METHOD

Facile synthesis of nucleoside 5'-(α -P-seleno)-triphosphates and phosphoroselenoate RNA transcription

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ABSTRACT

Phosphoroselenoate RNA (PSe-RNA) is nuclease resistant and has great potentials in X-ray crystal structure and function studies of noncoding RNAs and protein–RNA interactions. In order to conveniently synthesize PSe-RNA via transcription, we have developed a one-pot synthetic method for the nucleoside 5'-(α -P-seleno)-triphosphates (NTP α Se) analogs without protecting any functionality of the ribonucleosides. The NTP α Se diastereomers have been purified, fully characterized, and incorporated into RNAs by T7 RNA polymerase. The transcribed RNAs are diastereomerically pure, and the Se-derivatized ribozymes are generally active. Furthermore, we have established an affinity purification strategy by using immobilized boronate to conveniently purify NTP α Se analogs. Though the affinity-purified NTP α Se analogs are diastereomeric mixtures, they can be directly used in transcription without a significant impact on the transcription efficiency. Moreover, we found that the PSe-nucleotide is stable during polyacrylamide gel purification, indicating that the PSe-RNAs can be purified straightforwardly for crystal structural and functional studies.

Keywords: modified NTP α Se; selenium-derivatization of RNA; RNA transcription; RNA crystallography; hammerhead ribozyme

INTRODUCTION

The massive discoveries of noncoding RNAs in recent years, such as small regulatory RNAs (Lee et al. 1993; Makarova and Kramerov 2007) and the riboswitch RNAs (Gelfand et al. 1999), lead to a great demand for structural insights of the functional RNAs and RNA-ligand complexes (Ferre-D'Amare et al. 1998; Kieft et al. 1999; Batey et al. 2004; Macrae et al. 2006). In addition to crystallization, however, RNA crystallography has faced the other long-standing challenge: derivatization and phasing (Ke and Doudna 2004). To address these needs, several phasing-and-crystallization strategies have been developed recently (Ferre-D'Amare et al. 1998; Keel et al. 2007; Ferre-D'Amare 2010; Koldobskaya et al. 2011). In the case of a novel protein structure, the phase problem is usually solved by the selenomethionine (Se-Met) derivatization and multiwavelength anomalous dispersion (MAD) technique (Hendrickson 1991; Deacon and Ealick 1999). Inspired by the convenient Se-derivatization of proteins,

since 1998, Huang's research group has pioneered and developed the selenium derivatization of nucleic acids for X-ray crystallography (Carrasco et al. 2001; Caton-Williams and Huang 2008; Sheng and Huang 2010). We found that the selenium-derivatized RNAs and DNAs are stable and that the selenium derivatizations do not cause significant perturbation (Jiang et al. 2007; Salon et al. 2007, 2010; Sheng et al. 2007, 2010, 2011; Caton-Williams and Huang 2008; Olieric et al. 2009; Hassan et al. 2010).

There are two strategies to derivatize RNA with selenium for X-ray crystallography: indirect derivatization (noncovalent Se-derivatization via the seleno-protein binding) (Ferre-D'Amare et al. 1998; Rupert and Ferre-D'Amare 2001; Ferre-D'Amare 2010) and direct derivatization (covalent Se-derivatization via the oxygen replacement) (Caton-Williams and Huang 2008; Sheng and Huang 2010). The cocrystallization method using Se-Met-derivatized U1A protein (a RNA-binding protein) was developed by Doudna and coworkers (Ferre-D'Amare et al. 1998; Rupert and Ferre-D'Amare 2001; Ferre-D'Amare 2010). Although this U1A method has been used to solve several ribozyme structures, it is labor intensive. For direct derivatization of RNA via covalent incorporation of selenium, chemical approach (solid-phase synthesis) or enzymatic methods (RNA polymerase

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transcription) can be applied (Caton-Williams and Huang 2008; Sheng and Huang 2010). A general drawback of solid-phase synthesis for large-scale RNA preparation is the RNA length limit (usually <60 nt). To solve this problem, a T4 RNA ligase method using a synthetic 2'-Se-RNA was developed by Micura's group in order to prepare a longer 2'-Se-RNA. (Hobartner et al. 2005). However, due to the size limit and high cost associated with solid-phase synthesis, RNA transcription is a preferred approach for most molecular and structural biology laboratories. A large quantity of long RNA molecules can be easily prepared via transcription by T7 RNA polymerase (Ferre-D'Amare and Doudna 2001; Kieft and Batey 2004).

