Efficient Substrate Cleavage Catalyzed by Hammerhead Ribozymes Derivatized with Selenium for X-Ray Crystallography†

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ABSTRACT: Because oxygen and selenium are in the same group (Family VI) in the periodic table, the site-specific mutagenesis at the atomic level by replacing RNA oxygen with selenium can provide insights on the structure and function of catalytic RNAs. We report here the first Se-derivatized ribozymes transcribed with all nucleoside 5′-(α-P-seleno)triphosphates (NTPαSe, including A, C, G, and U). We found that T7 RNA polymerase recognizes NTPαSe Sp diastereomers as well as the natural NTPs, whereas NTPαSe Rp diastereomers are neither substrates nor inhibitors. We also demonstrated the catalytic activity of these Se-derivatized hammerhead ribozymes by cleaving the RNA substrate, and we found that these phosphoroselenoate ribozymes can be as active as the native one. These hammerhead ribozymes site-specifically mutagenized by selenium reveal the close relationship between the catalytic activities and the replaced oxygen atoms, which provides insight on the participation of oxygen in catalysis or intramolecular interaction. This demonstrates a convenient strategy for the mechanistic study of functional RNAs. In addition, the active ribozymes site-specifically derivatized by selenium will allow for convenient MAD phasing in X-ray crystal structure studies.

Functional RNAs play important roles in biological systems, including rRNA processing, mRNA editing, gene regulation, and RNA cleavage catalysis (*1*−*4*). X-ray crystallography is a powerful method for 3D structural and functional studies of large RNA molecules (*5*−*7*). In addition to crystallization (*8*, *9*), however, heavy atom derivatization for phase determination is still the major problem in novel structure determination of nucleic acid molecules, especially RNAs, by X-ray crystallography (*6*−*8*). However, the selenomethionine strategy was developed in order to derivatize proteins and solve the phasing problem via multiwavelength anomalous dispersion (MAD). Recently over two-thirds of novel protein structures have been determined via the selenomethionine strategy (*10*, *11*), which has clearly revolutionized protein X-ray crystallography. Because selenium, sulfur, and oxygen are in the same group (Family VI) in the periodic table, we attempted to develop the selenium derivatization of nucleic acids by replacing oxygen with selenium for nucleic acid X-ray crystallography, similarl to the selenium derivatization of proteins by replacing sulfur with selenium (*10*, *11*).

To achieve this goal, our laboratory is in the process of developing selenium derivatization of nucleic acids for MAD phasing (*12*−*18*), and this novel derivatization methodology has been demonstrated in X-ray crystal structure studies of RNA and DNA molecules by several laboratories (*14*, *19*, *20*). To develop a general Se-derivatization strategy for the structural studies of functional RNAs, especially large ones, and to study the activity and structure after the selenium derivatization, we report here for the first time the successful enzymatic synthesis of Se-derivatized ribozymes using all four nucleoside 5′-(α-P-seleno)triphosphates (A, C, G, and U; Figure 1) and catalytic activities of these hammerhead ribozymes site-specifically derivatized with selenium. Our experimental results provide insights into the structure and catalytic mechanism and demonstrate a useful strategy for the study of ribozyme catalysis and structure.

