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Facilitation of RNA Enzyme Activity in the Molecular Crowding Media of Cosolutes

Shu-ichi Nakano,*,†,‡ Hisae Tateishi Karimata,‡,§,⊥ Yuichi Kitagawa,§ and Naoki Sugimoto*,†,‡,§

Faculty of Frontiers of Innovative Research in Science and Technology (FIRST), Frontier Institute for Biomolecular Engineering Research (FIBER), and Department of Chemistry, Faculty of Science and Engineering, Konan University, 7-1-20, Minatojima-minamimachi, Chuo-ku, Kobe, 650-0047, Japan

Received August 6, 2009; E-mail: shuichi@center.konan-u.ac.jp; sugimoto@konan-u.ac.jp

Abstract: Short RNA sequences exhibiting the activity of a target RNA cleavage are promising for cellular gene regulation and biosensor research, but the reaction media different from an aqueous solution may cause unanticipated molecular interactions and properties. In this study, we investigated the molecular crowding effects arising from steric crowding and altered solvent properties on the hammerhead ribozyme activity using water-soluble neutral cosolutes. Poly(ethylene glycol) (PEG) and other cosolutes at 20 wt % increased the RNA hydrolysis rate by a factor of 2.0−6.6 at 10 mM $MgCl_2$ and much more at lower $MgCl_2$ concentrations. Remarkably, although the cosolutes decreased the stability of the ribozyme stem helices, the thermal inactivation temperature of the ribozyme was significantly raised, resulting in a higher reaction rate, up to 270 times at 50 °C. More significantly, PEG decreased the metal ion concentration to perform the reaction even with a limiting Mg^{2+} or Na^+ concentration, facilitated the catalytic turnover, and activated a catalytically less active ribozyme sequence. These observations agreed that the cosolutes acted as an osmolyte stabilizing the water-release reaction of the RNA tertiary folding but destabilizing the water-uptake reaction of Watson−Crick base pairing. The opposite cosolute effect on the stabilities of RNA secondary and tertiary structures, which is fundamentally different from a protein folding, suggests how RNA stabilizes a tertiary structure and enhances the catalytic activity in molecular crowding media.

Introduction

Many of naturally occurring RNA enzymes, ribozymes, catalyze the site-specific phosphodiester bond cleavage of a target RNA sequence, and their folding and the catalytic activity are intimately related. The ribozyme reaction is commonly facilitated by divalent metal ions that stabilize the active conformation and may also play a direct role in the catalysis.[1−5] The hammerhead ribozyme is one of the smallest ribozymes, forming a core structure with three stems of base pairs (stems I, II, and III) surrounding the active center of conserved nucleotides. The reaction mechanism and the tertiary structure have been well-studied, and formation of the Y-shaped conformation in which stem I and stem II locate close in space is required for the catalysis. The truncated ribozyme motif, identified as a minimum sequence motif including the catalytically essential core of nucleotides at the junction of short base paired stem helices, cleaves a substrate RNA at high and moderate Mg^{2+} concentrations.[6−8] Recent studies of the crystallography and molecular dynamic simulations characterized Mg^{2+} as having a key structural role in the stabilization of the active conformation rather than a direct catalytic role.[9,10] This agrees with observations that the ribozyme activity appeared without a divalent metal ion when a high concentration of monovalent ions presents.[11−13] It is also reported that the ribozymes stabilizing the Y-shaped conformation cleave a target RNA efficiently even at relatively low Mg^{2+} concentrations.[9,14−18]

† Faculty of Frontiers of Innovative Research in Science and Technology.
‡ Frontier Institute for Biomolecular Engineering Research.
§ Department of Chemistry, Faculty of Science and Engineering.
⊥ JSPS Research Fellow.

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Because the hammerhead ribozyme is relatively small and exhibits a rapid cleavage of a target RNA in vitro, the ribozyme sequence acting in trans is often used for in vivo suppression of a target gene as a therapeutic tool.[19−22] The intracellular environment of a severe steric crowding among biomacromolecules and unique solvent properties such as a reduced number of free waters[23−27] may directly affect RNA interactions and the catalysis. The steric crowding and alterations of a solvent property are also suggested for the molecules immobilized on a surface of biosensor devices.[28−30] In vitro solutions containing a large amount of water-soluble neutral cosolutes, such as PEG (poly(ethylene glycol)), have been used to mimic the nonhomogeneous aqueous condition, and a number of investigations, especially regarding the protein folding, assembly, and enzyme activity, have been reported (summarized in ref 31). Intriguingly, protein−protein interactions were, in many cases, facilitated in cosolute-containing solutions. In contrast, our previous studies using DNA and RNA oligonucleotides indicated *destabilization* of Watson−Crick base pairs due to neutral cosolutes.[32−34] The difference in the cosolute effect between proteins and nucleotides is of great interest and significance.

We now report the hammerhead ribozyme activity in nonhomogeneous aqueous media by a neutral cosolute and demonstrate the cosolute effects to facilitate the ribozyme reaction in terms of reaction rate, metal ion binding, catalytic turnover, and formation of the catalytic form. The enhanced ribozyme activity can be accounted for by stabilization of the RNA tertiary structure while the stability of its stem helices of Watson−Crick base pairs is decreased. The observations suggest the significance of a less stable intermediate to increase a folding cooperativity into the ribozyme active structure. This strategy for the tertiary folding may be of great benefit to functionalization of RNAs under nonhomogeneous aqueous conditions, such as in a cell and on a biosensor device.

Experimental Section

Preparations of Nucleotides and Buffer Solutions. The hammerhead ribozyme sequences derived from *Schistosoma mansoni* (*S. mansoni*; Figures 1A and 5A) were enzymatically prepared by runoff transcription, using T7 RNA polymerase (GE Healthcare) and template DNA containing the T7 RNA polymerase promoter sequence (Hokkaido System Science). The substrate RNAs and an RNA−DNA chimeric oligonucleotide labeled with 6-FAM (6-carboxylfluorescein) at a 5′ end were synthesized by solid-phase chemical synthesis and purified by a polyacrylamide gel (acrylamide:bis(acrylamide) = 19:1) electrophoresis in 7 M urea. The short RNAs purified by HPLC (high-performance liquid chromatography) were used for the UV melting experiments.

