowing for a cooperative Mg²⁺ dependence at both cleavage sites (see Materials and methods section) revealed a higher Mg²⁺ dependence for cleavage at the c₀ site (Kₑ₁/₂ = 184 mM) than at the aberrant m₊₁ site (Kₑ₁/₂ = 129 mM), consistent with a severe metal ion binding defect for c₀ cleavage in the presence of LNA at nt −1 (the defect may include contributions from Kₑ_conf or Kₑ⁺; see Scheme 1). A double logarithmic plot of the same data illustrates that the kₒbs for c₀ cleavage was slower than that for m₊₁ cleavage at low [Mg²⁺], whereas this relation was reversed at high [Mg²⁺] (Figure 4F).

**pH dependence of c₀ and m₊₁ cleavage pathways**

In the hydrolysis reaction catalyzed by RNase P, a linear relationship of log kₒbs and pH with a slope of −1 is taken as evidence for the catalytic step being rate-limiting (8,9,11,20,22,42). This pH dependence, consistent with a single ionization, is thought to reflect ionization of a metal ion-coordinated water molecule acting as the nucleophile in this phosphodiester hydrolysis reaction (8,11,22). Analysis of the pH dependence of log kₒbs for cleavage of the LNA (T-1) ptRNA at sites c₀ and m₊₁ (Figure 5) revealed a slope of −0.9 up to pH 7 for c₀ cleavage, after which the curve inflected (Figure 5D), consistent with another step limiting the rate of the reaction above pH 7 (9). In contrast, cleavage at the m₊₁ site was linear over the entire pH range (5.5–8.1) but with a slope of only 0.45 (Figure 5D), indicative of another step than cleavage chemistry limiting the reaction at all tested pH values.

The pH variation experiments were performed at an intermediate Mg²⁺ concentration (60 mM). Interestingly, lowering the pH caused similar effects as lowering the Mg²⁺ concentration at neutral pH (Figure 4): at low pH, m₊₁ cleavage was faster than c₀ cleavage, while this relation was reversed above pH 7 (Figure 5D); and, related to this, at low versus higher pH (Figure 5A–C) the fraction of substrate cleaved at the c₀ site (Fₓ) was substantially lower at early than late time points.

**DISCUSSION**

**LNA and 2'-OCH₃ modifications at nt −1: blockage of steps after substrate ground state binding**

A 2'-OCH₃ modification at position −1 was previously reported as the most inhibitory substrate ribose modification in the reaction catalyzed by *E. coli* P RNA (8). In this previous study, a ptRNA with a single nucleoside as 5'-leader was miscleaved at the m₊₁ site as observed here. The substrate with a 1-nt 5'-leader precluded analysis of m₋₁ cleavage, but as shown here this site appears to be blocked by 2'-OCH₃ at nt −1 in *E. coli* P RNA-alone reactions with ptRNAs carrying longer 5'-leaders (Figure 2). The inhibitory strength of 2'-OCH₃ at −1 exceeded that of a 2'-deoxy modification, consistent with a steric effect caused by the extra methyl group (8).