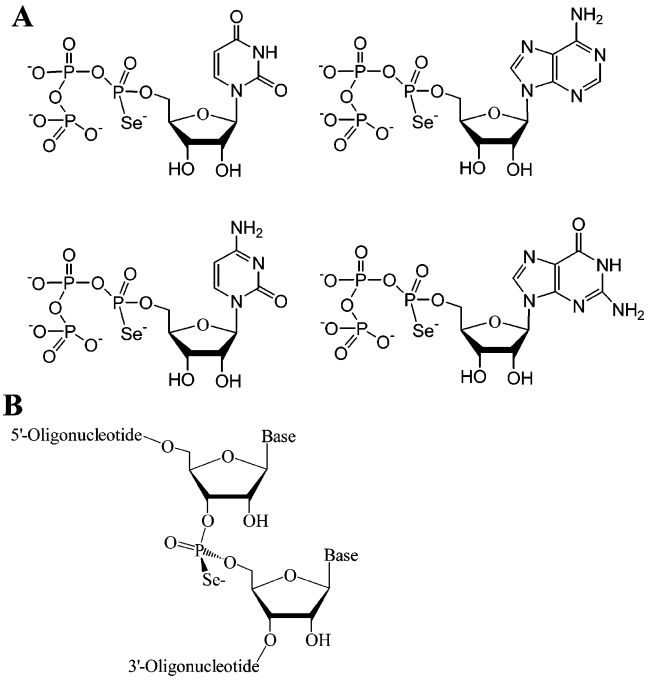

FIGURE 1: (A) NTPαSe structure. (B) Structure of Rp phosphoroselenoate RNA.

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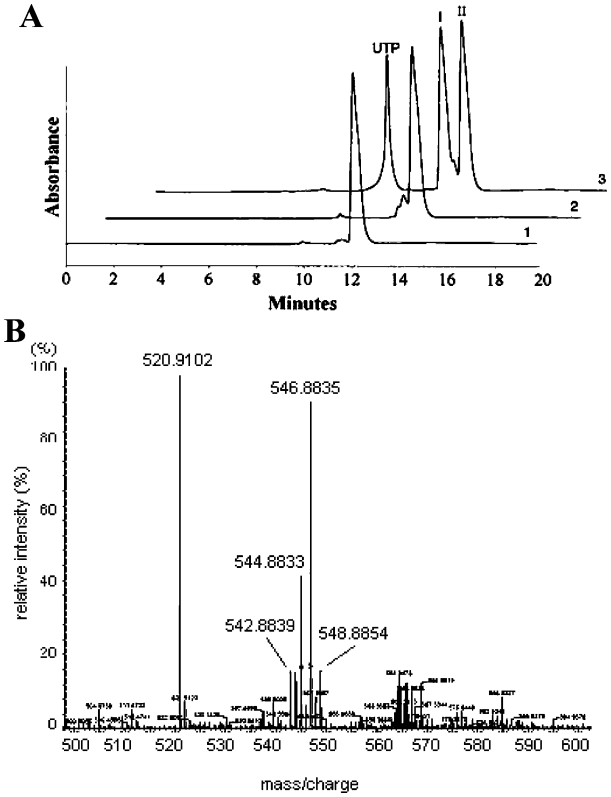

FIGURE 2: Analysis of UTPαSe by HPLC and HR-MS. (A) Typical HPLC profile: analysis of UTPαSe I and II, and normal UTP. (1) UTPαSe I; (2) UTPαSe II; (3) co-injection of UTP, UTPαSe I, and II. Their retention times were 9.7, 12.0, and 12.9 min, respectively. For the purpose of the HPLC presentation, traces 2 and 3 are shifted to the right. (B) HR-MS analysis of UTPαSe I (UTPαSe II is almost the same). Molecular formula: $C_9H_{15}N_2O_{14}P_3Se$; calculated isotopic mass $(M - H^+)^-$: 546.8823; measured mass: 546.8835.

MATERIALS AND METHODS

Oligonucleotides and Triphosphate Derivatives. The NTPαSe analogues were prepared as described (*17*, *18*), and all NTPαSe analogues were purified and then analyzed by HR-MS and RP-HPLC (Figure 2). A DNA template (55 nt, 5′-TGTACGTTTCGGCCTTTCGGCCTCATCAGGTTG-CCTATAGTGAGTC-GTATTACGC-3′,) was designed and synthesized on solid phase for in vitro transcription of the Se-modified hammerhead ribozymes using the NTPαSe analogues. T7 RNA polymerase promoter (top strand DNA, 5′-GCGTAATACGACTCACTATAG-3′) and an RNA substrate (5′-GGUCAUCUUUCCUAC-CUGUACGUCGUUGC-CUAA-3′) were also chemically synthesized.