All reagents for preparing the buffer solutions were purchased from Wako, except for Na_2EDTA (disodium salt of ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid) from Dojindo; PEG8000 from Sigma; Dextran10, Dextran70, and Ficoll70 from GE Healthcare; and 2-methoxyethanol and 1,2-dimethoxyethane from TCI and were used without further purification. The pH of a reaction buffer was adjusted after mixing a cosolute, and an apparent pK_a (acid dissociation constant) of the buffer solution was unchanged by cosolutes. The water activity was determined by the osmotic stressing method using vapor-phase osmometry (Wescor, 5520XR) or freezing point depression osmometry (Halbmikro, Typ Dig·L), with an assumption that the cosolutes do not directly interact with nucleotides.

Ribozyme Reactions under Single Turnover and Multiple Turnover Conditions. The rate of the ribozyme-catalyzed RNA cleavage was measured under single- and multiple-turnover conditions, in which the ribozyme was in excess of the substrate and vice versa, respectively. The single-turnover reaction was carried out with 2 μM ribozyme and 100 nM substrate RNA, and the multiple turnover reaction was done with 5 nM ribozyme and 100 nM substrate RNA. The reaction was carried out at 37 °C using a buffer solution consisting of 0.5−500 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-HCl (pH 7.0), 0.1 mM Na_2EDTA, and a neutral cosolute at 20 wt %, unless otherwise mentioned. With regard to the experiments without Mg^{2+}, 1 mM Na_2EDTA was included in the solution to chelate any contaminating divalent metal ions.

The sample was annealed at 60 °C and then incubated at the experimental temperature for 10 min, followed by the addition of $MgCl_2$ or NaCl to start the reaction, in which no pH change upon the $MgCl_2$ and NaCl additions was observed. The ribozyme reaction was quenched by more than a 5-times excess volume of the stop solution containing 80% formamide, 10 mM Na_2EDTA, and 2% blue dextran, and the product was immediately stored in a freezer at −30 °C until used. The solutions were then loaded on 20% polyacrylamide gel containing 7 M urea. After the gel electrophoresis using the running buffer consisting of 90 mM Tris-HCl, 90 mM boric acid, and 2 mM Na_2EDTA at pH 8.3 (TBE buffer), the fluorescence emission from 6-FAM was visualized and quantified by a fluorescent scanner (FUJIFILM, FLA-5100) using a 473 nm excitation laser and a 520 nm emission filter.

Nonenzymatic RNA Cleavage of the RNA−DNA Chimeric Oligonucleotide. The site-specific hydrolysis of an RNA−DNA chimeric strand of 5′-d(CCGGTGCGT)r(C)d(CTGGATTCCA)-3′ labeled with 6-FAM at the 5′ end was carried out at 60 °C because the reaction at 37 °C was too slow to measure the reaction rate. The oligonucleotide concentration was at 10 μM, and the reaction buffer contained 10 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-HCl (pH 7.0), 0.1 mM Na_2EDTA, and a cosolute at 20 wt %. The time-point data were collected from at least two independent experiments. Because the nonenzymatic hydrolysis was very slow, the reaction rate was calculated from a linearity of the time-dependent product yield plot, fitted to a linear regression approximating the exponential equation.

Calculation of the Kinetic Parameters. The observed rate constant (k) for the truncated ribozyme-catalyzed reaction was determined from a plot of the RNA fraction cleaved at the correct site (f) versus time (t), fitted to the single-exponential equation of $f = f_0 + (f_{max} - f_0)[1 - \exp(-kt)]$, where f_0 is the fraction at time zero and f_{max} is the fraction at an end point of the reaction. The linear approximation was applied to calculate the rate of very slow reactions. Because the kinetic trace of the extended ribozyme-

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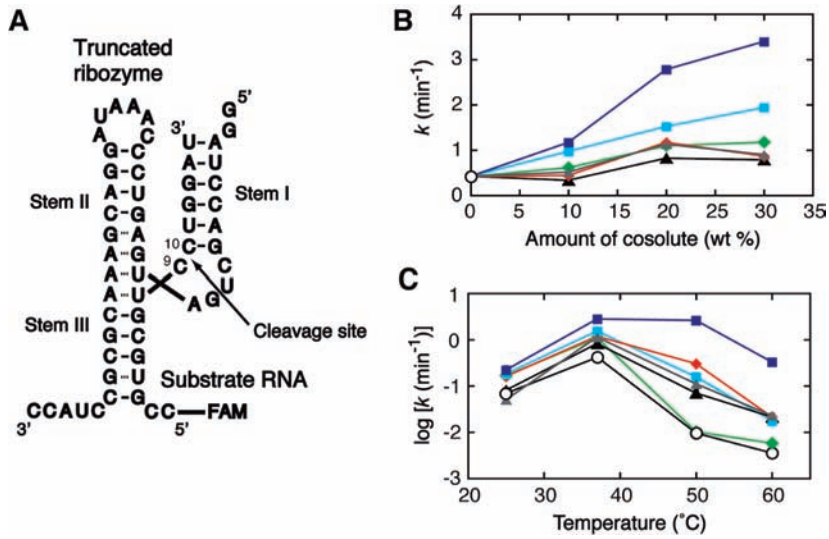

Figure 1. Truncated hammerhead ribozyme reaction at 10 mM $MgCl_2$. (A) Truncated ribozyme that cleaves a phosphodiester bond connecting C9 and C10 of the substrate RNA labeled with 6-FAM. (B) Influence of the amount of cosolutes on the reaction rate constant (k). The reaction was carried out at 37 °C, using ethylene glycol (green), PEG200 (cyan), PEG8000 (blue), Dextran10 (gray), Dextran70 (black), or Ficoll70 (red) as a cosolute. The 0 wt % data indicate the reaction without a cosolute (white). (C) Temperature dependences of the rate constant in the absence and presence of a cosolute at 20 wt %. The symbols are the same as those used in panel B.

catalyzed reaction deviated from the single-exponential fit, an initial velocity of the fast phase reaction (v_0) was evaluated. The steady-state kinetic parameters of K_m for the active structure formation and $k_{cat.}$ representing the rate constant at a saturating ribozyme concentration were determined from a plot of k versus the ribozyme concentration [R] changed from 1.0 to 15 μM, with the substrate RNA concentration at 100 nM, fitted to the steady-state kinetic equation of $k = k_{cat.}[R]/([R] + K_m)$.