Our results obtained with *E. coli* P RNA demonstrate that the transition state for canonical cleavage can be achieved in the presence of a fixed C3'-endo conformation at nt −1 (Figure 2B and C), indicating that a C2'-endo conformation prevailing at this position based on NMR analyses (26) is not an absolute requirement for catalysis to occur. It is unclear if the low efficiency of c₀ cleavage with LNA at −1 is primarily due to the steric effect of the extra methylene group or includes substantial contributions from the lack of ribose flexibility at this position. Also, the strong inhibitory effect of LNA might stem from the fact that the free electron pairs of the 2'-oxygen in LNA are orientationally constrained; this may impair the correct positioning of the Mg²⁺-hydrate complex containing the water molecule which we propose to protonate the 3'-oxyanion leaving group (Figure 6A). The moderate effect of LNA at −1 on substrate ground state binding (Table 2) relative to 2'-OCH₃ suggests that the extra methylene group causes less steric constraints than the 2'-OCH₃ group; likewise, the LNA (T-1) substrate was cleaved by *E. coli* P RNA at the c₀ site, whereas c₀ cleavage was not seen at all in the presence of the 2'-OCH₃ (T-1) modification (Figure 2). However, in contrast to the mild defect in substrate ground state binding, the high Mg²⁺ requirement for c₀ cleavage of LNA (T-1) ptRNA at saturating enzyme concentrations (Figure 4E) is consistent with a severe block in the binding of catalytically relevant Mg²⁺. Thus, the defect is on Kₑ_conf and/or kₒbs rather than on Kₑ⁺ (Scheme 1). It should also be noted that the effect of 2'-OCH₃ at −1 on ground state binding (30-fold at 15 mM Ca²⁺; Table 2) was still moderate compared with the complete blockage of cleavage at site c₀ for this modification. Thus, we favor a model according to which the extra methylene or methyl group causes a steric interference with the nearby bound catalytic Mg²⁺ during its re-coordination on the way to the transition state for...
cleavage (Figure 6). To which extent the defect is exerted at the level of $K_{\text{conf}}$ or bond breakage ($k_c$) cannot be discerned on the basis of our data.

Despite their similar chemical nature, the effects of LNA and 2'-OCH$_3$ substitutions at nt -1 differed in detail. Relevant towards understanding these differences could be the fact that the 2'-O-methylene group in LNA occupies a position in space that coincides only with one of the many positions that can be adopted by the 2'-OCH$_3$ group. The latter can rotate around the C2'-O2' bond, and

Figure 4. Processing of the LNA (T-1) substrate by *E. coli* P RNA at different Mg$^{2+}$ concentrations. (A–D) Kinetic analyses of the two parallel cleavage pathways leading to cleavage at site $c_0$ or $m+1$. Cleavage experiments were performed under single turnover conditions (5 mM P RNA, <1 nM ptRNA) in the presence of 1 M NH$_4$OAc, 50 mM PIPES, pH 7 and 60 mM Mg$^{2+}$. Plots representing selected Mg$^{2+}$ concentrations show cleavage at the canonical $c_0$ site (open circles) and site $m+1$ (filled triangles). The fraction of substrate reacted is expressed as the ratio of product at a given time [P] to the total amount of substrate [S]$_{\text{tot}}$ at the start of the reaction. The relative amount of cleavage taking place at the canonical site ($F_c$; open squares) was calculated as the amount of $c_0$ product divided by the sum of $c_0$ and $m+1$ cleavage products. (E) Observed rate constants for cleavage at sites $c_0$ and $m+1$ as a function of Mg$^{2+}$ concentration. Data were fitted to the Hill equation: $k_{\text{obs}} = (V_{\text{max}} \cdot [\text{Mg}^{2+}])/(K^0 + [\text{Mg}^{2+}])$, resulting in reasonable fits with $V_{\text{max}} = 0.08 \pm 0.003$, $K^0 = 0.066 \pm 0.02$ and $n = 1.61 \pm 0.12$ for $c_0$ cleavage, and $V_{\text{max}} = 0.054 \pm 0.003$, $K^0 = 0.03 \pm 0.017$ and $n = 1.71 \pm 0.21$ for $m+1$ cleavage; $K_{1/2}$ values were calculated as $K_{1/2} = \sqrt{K^0} = 184$ mM for $c_0$ and 129 mM for $m+1$ cleavage. (F) Double logarithmic presentation of the data shown in panel E to illustrate that cleavage at site $c_0$ was slower at low Mg$^{2+}$ concentrations but faster at high Mg$^{2+}$ concentrations relative to $m+1$ cleavage.
additional orientations result from the equilibrium between C3'- and C2'-endo sugar puckers (Figure 7). The orientation flexibility of the 2'-OCH3 group relative to the methylene group in LNA may explain the differential effects of the two substitutions on substrate ground state binding (Table 2). In addition, we recently showed that LNA at the C01 position stabilizes base pairing between T-1 and U73 (see Figure 1) (43). Thus, in the ground state the LNA (T-1) substrate is expected to adopt an extended acceptor stem structure to a larger degree than the 2'-OCH3 (T-1) substrate. However, based on our recent results, the breaking of an additional base pair at the end of the acceptor stem leaves the energetic barrier for reaching the transition state of the chemical step for cleavage at the canonical (c0) phosphodiester essentially unaffected (43). An effect on the aberrant m+1 and m-1 cleavage pathways cannot be excluded.