Transcription of the Hammerhead Ribozyme using NTPs and NTPαSe Analogues. The T7 RNA polymerase promoter and DNA template (Figure 3) were prepared by oligonucleotide solid-phase synthesis. Ampliscribe T7 Transcription Kit (Epicenter) was used for in vitro transcription, where the DNA template and the top strand were added in equal molar amounts to a cocktail containing DTT and the buffer. The cocktail was split into two equal portions. α-^{32}P-CTP was added to one portion, and α-^{32}P-ATP was added to the other portion. Each portion was split equally into three Eppendorf tubes. The α-^{32}P-CTP-containing mixture was split equally into three tubes labeled ATPαSe I, UTPαSe I, and NTP. The α-^{32}P-ATP-containing mixture was split equally into three tubes labeled CTPαSe I, GTPαSe I, and NTP. Equal molar amounts of NTPαSe I and NTP were added to each tube as labeled. (For example, to the ATPαSe I tube, ATPαSe I, CTP, GTP, and UTP were added.) Typical reactions were performed under the conditions of 0.1 μM top strand and template, NTP (1 mM), NTPαSe (1 mM), and 0.06 μL of enzyme per 1 μL of the reaction mixture. The reactions were initiated by the addition of T7 RNA polymerase and were incubated at 37 °C. Aliquots (3.5 μL each) were removed from the reaction mixture at the corresponding time points, and the transcription reactions were quenched by the addition of the same volume of the loading dye containing EDTA (100 mM) and placed on dry ice. The comparison experiments of NTPαSe I and II diastereomers were done in the similar way, where the incubation time at 37 °C was 1 h.

Kinase Reaction of the RNA Substrate. The RNA substrate (33 nt) was kinased by T4 polynucleotide kinase and γ-^{32}P-ATP for 1 h at 37 °C. After the kinase reaction, the excess γ-^{32}P-ATP and salts were removed by NaCl/EtOH precipitation.

Activity Study of the Se-Hammerhead Ribozymes. The ribozymes were transcribed for 1 h as previously discussed without the addition of any radioactive NTPs and desalted three times by centrifugation using a membrane (3000 Dalton cutoff). The ribozymes were concentrated and adjusted to the same concentration on the basis of the time-course transcription experiment (Figure 3C). A cocktail containing the Tris-Cl buffer (pH 7.6, 10 mM $MgCl_2$) and the ^{32}P-labeled RNA substrate was made, and it was split into 5 equal portions. The ribozymes transcribed with ATPαSe I, CTPαSe I, GTPαSe I, UTPαSe I, and NTPs were individually added to each portion to initiate the substrate digestion reaction incubated at 27 °C. Aliquots (3.5 μL each) were removed from the reaction mixture at the corresponding time points and quenched individually with the loading dye (3.5 μL) containing EDTA (100 mM) and placed on dry ice. The digestion reactions were analyzed by PAGE (Figure 4A). The gel image quantification was done by using a phosphorimager.

Se-Ribozyme Resistance to the Digestion of Snake Venom Phosphodiesterase I. The ribozymes were transcribed as previously discussed. The transcribed ribozymes were desalted three times by centrifugation using a membrane (3000 Dalton cutoff). To ensure equal molar concentrations, the samples were concentrated and adjusted to the same concentration. These modified and native ribozymes were then digested with snake venom phosphodiesterase I (0.001 U/μL, USB) in its buffer. Aliquots (3.5 μL each) were removed from the reaction mixture at the corresponding time points and quenched individually with the loading dye (3.5 μL) containing EDTA (100 mM) and placed on dry ice. The digestion reactions were analyzed by PAGE (Figure 5A).