Phosphoroselenoate modification has been introduced into DNA through solid-phase synthesis for the structure study by Egli and coworkers (Wilds et al. 2002). This PSe-DNA modification was successfully used to facilitate the phase determination of a homo-DNA structure (Egli et al. 2006). However, the PSe-DNA prepared by solid-phase synthesis was a mixture of Rp and Sp diastereomers, which needed to be separated. Separation of the diastereomers, which are formed at the chiral center of each Se-modified phosphate linker, requires anion exchange chromatography extensively. Since the diastereomer number increases exponentially by 2^n (n: the modification number), it is not practical to separate diastereomers generated by multiple phosphoroselenoates incorporated into DNA or RNA. To obtain diastereomerically pure PSe-nucleic acids, our research group has developed the enzymatic methods (Carrasco and Huang 2004; Carrasco et al. 2005; Brandt et al. 2006). In order to prepare phosphoroselenoate RNA (PSe-RNA) (Fig. 1) through transcription, we first synthesized the 5'-(α -P-seleno)-triphosphates (NTP α Se) analogs (Fig. 1; Carrasco et al. 2005; Brandt et al. 2006). Considering broad applications of the NTP α Se analogs and PSe-RNAs, it is necessary to develop a convenient strategy to synthesize the NTP α Se analogs and PSe-RNAs on a large scale. Herein, we report a novel synthetic method for the NTP α Se analogs without any nucleoside protection, which greatly reduces efforts in the synthesis and purification and increases the overall yield. We also developed a simple

purification strategy for NTP α Se and the affinity-purified NTP α Se analogs can be used directly to transcribe PSe-RNAs. Moreover, stability of the phosphoroselenoate modification was confirmed under the conditions of PAGE purification.

RESULTS

Nucleoside triphosphates, especially chemically modified ones, are normally synthesized by using protected nucleosides with free 5'-hydroxyl groups (Ludwig and Eckstein 1989; Carrasco and Huang 2004; Brandt et al. 2006). The protection is critical. If other hydroxyl groups (such as 2'-OH and 3'-OH) and amino groups on the nucleobases are not protected, they can cause formation of many by-products during the chemical synthesis of NTPs. These by-products are difficult to remove during the purification steps. In order to obtain a clean phosphorylation reaction with chemical selectivity on the 5'-hydroxyl group of the nonprotected nucleosides, we developed a selective phosphorylating reagent. This reagent (Fig. 2, 4) was generated in situ by reacting pyrophosphate (3) with 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (2), followed by addition of the nucleosides (1) to offer the cyclic phosphite (5). After selenization and hydrolysis, NTP α Se analogs (6) were successfully synthesized without any protection on the nucleosides. The HPLC analysis of the crude products indicated these 5'-triphosphates as the dominant products.

After the synthesis, NTP α Se analogs need to be purified from crude products before RNA transcription, otherwise salts and other organic impurities will inhibit the activity of RNA polymerase. In our previous study, NTP α Se analogs were purified by HPLC, which is labor-intensive and has a low capacity for larger-scale synthesis (Carrasco et al. 2005; Brandt et al. 2006). Thus, we developed the boronate affinity method for purification of NTP α Se analogs. Boronate has a high affinity to *cis*-diol-containing compounds (Batey et al. 1992; Liu and Scouten 2000). The *cis*-diols at the 2' and 3' positions of NTPs can interact with boronate strongly, which allows the separation of NTP α Se analogs from many other impurities. Boronate column purification of NTP α Se analogs offered satisfactory yields (Table 1). Moreover, as a comparison, we also purified NTP α Se analogs via HPLC (Supplemental Fig. S1) and performed RNA transcription (Fig. 3). Compared with HPLC, the boronate affinity column has a larger capacity (easily up to a 100 mg of NTP α Se) and requires less purification time and steps. The quality of NTP α Se analogs purified by the affinity column was examined by analytical HPLC. As a typical example, HPLC profiles of CTP α Se analogs before and after boronate column purification were shown in Supplemental Figure S2. After affinity purification, NTP α Se analogs (diastereomer mixtures) can be directly used for RNA transcription (Fig. 4). Full-length RNA products were observed with the affinity-purified NTP α Se analogs.

FIGURE 1. Chemical structures of the Se-derivatized nucleotides. (A) Nucleoside 5'-(α -P-seleno)triphosphates (NTP α Se, Sp and Rp). (B) Phosphoroselenoate RNA (PSe-RNA, Rp).

FIGURE 2. Facile synthesis of the NTPαSe analogs. (DMF) Dimethylformamide; (TBA) tributylamine.

All four NTPαSe analogs were synthesized via this facile route, and all eight diastereomers were purified and fully characterized by MS, HPLC, ^1H -, ^{13}C -, and ^{31}P -NMR (Supplemental Tables S1–S4; Supplemental Fig. S1). This is the first full characterization of all NTPαSe analogs. Based on the literature result on the phosphorothioates (Griffiths et al. 1987), Peak 1 (NTPαSe I) and Peak 2 (NTPαSe II) in HPLC profiles (Supplemental Fig. S1) were tentatively assigned as Sp and Rp, respectively. Eight diastereomers from the four NTPαSe analogs were individually examined using T7 RNA polymerase (Fig. 3). Consistent with our previous report (Carrasco et al. 2005; Brandt et al. 2006), clearly, T7 RNA polymerase can efficiently recognize the NTPαSe I diastereomers (Sp) at the same level as the native NTPs (Fig. 3), while the NTPαSe II diastereomers (Rp) were not recognized. Our transcription data with several different DNA templates indicated that the PSe-RNA transcription efficiency and yields are similar to the native ones. Since T7 RNA polymerase only recognizes Sp-NTPαSe diastereomers and Rp-NTPαSe diastereomers are neither substrates nor inhibitors (Fig. 3), RNAs transcribed with the affinity-purified NTPαSe analogs are diastomerically pure (Fig. 4).