Regarding metal-dependent substrate ground state binding to E. coli P RNA, 2'-H and 2'-deoxy substitutions at nt -1 were most deleterious, whereas LNA and 2'-F at this position had, overall, mild effects. We infer (i) that the absence of an H-bond donor function at the 2'-position of nt -1, as with LNA, is not crucial for substrate ground state binding; (ii) an extra 2'-methyl(ene) group has a mild effect if its position is constrained as in LNA; (iii) the mild effect of 2'-F can be explained in several ways; we favor the idea that the electronegative 2'-fluorine, as 2'-OH or LNA but in contrast to 2'-H, can accept a hydrogen bond from a metal ion-coordinated water molecule (20); this should also be the case for 2'-OCH3, but here steric interference seems to dominate; (iv) a locked C3'-endo sugar pucker at nt -1 is compatible with high affinity ground state binding, indicating that the sugar pucker at this position has not to be constrained in C2'-endo for ground state binding, a possibility that arose from our previous NMR study (26). The observed effects were similar for B. subtilis P RNA, but apart from its lower substrate affinity, this RNA was less sensitive to the 2'-deoxy substitution at nt -1 (Table 2). However, sensitivity to the 2'-deoxy modification emerged in the B. subtilis holoenzyme reaction, where the effect of 2'-H even exceeded that of 2'-OCH3. Possibly, the 2'-deoxy effect was masked in the B. subtilis P RNA-alone context owing to the generally low substrate affinity of this RNA in the absence of the protein cofactor. In summary, our results are consistent with the idea that acceptance of a hydrogen bond from a metal ion-coordinated water molecule is a key feature of the 2'-substituent at nt -1 during substrate ground state binding.
Figure 6. Transition state models for E. coli RNase P RNA-catalyzed phosphodiester hydrolysis at the canonical c₀ site with a ribose (A) or LNA (B) at nt −1. The model in (A) [adapted from ref. (55)] predicts that both Mgᴬ and Mgᴮ directly coordinate to the pro-Rp oxygen, with Mgᴬ coordinating the OH⁻ nucleophile, and Mgᴮ interacting with the 3'-oxygen and the 2'-hydroxy group at nt −1 via a water molecule of its hydration shell; we propose this water molecule to donate the proton to the 3'-oxanyion leaving group. The ribose at nt −1 is assumed to mainly populate the 2'-endo sugar pucker based on NMR analyses of acceptor stem mimics (26). (B) Here, the −1 nucleotide is LNA with the sugar moiety fixed in the C3'-endo conformation; the steric interference caused by LNA or 2'-OCH₃ at position −1 (indicated by the dashed curved line) likely disrupts the binding site for Mgᴮ. The model differs from others [e.g. (56)] by assuming that only two instead of three metal ions are essential; also, in our model Mgᴮ is proposed to simultaneously interact with the pro-Rp oxygen, the 3'-oxygen and the 2'-OH, whereas the latter interaction is assigned to the third metal ion in the model by Kazantsev and Pace (56). Group I intron ribozymes have a similar size as bacterial P RNA and also catalyze phosphoryl transfer reactions yielding products with 3'-OH termini. The two-metal-ion mechanistic model we propose here is essentially identical to that for the second step of group I intron splicing inferred from a crystal structure of the Azotobacter sp. ptRNA¹⁰⁰ group I intron (57), with two differences: (i) the contact of Mgᴮ to the 2'-OH is outer-sphere in the RNase P reaction, but inner-sphere in the group I intron reaction; (ii) the nucleophile is a hydroxide in the RNase P reaction, but the terminal 3'-OH of the 5'-exon in the second step of the group I intron reaction (57). Despite striking similarities, one mechanistic difference has been observed in phosphoryl hydroxysterase reactions catalyzed by the Tetrahymena group 1 intron-derived ribozyme and E. coli P RNA: whereas a 2'-amino modification at ptRNA position −1 inhibited cleavage at the canonical site with increasing protonation of the 2'-amine in the P RNA reaction (consistent with electrostatic repulsion of a metal ion), the reverse was seen with the Tetrahymena ribozyme; in the latter reaction, protonation of the 2'-amine at equivalent position rather stimulated cleavage at the canonical site (20,58). The molecular basis of this difference is not clear at present.