Se-Ribozyme (after Use in RNA Substrate Cleavage) Resistance to the Digestion of Snake Venom Phosphodiesterase I. The ^{32}P body-labeled ribozymes transcribed with NTPs and UTPαSe I were prepared as discussed previously in the transcription study section. The radioactive reagent, NTPs, and salts were removed three times by centrifugation using a membrane (3000 Dalton cutoff). These modified and native ribozymes were used to digest the RNA substrate for 1 h at 27 °C, as described in the RNA substrate digestion section. After digestion, the ribozymes were recovered three

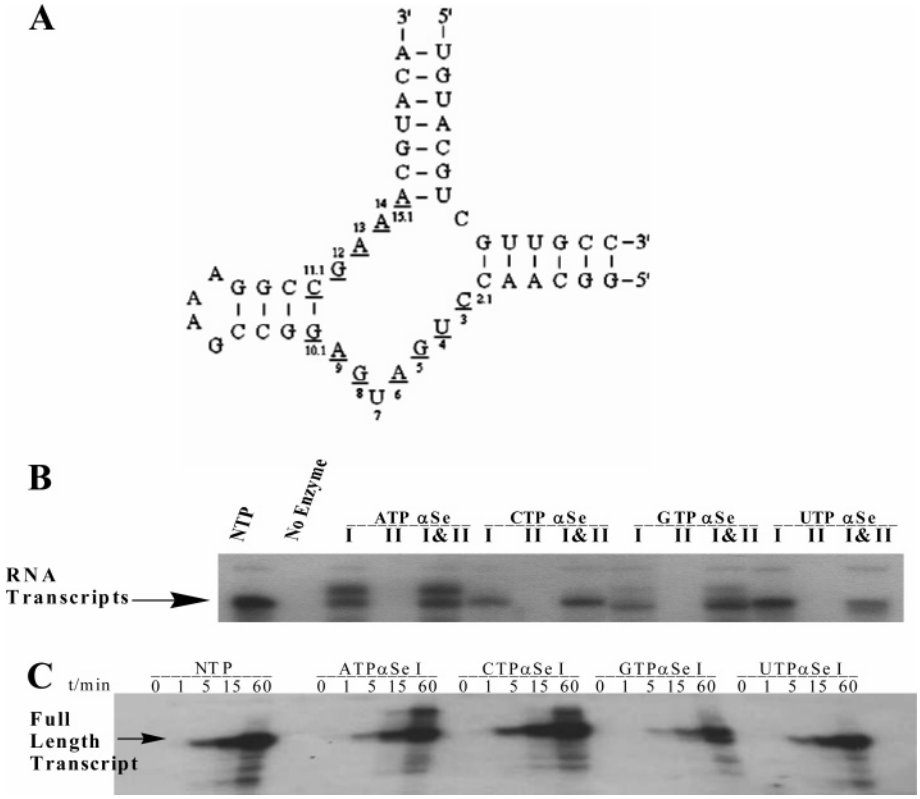

FIGURE 3: (A) Secondary structure of the hammerhead ribozymes; the underlined sequences are highly conserved. (B) Transcription with native NTP, ATPαSe I and II, CTPαSe I and II, GTPαSe I and II, and UTPαSe I and II. (C) Time-course experiment of the transcription with native NTP, ATPαSe I, CTPαSe I, GTPαSe I, and UTPαSe I.