The Mg^{2+} concentration showing the half-maximum rate of the cleavage ($[Mg^{2+}]_{1/2}$) was obtained from a plot fitted to the equation of $k = k_{Mg,sat.}\{[Mg^{2+}]^n/([Mg^{2+}]_{1/2}{}^n + [Mg^{2+}]^n)\}$, where k is the observed rate constant at the examined magnesium ion concentration ($[Mg^{2+}]$), $k_{Mg,sat.}$ is the maximum rate constant at a saturating Mg^{2+} concentration, and n is the number of cooperatively bound magnesium ions that affect the reaction rate.

Measurement of the Thermal Melting Temperature of Oligonucleotide Structures. The UV absorbance of RNA oligonucleotides was measured by a Shimadzu 1700 spectrophotometer equipped with a temperature controller. The UV melting curve was measured with 40 μM as an RNA strand concentration in the buffer containing 10 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-HCl (pH 7.0), 0.1 mM Na_2EDTA, and a cosolute at 20 wt %. The melting temperature (T_m), at which half of the secondary structure was denatured, was determined from the melting curve monitored at 260 nm with a heating rate at 0.5 °C min^{-1}.

Results

Increase in the Reaction Rate and Reaction Temperature of the Hammerhead Ribozyme due to Cosolutes. Because the truncated hammerhead ribozyme acting in trans has been well-characterized,[35] we used the truncated ribozyme cleaving a phosphodiester bond connecting C9 and C10 of the substrate RNA labeled with fluorescein (Figure 1A). The rates of a single-turnover reaction with 10 mM $MgCl_2$ at 37 °C were compared in the absence and presence of a neutral cosolute of ethylene glycol, PEG with an average molecular weight (MW_{av}) of 200 or 8000 (PEG200 or PEG8000, respectively), Dextran10 ($MW_{av} = 1 \times 10^4$), Dextran70 ($MW_{av} = 7 \times 10^4$), or Ficoll70 ($MW_{av} = 7 \times 10^4$). We found that the cosolutes increased the rate of RNA hydrolysis at the correct site. The monophasic kinetic traces showing a product yield approaching about 80% provided 2.0−6.6 times greater rate constants in the presence of the cosolutes at 20 wt % (Table 1), and a faster reaction rate was obtained with a greater amount of ethylene glycol and PEGs (Figure 1B). Investigations using PEGs of various molecular weights indicated a greater rate constant with the larger-sized PEGs but the largest PEGs of PEG8000 and PEG20000 showed almost the same value. In addition, no substantial conformational change in the ribozyme-substrate complex upon the addition of PEG8000 was suggested from the CD (circular dichroism) spectra measured with an uncleavable substrate containing the deoxyribonucleotide (dC) substitution at C9 (see the Supporting Information).

As reported for other hammerhead ribozymes,[36−39] the rate-limiting proton transfers attributed to the RNA transesterification was verified for our truncated ribozyme from the pH-dependent rate constant data both in the PEG-free and PEG-containing solutions (0.42 min^{-1} at pH 7.0 and 1.8 min^{-1} at pH 7.5 in the

Table 1. Rate Constants for the Truncated Hammerhead Ribozyme Reaction at 10 mM $MgCl_2$ in the Absence and Presence of 20 wt % Cosolute[a]

cosolute	truncated ribozyme reaction (min^{-1})	cosolute	truncated ribozyme reaction (min^{-1})
none	0.42 ± 0.05 (<0.002)	Ficoll70	1.2 ± 0.1
ethylene glycol	1.1 ± 0.2	glycerol	1.1 ± 0.2
PEG200	1.5 ± 0.2	1,3-propanediol	0.87 ± 0.08
PEG8000	2.8 ± 0.	2-methoxyethanol	1.5 ± 0.2
Dextran10	1.1 ± 0.2	1,2-dimethoxyethane	2.0 ± 0.4
Dextran70	0.82 ± 0.08		

[a] The reaction was carried out at 37 °C. The values in parentheses are the data obtained in a Mg^{2+}-free solution containing 1 mM EDTA.

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Table 2. Rate Constants for the Hydrolysis of an RNA−DNA Chimeric Strand at 10 mM $MgCl_2$ in the Absence and Presence of 20 wt % Cosolute[a]

cosolute	hydrolysis rate (10^{-5} min^{-1})	cosolute	hydrolysis rate (10^{-5} min^{-1})
none	2.4 ± 0.1 (0.052 ± 0.021)	Ficoll70	0.86 ± 0.04
ethylene glycol	0.83 ± 0.05	glycerol	1.3 ± 0.1
PEG200	1.6 ± 0.1	1,3-propanediol	0.72 ± 0.03
PEG8000	1.3 ± 0.2	2-methoxyethanol	0.79 ± 0.03
Dextran10	0.74 ± 0.02	1,2-dimethoxyethane	0.54 ± 0.03
Dextran70	0.47 ± 0.02		

[a] The hydrolysis of 5′-d(CCGGTGCGT)r(C)d(CTGGATTCCA)-3′ was performed at 60 °C. The values in parentheses are the data obtained in a Mg^{2+}-free solution containing 1 mM EDTA.

absence of a cosolute, and 2.8 min^{-1} at pH 7.0 and 4.0 min^{-1} at pH 7.5 in the presence of 20 wt % PEG8000). These values fall within the range of those reported for the truncated ribozymes exhibiting a rate-limiting chemical step.[35]

The ribozyme activity was significantly reduced at high temperatures by thermal denaturation of the catalytic form, but cosolutes efficiently protected from the ribozyme inactivation by heat (Figure 1C). For example, 20 wt % PEG8000 increased the reaction rate at 50 °C by a factor of 270 compared with the reaction in a cosolute-free solution at the same temperature, and an efficient reaction completing the RNA hydrolysis within 10 min was observed even at 60 °C. This result suggests that the ribozyme active conformation is stabilized by the cosolutes.