Cleavage fidelity

For simple class I tRNAs, such as the ptRNA¹⁰⁰ used here or B. subtilis ptRNA¹⁰⁰ (22), it has previously been discussed that interactions between E. coli P RNA and the ptRNA cleavage site are of redundant nature. For example, base identity switches at nt −1 that weaken the interaction with A248 of E. coli P RNA, 2'-substitutions at nt −1 (e.g. 2'-H or 2'-F), an Rp-phosphorothioate substitution at the scissile phosphodiester or a C₂₋₂/C₂₋₁ or C₂₋₁/C₂₋₂ mutation in the CCA-binding site of P RNA do not by themselves lead to substantial miscleavage (22). However pairwise modification, such as 2'-deoxy modification at nt −1 combined with an A248U or G292C mutation resulted in substantial miscleavage (20,22). In contrast, LNA and 2'-OCH₃ at nt −1 induced severe miscleavage in the absence of any other modification, emphasizing the deleterious effect of the extra methyl(ene) group on cleavage chemistry or a preceding conformational rearrangement of E•S complexes (Scheme 1).

Differences in the Mg²⁺ and pH dependence of the correct (c₀) and mis-cleavage (m₊₁) pathways

Harris and coworkers recently demonstrated communication between a 2'-deoxy modification at nt −1 and weakening of the A248/nt −1 contact. Alterations at both sites, such as combination of 2'-deoxy U-1 ptRNA¹⁰⁰ with the A248U mutant E. coli P RNA, resulted in cleavage at the c₀, m₊₁ and also at the m₊₂ site (22). When studying the proportion of cleavage events at the c₀ and m₊₁ site as a function of the Mg²⁺ concentration, the authors observed increased utilization of the m₊₁ site with increasing Mg²⁺ concentration. They concluded that the pathway leading to cleavage at the c₀ site exhibits a greater apparent affinity for Mg²⁺ than the pathway leading to cleavage at the m₊₁ site. We performed a similar analysis for the m₊₁ site in the presence of LNA at −1 (Figure 4) and observed a reverse correlation: here the amount of cleavage at the c₀ site increased with increasing Mg²⁺, indicating that the LNA modification lowers the apparent affinity for Mg²⁺ of the canonical cleavage pathway below that of the pathway leading to m₊₁ cleavage.