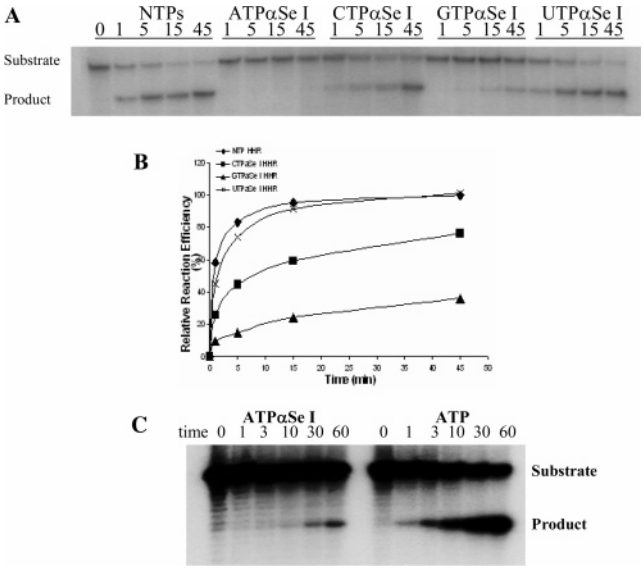

FIGURE 4: Catalysis and analysis of the modified and native hammerhead ribozymes: 5′-GGCAACCUGAUGAGGCCGAAAG-GCCGAAACGUACA-3′. The highly conserved sequences are underlined. (A) Time-course ribozyme digestion of the RNA substrate: 5′-GGUCAUCUUUCCUACC-UGUACGUCGUUGC-CUAA-3′. (B) Plot of the catalytic experimental results, relative to the activity of the native ribozyme. (C) Time-course Mn^{2+} rescue experiment of the ribozymes transcribed with ATPαSe I and ATP.

times by centrifugation using a membrane (3000 Dalton cutoff). These modified and native ribozymes were then digested with snake venom phosphodiesterase I (0.001 U/μL, USB) in its buffer. Aliquots (4.5 μL each) were removed from the reaction mixture at the corresponding time points and quenched individually with the loading dye (4.5 μL) containing EDTA (100 mM) and placed on dry ice. The digestion reactions were analyzed by PAGE (Figure 5B).

RESULTS

To investigate T7 RNA polymerase recognition of NTPαSe analogues and each diastereomer pairs, we separated two diastereomers of each NTPαSe by HPLC. The HPLC separation of UTPαSe diastereomers is shown as an example in Figure 2A, and the fast- and slow-moving peaks on HPLC were termed as NTPαSe I and II, respectively; both diastereomers of UTPαSe were also analyzed by HR-MS (Figure 2B). We then performed the in vitro transcription of the hammerhead ribozymes using each NTPαSe I and II diastereomer (Figure 3). The experimental results indicate that all NTPαSe I diastereomers are well recognized as substrates by T7 RNA polymerase, and all NTPαSe II diastereomers are neither substrates nor inhibitors (Figure 3B). Because all NTPαSe I and II diastereomers underwent the same treatment during purification, storage, and transcription, it is unlikely that the observed RNA transcripts from NTPαSe I were due to the oxidation of NTPαSe I by air, which converts NTPαSe to normal NTP (*17*). Because RNA transcripts with N + 1 and even N + 2 are often observed in transcription and T7 RNA polymerase prefers to incorporate ATP as extra nucleotides in this nontemplated incorporation, we also observed that ATPαSe was incorporated more than other NTPαSe at the 3′ terminus. To further compare their efficiency as substrates with the natural NTPs, we also carried out time-course transcription using all NTPαSe I analogues (Figure 3C). We found that T7 RNA polymerase recognizes NTPαSe I analogues as well as natural NTPs, and NTPαSe incorporation efficiency is at the

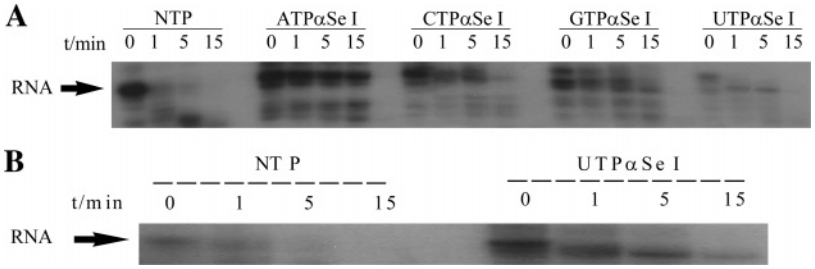

FIGURE 5: Se-Ribozyme resistance to the digestion of snake venom phosphodiesterase I. (A) PAGE analysis of the digestion. (B) Snake venom phosphodiesterase I digestion of the modified and native ribozymes transcribed with NTP and UTPαSe I; these ^{32}P-labeled ribozymes were digested after use in their substrate cleavage reactions.

same level as that of natural ones. The transcription yields of ATPαSe, CTPαSe, GTPαSe, and UTPαSe, relative to that of the corresponding NTP, are 100%, 151%, 70%, and 139%, respectively.