Ribozyme Active Conformation Stabilized but Watson−Crick Base Pairs Destablized by Cosolutes. We also examined small neutral cosolutes of glycerol, 1,3-propanediol, 2-methoxyethanol, and 1,2-dimethoxyethane as the additive in a reaction buffer. Although these cosolutes are relatively small (MW = 76−90), the ribozyme reaction was accelerated and the rate constant with 1,2-dimethoxyethane was greater than those with polysaccharides of Dextran and Ficoll (Table 1). For comparison with a nonenzymatic reaction, the specific base-catalyzed hydrolysis of an RNA phosphodiester bond was examined. The phosphodiester bond of a structurally unconstrained ribonucleotide is hydrolyzed in the presence of a divalent ion, and an RNA−DNA chimeric oligonucleotide containing a single ribonucleotide is useful for evaluating the RNA transesterification.[40] Our experiments using the single-stranded RNA−DNA chimeric strand derived from the substrate RNA sequence showed no acceleration of the Mg^{2+}-mediated RNA hydrolysis by cosolutes (Table 2). These data indicate that the cosolutes enhance only the ribozyme-catalyzed reaction.

Enzyme kinetic parameters for the ribozyme reaction were analyzed on the basis of the simple two-step mechanism of an active structure formation followed by transesterification. In the absence of a cosolute, the steady-state kinetic parameter of K_m for the active structure formation and $k_{cat.}$ representing the rate constant at a saturating ribozyme concentration were 11 μM and 4.5 min^{-1}, respectively. We observed decrements in K_m in the cosolute-containing solutions (Table 3) in which PEG8000 showed the smallest K_m of 0.76 μM among the solutions and 1,2-dimethoxyethane showed a relatively small value of 0.99 μM. On the other hand, $k_{cat.}$ varied only slightly due to the cosolutes (2.8−4.5 min^{-1}), indicating that K_m rather than $k_{cat.}$ was responsible for the accelerations of the ribozyme reaction.

In contrast to the enzyme kinetic parameter, destabilizations of the RNA secondary structure were suggested from the thermal denaturation studies using oligonucleotides. We examined RNA sequences corresponding to the stem II base pairs and a hairpin loop (hpRNA), stem I base pairs (6-mer dsRNA), stem III base pairs (5-mer dsRNA), and stems I and III base pairs (11-mer dsRNA) of the ribozyme/substrate complex (Figure 2A). The cosolutes at 20 wt % decreased the T_m (melting temperature) of the hpRNA by 5.9−14.3 °C, the 6-mer dsRNA by 2.9−9.9 °C, and the 11-mer dsRNA by 1.2−8.6 °C (Figure 2B). In addition, although the T_m of the 5-mer dsRNA was too low (<20 °C) to accurately determine, the cosolute-induced destabilization was evident from a shape of their melting curves.

Table 3. Steady-State Kinetic Parameters for the Truncated Ribozyme Reaction with 10 mM $MgCl_2$ at 37 °C

cosolute at 20 wt %	K_m (μM)	$k_{cat.}$ (min^{-1})
none	11 ± 3	4.5 ± 0.7
ethylene glycol	3.0 ± 0.9	2.8 ± 0.3
PEG200	1.8 ± 0.4	3.0 ± 0.2
PEG8000	0.76 ± 0.24	3.5 ± 0.3
glycerol	7.8 ± 1.1	4.0 ± 0.3
1,3-propanediol	3.5 ± 0.6	2.8 ± 0.2
2-methoxyethanol	2.3 ± 0.5	3.1 ± 0.2
1,2-dimethoxyethane	0.99 ± 0.18	2.8 ± 0.1

Binding Properties of Metal Ions Altered by Cosolutes. PEG8000 accelerated the ribozyme reaction much more at lower $MgCl_2$ concentrations in which the rate was very slow without a cosolute. Figure 3A shows $MgCl_2$ concentration profiles, indicating faster reaction rates in the PEG8000-containing solution than in the cosolute-free solution at low Mg^{2+} concentrations (e.g., by a factor of 16 at 3 mM Mg^{2+}), but both solutions gave the same plateau of a rate constant ($k_{Mg,sat.}$) at 3.4 min^{-1}. Remarkably, the Mg^{2+} concentration showing a half-maximum rate of RNA hydrolysis, $[Mg^{2+}]_{1/2}$, was changed from 38 mM in the absence of a cosolute to 3.1 mM in the presence of 20 wt % PEG8000, while the slope in a low Mg^{2+} concentration range reflecting the binding cooperativity remained close to 1.

The hammerhead ribozyme can be active without divalent metal ions when the tertiary structure is formed with a high concentration of a monovalent metal ion (e.g., 4 M).[11−13] We carried out the reaction using high and moderate concentrations of NaCl but no $MgCl_2$. Although the hydrolysis rate was much slower than the reaction with 10 mM $MgCl_2$, monophasic kinetic traces gave a product yield of about 80%. A rate-limiting chemical step was suggested from the pH-dependent rate constant data at 1 M NaCl (0.21 h^{-1} at pH 7.0 and 0.82 h^{-1} at pH 8.0 in the absence of a cosolute, and 0.93 h^{-1} at pH 7.0 and 4.3 h^{-1} at pH 8.0 in the presence of 20 wt % PEG8000), as demonstrated for the reaction with Mg^{2+}. Figure 3B shows linear plots of the rate constant data over the examined NaCl concentration range for both solutions. Their slopes were quite different, resulting in greater enhancements of the reaction rate by PEG8000 at lower NaCl concentrations, e.g., about 100 times at 300 mM NaCl. We also carried out the reaction near the physiological salt concentration, using a solution containing 0.5 mM $MgCl_2$ and 100 mM NaCl. Under this condition, no reaction was observed without a cosolute, but there was a substantial ribozyme activity of cleaving about 50% of the substrate RNA within 1 h in the presence of PEG8000 at 20 wt %. Although it is regarded that the catalytic activity of the truncated hammerhead ribozyme is observed only at high and moderate Mg^{2+} concentrations[6−8] or a very high concentration of a monovalent ion,[11−13] efficient ribozyme activity emerged with limiting salt concentrations in the PEG-containing solution.