The same authors (22) observed that Fc remained essentially constant over time in E. coli P RNA cleavage of ptRNAs with 2'-deoxy modifications at nt −1 and/or mutations at A248 of P RNA. Thus, they assumed that cleavages at the canonical and aberrant site (m₊₁) in their case) follow the same single exponential decay rate (kobs) calculated from the sum of the c₀ and m₊₁ site cleavage products accumulating at individual time points. Using Fc and kobs, the apparent first-order rate constant for
cleavage ($k_{\text{app}}$) at a particular site was calculated according to $k_{\text{app}[c_0]} = F_c \times k_{\text{obs}}$, and $k_{\text{app}[m+1]} = (1 - F_c) \times k_{\text{obs}}$ [for details, see ref. (22)]. Although this assumption also holds for our data obtained at Mg$^{2+}$ concentrations between 60 and 200 mM (Figure 4F), it is not applicable particularly to the $F_c$ and $k_{\text{obs}[c_0]}$ data obtained under conditions of low [Mg$^{2+}$] and low pH (see Figures 4A, F and 5A). Changes in $F_c$ and $k_{\text{obs}[c_0]}$ may include contributions from the equilibria described by $K_{c_0}$ and $K_{\text{conf}[c_0]}$ (and the corresponding forward and back rate constants) as well as $k_{c_0}$ (see Scheme 1). Our findings ($F_c$ increases with [Mg$^{2+}$], $k_{\text{obs}[c_0]} < k_{\text{obs}[m+1]}$ at low [Mg$^{2+}$] and pH, $k_{\text{obs}[c_0]} > k_{\text{obs}[m+1]}$ at high [Mg$^{2+}$] and high pH) can be explained within the following framework (also illustrated in Scheme 2): When enzyme and substrate are mixed, rapid equilibria of (E/S)c0 and (E/S)m+1 form, which commit the complexes to reaction along the different pathways. Partitioning between (E/S)c0 and (E/S)m+1 is sensitive to the Mg$^{2+}$ concentration. At low [Mg$^{2+}$], the ratio of equilibrium binding constants $K_{c_0}/K_{m+1}$ (Scheme 1) exceeds that at higher [Mg$^{2+}$], explaining why $F_c$ increased with [Mg$^{2+}$] (Figure 4A–D). The rate constant for bond breakage at the c$_0$ site ($k_{c_0}$) and/or the preceding conformational step ($k_{\text{conf}[c_0]}$; see Scheme 1) have a higher Mg$^{2+}$ requirement than that for m$_1$ cleavage (Figure 4F). Finally, different steps are rate-limiting for cleavage at sites c$_0$ and m$_1$, as inferred from the different pH dependencies between pH 5.5 and 7.0 (Figure 5D).

**Suppression of m$_1$ cleavage**

Another idiosyncrasy of LNA or 2'-OCH$_3$ at -1 is the complete absence of cleavage by E. coli P RNA at the m$_1$ site (Figure 2B and C), which represents the prevailing miscleavage site [e.g. (44)]. Pan and colleagues showed that a 2'-deoxy substitution at -1 in ptRNA not only decreased the cleavage rate constant by B. subtilis P RNA 240-fold for the c$_0$ site but also 470-fold for the m$_1$ site and 210-fold for another aberrant site (between nt +2 and +3). This finding suggested a contact between the ribozyme and the ptRNA substrate 2'-OH at -1 which promotes a conformational rearrangement required for catalysis at both the c$_0$ as well as at aberrant sites, such as the m$_1$ site (19,24). Blockage of cleavage at the unmodified m$_1$ site owing to LNA or 2'-OCH$_3$ at -1 would be consistent with the findings of Loria and Pan.

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**Scheme 2.** Illustration of the differential effects of Mg$^{2+}$ concentration on the parallel pathways for cleavage of the LNA (T-1) substrate by E. coli P RNA, using a simplified version of Scheme 1. At low [Mg$^{2+}$], the proportion of (E/S)c0 complexes is lower than at high [Mg$^{2+}$] (indicated by differences in letter size; also, increasing [Mg$^{2+}$] increases the observed rate constant for cleavage at the c$_0$ site, $k_{\text{obs}[c_0]}$, more than $k_{\text{obs}[m+1]}$; changes in $k_{\text{obs}}$ may include effects on $K_{\text{conf}}$ and/or the chemical step ($k_{c_0}$ and $k_{m+1}$, respectively; see Scheme 1).
(19,24). Based on their model, utilization of the m +1 site in *E. coli* P RNA-catalyzed cleavage of ptRNA LNA (T-1) or 2'-OCH$_3$(T-1) may suggest that the 2'-OH group at nt +1 took over the role of that at nt −1 in promoting the conformational step preceding catalysis (Scheme 1). Likewise, redirection of cleavage from the m +1 to the m −1 site in the 2'-OCH$_3$(T-1) substrate when cleaved by the *E. coli* RNase P holoenzyme (Figure 2C) may be due to the protein cofactor allowing utilization of the 2'-OH at nt −2 as anchor point for the conformational step (24).