To study the catalytic activities of these Se-ribozymes transcribed with NTPαSe I diastereomers, we performed catalytic reactions using a kinased 33 nt RNA substrate (Figure 4). After transcription, the catalytic investigation was done through a comparison of the Se-modified ribozymes and the native one in the presence of Mg^{2+} (10 mM) as the metal ion. Substrate digestion was initiated by the addition of the native or modified ribozymes. Our experiment results reveal that the hammerhead ribozymes transcribed with UTPαSe I and CTPαSe I (Figure 4A and B) have the same level of efficiency as the native one (98% and 76% of the native ribozyme) and that the GTPαSe I-transcribed ribozyme has only 30% of native activity, whereas the ATPαSe I-transcribed ribozyme has very low activity (less than 0.1% of native activity). The activity of the ribozyme transcribed with ATPαSe I was accurately determined by extending the digestion time to 24 h. However, the catalysis of the ATPαSe-transcribed ribozyme can be rescued by nearly 200-fold when Mg^{2+} is replaced with Mn^{2+} in the reaction medium (Figure 4C and Supporting Information), although the activity enhancement of the GTPαSe-transcribed ribozyme is small (less than 1-fold) when Mg^{2+} is replaced with Mn^{2+} in the reaction medium (Supporting Information).

Our previous MS study indicated that T7 RNA polymerase can incorporate the selenium modification into RNA (*18*), and the transcription difference (Figure 3B) between NTPαSe I and II diastereomers also suggests the incorporation of the phosphoroselenoate modification. To further confirm the presence of Se on the ribozymes, all ribozymes transcribed with NTPαSe I were subjected to digestion by snake venom phosphodiesterase I, which successively degrades both DNA and RNA from the 3′ to the 5′ direction. We observed the reduction of the enzymatic digestion by 5−10-fold among the Se-modified RNAs (Figure 5A), suggesting the presence of the phosphoroselenoate groups in the RNAs. In addition, to confirm the stability of the selenium functionality in RNA substrate cleavage, we also performed enzymatic digestion after the cleavage reaction. We found that the Se-ribozyme, transcribed with UTPαSe I and used after the RNA substrate cleavage, showed resistance to enzymatic digestion (5-fold, Figure 5B), whereas the native one showed no resistance. The digestion resistance of the Se-ribozyme before and after substrate cleavage confirms the presence of the stable phosphoroselenoate modifications. This enzymatic digestion resistance is consistent with the resistance of DNA and RNA phosphoroselenoates (*17*, *18*), phosphorothioates (*25*), and boranophosphates (*26*).

DISCUSSION

Our transcription experiments indicate that T7 RNA polymerase only recognizes NTPαSe I diastereomers, and all NTPαSe II diastereomers are neither substrates nor inhibitors (Figure 3), which suggests that the separation of diastereomers I and II of each NTPαSe is not necessary for transcription. In addition, because T7 RNA polymerase only recognizes the Sp diastereomer of 5′-(α-P-thio)triphosphates (*22*, *23*), we tentatively assign the Sp configuration to all NTPαSe Is and the Rp configuration to all NTPαSe IIs. This selective recognition of the Sp and Rp diastereomers also indicates that the atom (O or Se) in the pro-Rp position is probably involved in the polymerase interaction and/or catalysis, whereas the atom in the pro-Sp center is not. The inversion of configuration at the phosphorus center upon incorporation, reported in the literature (*22*−*24*), suggests that these phosphoroselenoate ribozymes have an Rp configuration. Naturally, the transcribed Se-ribozymes are diastereomerically pure, which is an advantage over the chemical synthesis of phosphoroselenoate nucleotides (*19*). As NTPαSe analogues behave the same as natural NTPs, large-scale transcription with NTPαSe analogues has demonstrated that it is possible to prepare large quantities (milligrams) for X-ray crystallography.