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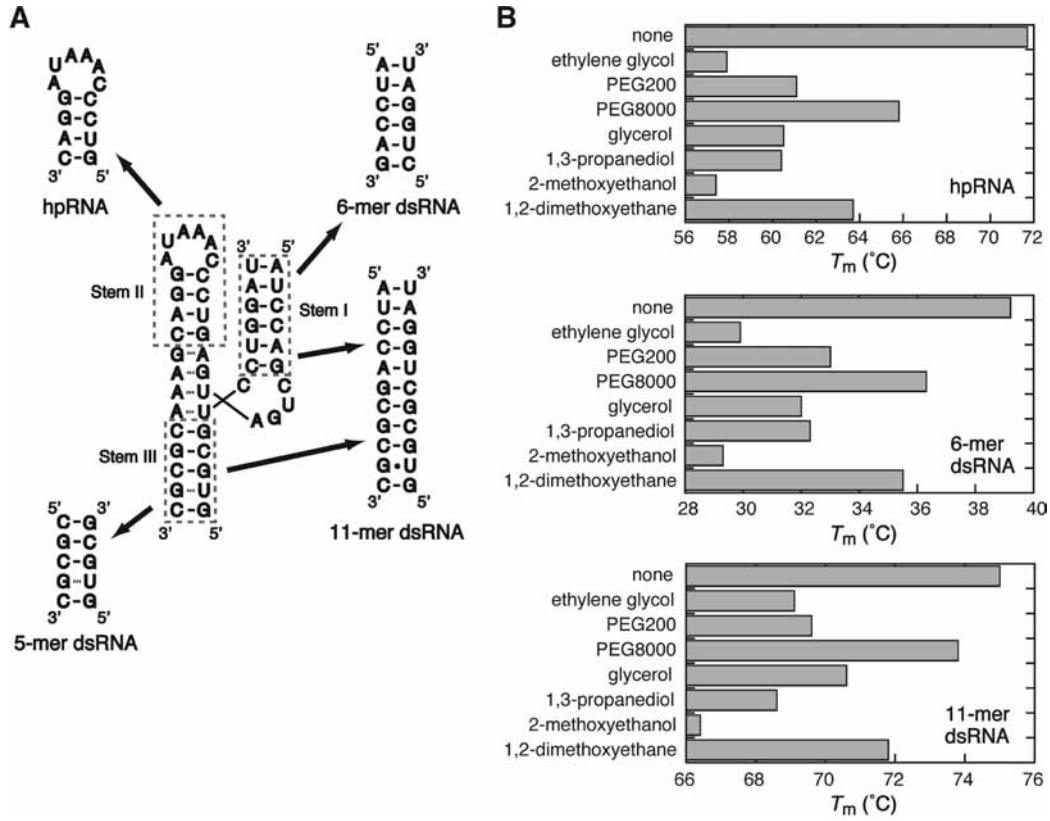

Figure 2. Thermal stability of RNA secondary structures derived from the truncated hammerhead ribozyme/substrate complex in the buffer containing 10 mM $MgCl_2$. (A) Oligomer RNA sequences of the hpRNA corresponding to the hairpin loop and base pairs in stem II, and the 5-mer, 6-mer, and 11-mer dsRNAs corresponding to the base pairs in stems I and III. (B) Comparisons of T_m values of 40 μM hpRNA, 6-mer dsRNA, or 11-mer dsRNA, obtained in the absence and presence of a cosolute at 20 wt %. The T_m values of the 5-mer dsRNA were too low (<20 °C) to be accurately determined.

Ribozyme Turnover Accelerated by Cosolutes. The hammerhead ribozyme activity was further investigated under a multiple turnover condition with 10 mM $MgCl_2$ using a 20-times excess of the substrate RNA over the ribozyme. As observed for the single-turnover reaction, the neutral cosolutes increased the RNA hydrolysis rate (Figure 4A). A rate-limiting release of the cleaved RNA fragments for the ribozyme turnover was suggested from observations of a burst phase of 2−4% that reflects the product during the first turnover (at most 5% in this case), more than 200-times slower rate than a single-turnover reaction, and the rate constant data invariable with pH (Figure 4B). The faster release of the cleaved RNA fragments in cosolute-containing solutions agreed with a decreased stability of the stem I and stem III base pairs (Figure 2) and a previous report that PEG increased the dissociation rate of Watson−Crick base pairs.[41] Remarkably, although less ribozyme activity was detected at 50 °C without PEG (0.00042 min^{-1}), the reaction rate at 50 °C (0.018 min^{-1}) in the PEG8000-containing solution was twice as fast as that at 37 °C (0.0091 min^{-1}), caused by a faster dissociation rate of the cleaved RNA fragments at a higher temperature.

Cosolute Effects on RNA Refolding by Examinations with the Less Active Hammerhead Ribozyme Sequence. There are reports of the hammerhead ribozyme exhibiting inefficient activity, due to a formation of inactive conformers that need the refolding involving slow conformational rearrangements for the RNA transesterification.[35] To study cosolute effects on the RNA refolding into the catalytic active conformation, we prepared a less active ribozyme sequence indicated in Figure 5A, a design based on the extended hammerhead ribozyme motif showing an inefficient activity.[20] The extended ribozyme motif forms an internal loop with the substrate RNA in stem I, and utilization of a similar dynamic catalytic mechanism for the catalysis has been suggested between the truncated and extended ribozyme motifs.[42,43] However, the nucleotides forming stem III and the length of stem I in our ribozyme were different from those of the highly active ribozymes, resulting in a substantially decreased reaction time. Although the extended ribozyme motif often exhibits a high activity (a half-life within 10 s at 10 mM $MgCl_2$ and 37 °C),[9,14−18] our extended ribozyme cleaved the substrate RNA through a non-single-exponential function of time, and the rate of a fast phase reaction was, as expected, relatively slow (a half-life of about 1 min at 10 mM $MgCl_2$ and 37 °C was similar to that reported with the ribozyme of a similar sequence.[20]). Moreover, the product yield was low at 10 mM $MgCl_2$ (about 40% after 15 min) and very low at 0.5 mM $MgCl_2$ (about 10% after 15 min), suggesting an existence of alternate kinetically trapped conformers that hinder the catalysis. Importantly, PEG8000 increased the product yield as well as the rate of the fast phase reaction. The half-life of the fast reaction in the presence of 20 wt % PEG8000 was within 10 s, and the reaction yield after 15 min became greater than 50% even with 0.5 mM $MgCl_2$ (Figure 5B).