**pH dependence of c$_0$ and m +1 cleavage by *E. coli* P RNA**

The shallow pH dependence of log $K_{obs}$ for cleavage at the m +1 site is consistent with one or more slow steps upstream of the hydrolysis step in the miscleavage pathway, such as conformational rearrangements. Our findings and those of others (22,45) indicate that cleavage at different sites (c$_0$, m$_{-1}$, m$_{+1}$ or others) has different pH and metal ion dependencies and involves different contributions from rate-limiting steps. Accordingly, E–S complexes leading to cleavage at the different sites are structurally distinct. Cleavage at the c$_0$ site has usually the highest apparent metal ion affinity, which still pertains to ‘mild’ modifications, such as 2'-OCH$_3$ at nt −1. However, more deleterious modifications, such as LNA or 2'-OCH$_3$ at −1, can break this rule (Figure 4E). Considering that LNA at nt −1 had little effect on substrate binding in the ground state, this modification is a paradigm for a modification that specifically inhibits formation of later steps along the cleavage pathway.

**Cleavage of modified substrates by *B. subtilis* P RNA**

Loria and Pan (19) have studied processing of yeast ptRNA$^{\text{Phe}}$ by *B. subtilis* P RNA, representing a tRNA with a canonical 7-bp acceptor stem as the ptRNA$^{\text{Gly}}$ used in our investigation (Figure 1). They observed substantial miscleavage of ptRNA$^{\text{Phe}}$, particularly at the m$_{-1}$ site and even in the absence of any ribose modifications. In contrast, we did not observe significant miscleavage by *B. subtilis* P RNA for any of the ptRNA$^{\text{Gly}}$ variants used here, except for the LNA (T-1) substrate which gave rise to cleavage at the m$_{+1}$ site (Table 4). Even the 2'-OCH$_3$(T-1) variant was cleaved exclusively at the c$_0$ site (Figure 3), in contrast to *E. coli* P RNA which entirely miscleaved this substrate. Thus, we observed little propensity of *B. subtilis* P RNA to miscleave the ptRNA$^{\text{Gly}}$ substrate. The discrepancy to the previously observed miscleavage of yeast ptRNA$^{\text{Phe}}$ (19) may be explained by the fact that yeast ptRNA$^{\text{Phe}}$ has a rather labile acceptor stem with 3A–U and 1G–Ubp in a row in its lower part; in comparison, the acceptor stems of *B. subtilis* tRNA$^{\text{Phe}}$ molecules consist of 6G–C and a single A–Ubp, which is more typical of bacterial tRNAs. Low rigidity of the tRNA body is expected to cause conformational heterogeneity, which would explain increased miscleavage. This further raises the question if miscleavage at a specific site necessarily proceeds along the same mechanistic pathway for ptRNAs with rigid and less rigid tRNA bodies.

**Cleavage of modified substrates by the *E. coli* RNase P holoenzyme**

In several previous analyses, essentially identical miscleavage patterns were observed in *E. coli* P RNA-alone and holoenzyme reactions. This pertains, for example, to the disruption of the 3'-CCA interaction by mutation (36,46), or to reactions utilizing a A248U mutant P RNA and a ptRNA with a 2'-deoxy-U at nt −1 (41). However, in a study investigating the effect of a substrate 2'-amino modification at nt −1 on cleavage by *E. coli* RNase P (RNA), the P protein suppressed miscleavage at the m$_{-1}$ site and redirected cleavage to the c$_0$ site (21). This suggests that effects of the P protein on cleavage fidelity depend on the specific structural alteration.