Our catalytic study using the selenium-modified ribozymes shows that the hammerhead ribozymes transcribed with UTPαSe and CTPαSe have the same level of activity as that of the native ribozyme. This is probably due to the 5′-phosphate nonbridging pro-Rp oxygen atoms of U and C located in the highly conserved sequences (U4 and C3 and C11.1, the underlined sequences in Figure 3A) just in contact with solvent water molecules, which is consistent with determined structures (*27*, *28*). However, because of the involvement of the 5′-phosphate pro-Rp oxygen atoms of As in the conserved sequences (A_9, A_{13}, and A_{14}; *29*, *30*), the replacement of the oxygen with selenium results in over a 1000-fold reduction in efficiency. The replacement of Mg^{2+} with Mn^{2+} in the reaction medium rescued the catalysis by nearly 200-fold (Figure 4C), suggesting the metal cation interaction with these specific sites. In the case of GTPαSe I, though there are three Gs in the highly conserved sequences, the transcribed ribozyme displayed an efficiency that is only 3-fold lower than that of the native one. This minimum efficiency disruption and the Mn^{2+} nonrescuing property suggest that these 5′-phosphate pro-Rp oxygen atoms of the Gs are not involved in catalysis.

Our enzymatic digestion experiments of the Se-ribozymes, before and after ribozyme catalysis, have clearly indicated the presence of the stable selenium substitution (phosphoroselenoate modification). The resistance is consistent with the resistance of DNA and RNA phosphoroselenoates (*17*, *18*), phosphorothioates (*25*), and boranophosphates (*26*). Clearly, this systematic and convenient modification of the ribozyme with each NTPαSe I can assist the study and determination of its conserved sequences. This method is especially useful in elucidating the role of the pro-Rp oxygen on the 5′-phosphate of the modified nucleotide, that is, as to whether it participates in catalysis. In addition, the most active Se-ribozymes, with the structure most similar to that of the native ribozyme, can be easily identified through a quick activity test and will be the ideal candidates for X-ray crystal structure studies. This RNA derivatization strategy can also be applied to the derivatization of RNA−protein complexes by labeling the nucleic acid molecules with selenium instead of the protein counterparts because the derivatization and purification of nucleic acids are much quicker and easier than those of proteins.

In conclusion, we have shown that all NTPαSe I analogues are efficient substrates as well as natural NTPs for T7 RNA polymerase, whereas the NTPαSe II analogues are not recognized. Because all II diastereomers are neither substrates nor inhibitors, the separation of the mixture of diastereomer I and II is not necessary for the efficient transcription of diastereomerically pure RNAs. More importantly, these hammerhead ribozymes transcribed using different NTPαSe analogues display catalytic activities at a variety of levels, which are consistent with the roles of the replaced pro-Rp oxygen atoms in structure and catalysis. Therefore, this modification on the backbone with phosphoroselenoate is useful in the catalytic mechanism study and in the identification of highly conserved sequences of any functional RNA molecules. In addition, the most active ribozymes derivatized with Se can be identified via a simple and quick transcription and activity screening using different NTPαSe analogues. Obviously, this strategy of RNA derivatization with selenium also has great potential as a general approach for X-ray crystal structure studies of functional RNAs via MAD or single wavelength anomalous dispersion (SAD) phasing.

SUPPORTING INFORMATION AVAILABLE

Synthesis and purification of NTPαSe analogues and catalysis of the modified and native hammerhead ribozymes using Mn^{2+} as the metal cation. This material is available free of charge via the Internet at http://pubs.acs.org.

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