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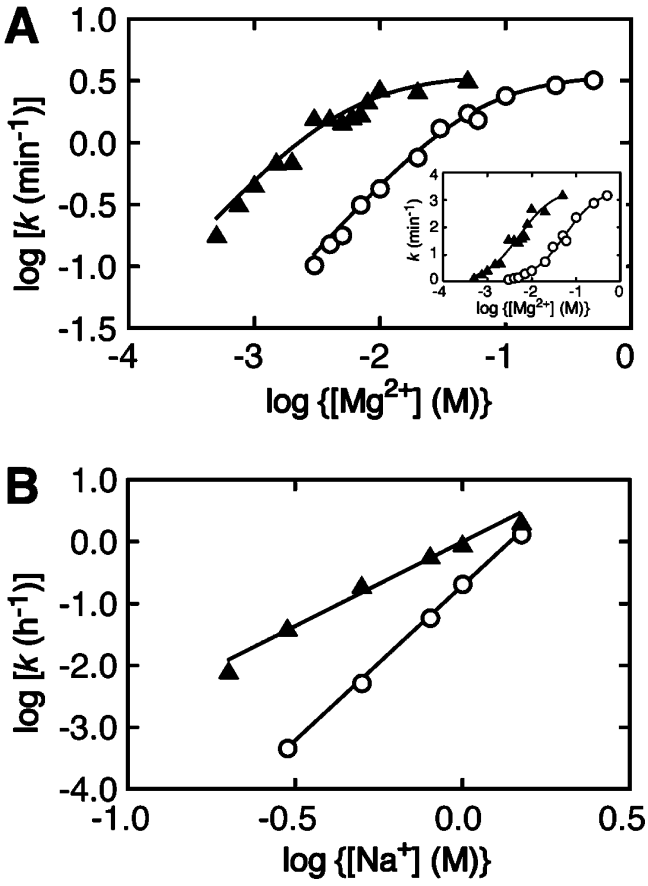

Figure 3. Cation concentration profiles for the reaction rate of the truncated ribozyme in the absence (circles) and presence of 20 wt % PEG8000 (triangles) at 37 °C. The fits to a theoretical equation are also indicated. (A) Mg^{2+} concentration profiles, providing $[Mg^{2+}]_{1/2}$ of 38 mM without a cosolute and 3.1 mM with PEG and the numbers of cooperatively bound Mg^{2+} of 1.1 for both solutions. The $k_{Mg,sat.}$ indicating the rate constant at a saturating Mg^{2+} concentration was 3.4 min^{-1} for both solutions. (B) Na^+ concentration profiles in the absence of Mg^{2+}, indicating the binding cooperativity of Na^+ of 5.0 without a cosolute and 2.7 with PEG.

We also carried out experiments using the reaction buffer containing only 50 mM NaCl, which was inefficient for the ribozyme folding. However, the extended ribozyme exhibited the cleavage activity when the reaction buffer contained PEG8000 or other cosolutes: While there was no detectable product after a 36 h reaction without a cosolute, the ribozyme activity was observed in the cosolute-containing solutions (e.g., the reaction yield of 26% for a 24 h reaction with 20 wt % PEG8000), and a PEG size dependency of the reaction rate was indicated (Figure 5C). This observation suggests a catalytic pathway with indirect involvement of Mg^{2+} [9,10,44] and the cosolutes, especially PEG, acting as a stabilizer for the ribozyme folding into the catalytic form.

Discussion

Cosolute Effects on the RNA Transesterification. We demonstrate here neutral cosolute-induced facilitations of the hammerhead ribozyme activity in terms of reaction rate, catalytic turnover, and formation of the catalytically active form. The cosolute effect on the reaction rate was remarkable. For example, we observed accelerations of the RNA hydrolysis rate up to 16 times in 3 mM $MgCl_2$, 100 times in 300 mM NaCl, 270 times at 50 °C, and 9 times for the multiple turnover reaction. We carried out experiments using a variety of neutral cosolutes mostly at 20 wt % that generated steric crowding among molecules and changed the solvent properties; however, a small amount of cosolutes (e.g., 1 wt %) showed no influence on the reaction (see the Supporting Information). The cosolute-induced accelerations of the ribozyme reaction, but not the nonenzymatic RNA cleavage, suggest no contribution from contaminants, if any, in the cosolutes to the ribozyme reaction. No significant contamination of divalent metal ions was also proved by the experiments using a high concentration of EDTA (see the Supporting Information). It is mentioned that no ligation activity with the cleaved RNA fragments was observed both in the absence and presence of the cosolutes (data not shown).

The rate-limiting RNA transesterification was suggested for the truncated ribozyme-catalyzed reaction even in the presence of a cosolute. Because the $k_{cat.}$ data shown in Table 3 were changed less by the cosolutes, acceleration of the transesterification step is unlikely, which agreed with no enhancement of the hydrolysis rate of an RNA−DNA chimeric strand. It is mentioned that decreased rates due to cosolutes were obtained with the chimeric nucleotide, possibly arisen from influences on the backbone conformation of unpaired nucleotides, as suggested in our previous study.[34] The observation that the values of $k_{Mg,sat.}$ in the absence and presence of PEG8000 are identical (Figure 3A) supports the conclusion that the chemical step is not responsible for the acceleration of the ribozyme reaction. Rather, a cosolute-assisted stabilization of the active form was strongly suggested from the increased thermal inactivation temperature (Figure 1C) and the decreased steady-state kinetic parameter of K_m (Table 3), and destabilization of the enzyme−substrate complex to have a higher probability of forming the catalytically active form due to an increased flexibility is unlikely.