The cleavage site of the 2'-OCH$_3$(T-1) substrate was almost entirely shifted from the m$_{-1}$ to the m$_{+1}$ site in the *E. coli* holoenzyme versus P RNA-alone reaction (Figure 2C, lanes 2 and 5). With LNA at −1, also some cleavage at the m$_{-1}$ site appeared in the presence of the protein cofactor (Figure 2C, lanes 9 and 12). Evidently, the protein, by providing additional substrate binding energy and by enhancing the affinity of key metal ions, modulates the activation barriers of pathways leading to cleavage at different sites. The differences in monovalent and di-valent salt conditions used in RNA-alone and holoenzyme reactions may also have contributed to the observed protein-dependent changes, resulting in differential effects on the individual miscleavage pathways. Specifically, the shift of cleavage site for the 2'-OCH$_3$(T-1) substrate may be explained by (EoS)$m_{+1}$ complex formation favored over that of (EoS)$m_{-1}$ complexes in the context of the holoenzyme reaction, and/or by a differential effect of the protein on the individual equilibrium docking steps [(EoS)$^\ast$; Scheme 1] preceding catalysis (24). The protein might also affect active site architecture more profoundly than as yet thought.

**Differences between *E. coli* and *B. subtilis* RNase P (RNA)**

Architectural differences between bacterial P RNAs of type A (for 'ancestral') and type B (for 'Bacillus') (33) are associated with biochemical differences. The *B. subtilis* RNase P holoenzyme binds ptRNA with a much higher affinity than mature tRNA, but this discrimination against mature tRNA is much less pronounced for *E. coli* RNase P (34,35). RNase P enzymes containing the *B. subtilis* P RNA are less prone to aberrant cleavage than its *E. coli* counterpart (37, this study). The *E. coli* protein reduces the Mg$_{2+}$ concentration requirement for tertiary structure formation of P RNA and increases the melting temperature at physiologically relevant Mg$_{2+}$ concentrations (37). In contrast, the *B. subtilis* protein does not exert such stabilizing effects on its cognate or *E. coli* P RNA (37). Taking these reported differences between the two RNase P model systems into account, it is not surprising that we saw differences and commonalities (for details, see Results). Differences included a stronger defect caused
by 2'-H at nt -1 on the \textit{B. subtilis} versus \textit{E. coli} holoenzyme, and deviating cleavage site selection for the 2'-OCH$_3$ substrate; major commonalities included the severe catalytic block by LNA and 2'-OCH$_3$ and, for the LNA substrate, the occurrence of low efficiency parallel cleavage pathways leading to hydrolysis at the c$_0$ and m$_{+1}$ sites. The differences illustrate the complex interplay of functional groups and of thermodynamic as well as kinetic parameters in this RNA-catalyzed and protein-assisted phosphodiester hydrolysis reaction. Compared with protein enzyme orthologs with rigid and strongly conserved active sites, type A and B RNase P enzymes appear more as homologs that still catalyze the same reactions, but with distinct architectural and non-identical mechanistic concepts.

The P protein is unable to compensate the catalytic block by LNA

The protein subunit of bacterial RNase P increases substrate affinity and specificity (47,48), stabilizes the local RNA conformation (37), preorganizes S- and C-domain orientation (24) and increases the affinity of catalytically important divalent metal ions (11). For \textit{B. subtilis}, it has been shown that the P protein interacts with the 5'-leader of tRNA precursor thus increasing substrate affinity (37,49) while reducing the contact area to the T stem-loop region of tRNA substrates relative to P RNA alone (50). Recently, the binding affinity and processing efficiency of a set of \textit{E. coli} ptRNA substrates with different 5'-leaders and bodies was investigated in the absence and presence of the protein cofactor (51). The results indicated that the protein confers the capacity to the \textit{E. coli} RNase P holoenzyme to bind all 5'-pre-tRNAs with uniform affinity. Apparently, this capacity was achieved during evolution by combining suboptimal tRNA structures with 5'-leader sequences that confer high ptRNA affinity (51). Such a thermodynamic compensation effect was also seen here for binding of the 2'-OCH$_3$ (T-1) ptRNA, where defects of binding to \textit{E. coli} and \textit{B. subtilis} P RNA were alleviated by the presence of the protein cofactor (Table 2). In contrast, the dramatic inhibitory effect on P RNA catalysis caused by LNA and 2'-OCH$_3$ at nt -1 remained in the holoenzyme reaction, indicating that these modifications block E*S$_0$ formation and/or the bond breakage step itself. Finally, our results demonstrate that LNA enriches the nucleotide analog toolbox for mechanistic enzyme studies.

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