Influence of Cosolutes on the RNA Folding. Cosolute effects on the RNA folding are elucidated from our results because the ribozyme activity is intimately related to the folding into an active structure accompanied by metal ion binding. It has been identified that at least two Mg^{2+} are involved in the folding of the hammerhead ribozyme, a tightly associated Mg^{2+} for forming consecutive G/A mismatches at a proximal position of the cleavage site ($[Mg^{2+}]_{1/2}$ about 100 μM) and a weaker coordinated Mg^{2+} for forming the catalytically active Y-shaped conformation ($[Mg^{2+}]_{1/2}$ in a millimolar range or higher).[13,16,37] We found that PEG8000 did not affect the slope of the log k − log $[Mg^{2+}]$ plot in a low Mg^{2+} concentration range ($n = 1.1$) representing noncooperative binding of Mg^2 but decreased $[Mg^{2+}]_{1/2}$ from 38 to 3.1 mM (Figure 3A). It is thus likely that PEG8000 decreases the magnesium ion concentration required for adopting the Y-shaped conformation. On the other hand, sodium ion concentration profiles shown in Figure 3B indicated that PEG8000 decreased the number of cooperatively bound Na^+ from 5.0 to 2.7, to shield the electrostatic repulsion among phosphate groups close in space. The cosolute-induced reduction of the sodium ion condensation has also found for Na^+ diffusely bound to base pairs.[33,34,45] Therefore, it is possible that the hammerhead ribozyme reaction can be accelerated by diffusely bound sodium ions when no divalent metal ions are present.

The slow and multiphasic kinetic behavior of our extended ribozyme suggests an existence of alternate kinetically trapped

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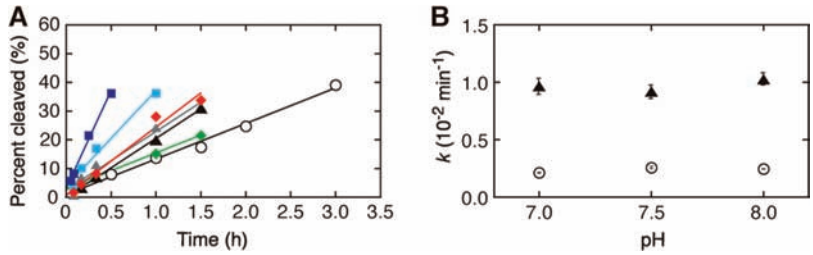

Figure 4. Multiple turnover reactions by the truncated ribozyme at 10 mM $MgCl_2$ and 37 °C. (A) Kinetic traces obtained with various solutions. The symbols are the same as those used in Figure 1B. (B) Rate constant data at different pHs obtained in the absence (circles) and presence of 20 wt % PEG8000 (triangles).

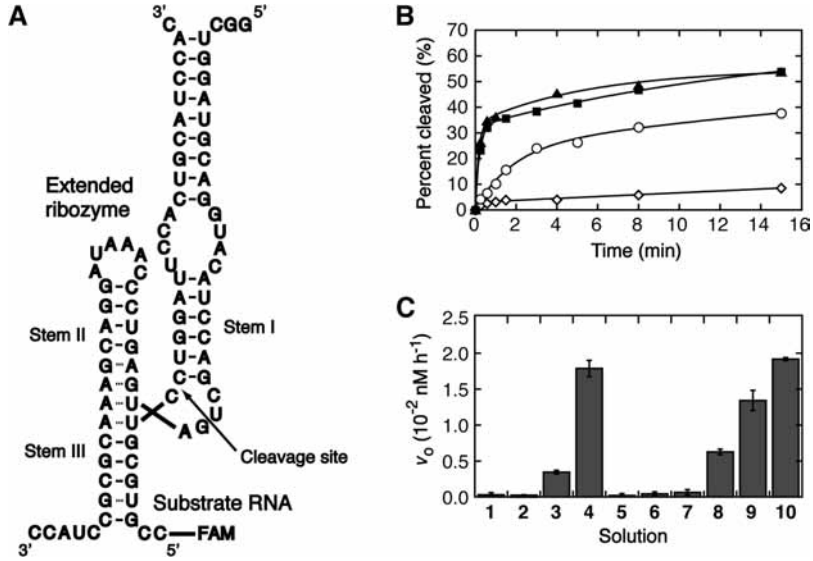

Figure 5. Influence of 20 wt % cosolutes on the catalytically less active ribozyme reaction at 37 °C. (A) The extended ribozyme sequence exhibiting an inefficient activity. (B) Kinetic traces obtained at 10 mM $MgCl_2$ with PEG8000 (squares) and without PEG8000 (circles), and at 0.5 mM $MgCl_2$ with PEG8000 (triangles) and without PEG8000 (diamonds). (C) Comparison of the initial velocity (v_0) without Mg^{2+}, but with 50 mM NaCl and 1 mM EDTA. Solutions represented by the numbers indicate the reaction in the absence (**1**) or presence of ethylene glycol (**2**), PEG200 (**3**), PEG8000 (**4**), Dextran10 (**5**), Dextran70 (**6**), Ficoll70 (**7**), PEG2000 (**8**), PEG4000 (**9**), or PEG20000 (**10**).

conformers either on or off the pathway that hinder the catalysis.[17,35] Nevertheless, PEG8000 effectively increased the amount of catalytically active species, in addition to the reaction rate (Figure 5B). Because the cosolutes decreased the stability of Watson−Crick base pairs, energetic costs for conformational rearrangements involving a dissociation of "wrong" base pairs were presumably lowered in cosolute-containing solutions, and this facilitated inactive conformers to refold into the catalytic form. The destabilization of Watson−Crick base pairs also explains cosolute-induced accelerations of the ribozyme turnover demonstrated in Figure 4A. The study of a thermophilic ribozyme in the catalytic domain of ribonuclease P suggested the significance of a less structured intermediate and increased cooperativity to achieve the higher structural stability,[46] and this has been observed in model triplex systems as well, which allows stronger base pairing to increase tertiary structure stability.[47] Because the neutral cosolutes destabilize base-paired structures as an intermediate of the tertiary folding, neutral cosolutes are beneficial for the hammerhead ribozyme to increase the folding cooperativity and a stability of the catalytically active form. It is emphasized that adding a neutral cosolute is a quite simple method to enhance the ribozyme activity, and it would also be useful to stabilize RNA tertiary structures.

Importance of Cosolutes as an Osmolyte. According to the data obtained in this study, excluded volume effect by cosolutes, which increases the molecular activity,[25] is not the major determinant for the enhancements of the ribozyme activity. The solution viscosity, which is the highest for Dextran70 and lower for the small-sized cosolutes, also does not account for our observations. Previous studies using oligonucleotides indicated that the formation of Watson−Crick base pairs was a water-uptake reaction, and the base pair stability was decreased in neutral cosolute-containing solutions because the cosolutes reduce the water activity of a solution.[32−34] A water-release reaction for the hammerhead ribozyme-catalyzed RNA hydrolysis was suggested by the high hydrostatic pressure approach, for which desolvation of the stem helices and metal ions was supposedly associated with organization of the three stems to form the Y-shaped conformation.[48] Accordingly, the cosolute effects on the hammerhead ribozyme reaction possibly arise from cosolutes acting as an osmolyte that stabilizes the water-release reaction of the tertiary structure formation (discussed further in the Supporting Information), which is in contrast to

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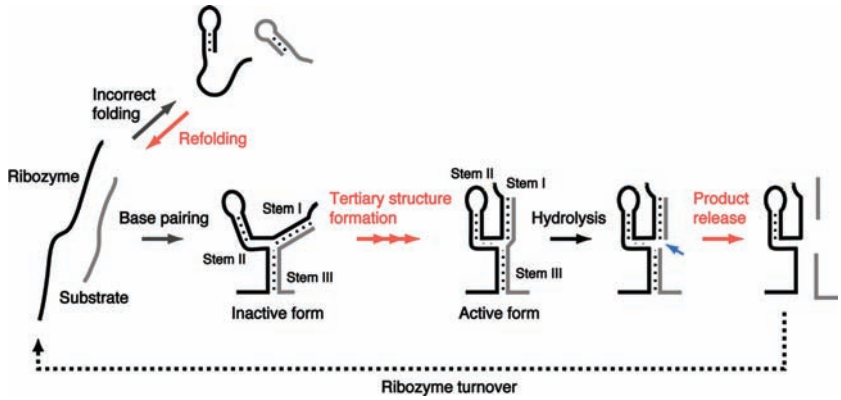

Figure 6. Simplified reaction mechanism of the hammerhead ribozyme-catalyzed RNA cleavage. The cleavage site is indicated by a blue arrow, and the reaction steps accelerated by a neutral cosolute are indicated in red. The scheme is drawn with the truncated ribozyme, but a similar mechanism can be drawn with the extended ribozyme.

destabilization of the water-uptake reaction of the secondary structure formation by Watson−Crick base pairs. On the other hand, there are several reports describing the osmolyte-induced stabilization of protein secondary structures as well as their tertiary structures.[49−52] Thus, it is likely that the cosolute effect on the RNA folding is fundamentally different from that of proteins, originating from different water participations in their reactions. Because the conformation correctly folded into the catalytically active form is supposed to sufficiently release water molecules that overcome the number of waters bound upon the base pair formations of stem helices, the reduced water activity is also favorable for refolding inactive conformers into the active form. As a consequence of the participation of water molecules in the catalytic reaction mechanism, the reaction rate, the turnover rate, and the amount of the correctly folded RNA can be increased in the reduced water activity media by cosolutes (Figure 6).

Implications of the Ribozyme Reaction under Osmotic Pressure. The cosolute study reveals the physical properties of biomolecules under nonhomogeneous aqueous conditions. We examined the buffer solutions that mimic physiological media in terms of ionic strength, ionic composition, and molecular crowding under osmotic pressure. The truncated hammerhead ribozyme showed efficient activities in the physiological salt concentration (e.g., 0.5 mM $MgCl_2$ and 100 mM NaCl) only under osmotic pressure. The efficient reactions at the physiological salt concentration imply an ability of the hammerhead ribozyme to cleave a target RNA in a living cell where the number of free water molecules is fairly limited. Moreover, recent discoveries of the self-cleaving ribozymes functioning as regulatory cellular ribozymes, such as a metabolite-dependent ribozyme of *GlmS*[53] and a cotranscriptional cleavage ribozyme of CoTC,[54] and self-cleaving ribozyme sequences encoded in human genome[55,56] have raised the versatility of RNA enzymes in biology. Because the RNA folding into the "functional" tertiary structure is, in many cases, accompanied by water release and metal ion binding, these cellular ribozymes may exhibit a raised activity in the intracellular media. The hammerhead ribozyme reaction to cleave a target RNA in reduced water activity media indicates the reaction not only in living cells but also on a biosensor surface on which nucleotides are immobilized,[57,58] where the amount and mobility of water molecules are restricted. The cosolute effects by very simple molecules acting as an osmolyte also suggest how RNA enhances the catalytic activity in poorly functioning primordial cells. The reduced requirement of Mg^{2+} for the catalysis and an effective ribozyme activity at high temperature when cosolutes are used has great advantages in biotechnology uses. It is a convenient method to eliminate nonspecific RNA hydrolysis during a ribozyme reaction that the reaction is performed at a high temperature in the presence of a low concentration of Mg^{2+} and a cosolute. In addition, the reduced water activity would affect the reaction efficiency and the rate on a surface of biosensor devices, such as microarray chips.[28−30] The data presented here offer a broad utility of cosolute-containing solutions in the area of RNA technologies, such as for cellular gene regulation and biosensor researches for high-throughput gene expression assays and genomic analysis.

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Supporting Information Available: Kinetic traces and rate constant data for the hammerhead ribozyme reaction, CD spectra, influences of a small amount of macromolecules and the buffer content on the reaction rate, and relashionships between the ribozyme activity (k and K_m) and the water activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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