A PSe-RNA (22 nt) containing six Se atoms was first prepared by RNA transcription using UTPαSe I, and its integrity was confirmed by MS (Fig. 5A). Two hammerhead ribozymes (Fig. 6, HHN and HHM) were used later for examining the transcriptional incorporation of NTPαSe analogs. The wild-type hammerhead ribozyme (HHN) transcribed using all native NTPs could cleave itself in the transcription condition (Fig. 7). The mutant hammerhead ribozyme (HHM) is a hammerhead mutant that has a very low turnover rate. The majority of HHM transcribed could not cleave itself (Fig. 7). Low self-cleavage activities of PSe-RNA HHM (the Se-C and Se-U mutant ribozymes) were also observed (Fig. 3), while the Se-A and Se-G mutant ribozymes were not active. This is also consistent with literature that the A and G nucleotides play more critical roles in the hammerhead ribozyme, especially in the active site. Due to the presence of the A at the cleavage site and the five As in the highly conserved sequences of HHN (Fig. 6), HHN transcribed by ATPαSe showed no self-cleavage activity (Fig. 7, lane N of A). Unsurprisingly, HHN transcribed with the other Sp-NTPαSe

(CTPαSe, GTPαSe, or UTPαSe) has self-cleavage activity (Fig. 7). The catalytic activity of the Se-derivatized ribozymes suggests that the selenium modifications on nonessential phosphates do not cause significant structural and functional perturbations. Furthermore, the inhibition of the ribozyme self-cleavage by ATPαSe incorporation (Fig. 7, lane N of A) indicates that the PSe-RNA is stable under the transcription conditions without deselenization, since deselenization will result in formation of the self-cleaving wild-

type ribozyme. Moreover, the UTPαSe-transcribed ribozyme (HHM) containing 15 Se atoms was confirmed by MALDI-TOF (Fig. 5B).

To investigate stability of the phosphoroselenoate moiety during PAGE purification, pure ATPαSe I was loaded on a polyacrylamide gel for electrophoresis and recovered from gel. The original and recovered ATPαSe was analyzed by HPLC by comparing it with native ATP (Supplemental Fig. S3). HPLC analysis showed that the phosphoroselenoate moiety was quite stable under PAGE purification and recovery conditions, since only an insignificant amount of ATPαSe (<2%; profile 4 in Supplemental Fig. S3) was oxidized to native ATP after the PAGE purification. This suggests that the PSe-RNAs can be purified by ordinary polyacrylamide gel, which is the most common purification method used for preparing large-scale RNAs in X-ray crystallography. As a matter of fact, PSe-RNA is more stable in the presence of magnesium salt (or even in a solution with RNase contamination) than native RNA (Carrasco et al. 2005; Brandt et al. 2006). For example, after storing long

TABLE 1. NTP and NTPαSe yields from boronate affinity column purification

Compounds	pH of loading buffers ^a	Column recovery yield (%) ^b	Column purification yield (%) ^c
ATP	9.5	94	
ATPαSe	9.5		88
CTP	10.5	92	
CTPαSe	10.5		92
GTP	9.5	94	
GTPαSe	9.5		59
UTP	9.5	92	
UTPαSe	9.5		86

^aThe column loading buffer: 1 M triethylamine bicarbonate (TEABC), pH as stated in the table; the elution buffer: 0.05% acetic acid at pH 3.5.

^bColumn recovery yields were calculated by UV determination and using the pure NTPs that were loaded and recovered from the affinity column.

^cColumn purification yields were calculated by UV determination and by using the crude Se-NTPs that were loaded and purified from the affinity column.

FIGURE 3. Enzymatic incorporation of NTP α Se analogs purified by HPLC to (hammerhead mutant) HHM RNA. Minor cleavage (the small faster-moving bands) was observed with the native, Se-C and Se-U ribozymes. NTP (all native nucleoside triphosphates), A₁ (ATP α Se I), A₂ (ATP α Se II), A_{1&2} (I and II; I vs II = 1:1); C₁ (CTP α Se I), C₂ (CTP α Se II), C_{1&2} (I and II; I vs II = 1:1); G₁ (GTP α Se I), G₂ (GTP α Se II), G_{1&2} (I and II; I vs II = 1:1); U₁ (UTP α Se I), U₂ (UTP α Se II), U_{1&2} (I and II; I vs II = 1:1).

Se-RNAs for 4–5 mo in the freezer (with 1 mM DTT), MALDI-MS analysis still confirmed their integrity.

DISCUSSION

Nucleotide triphosphates are common biological molecules in all life forms, and their analogs have wide applications for scientific and therapeutic discoveries. Our facile synthesis and purification methods for the NTP α Se analogs enable scale-up preparation of PSe-RNAs for X-ray crystallography and other potential applications. Our experimental research indicates that only NTP α Se I could be incorporated into RNAs by T7 RNA polymerase. Moreover, unless the cleavage site is modified, the Se-derivatized ribozymes are generally active, indicating that the Se-modification does not significantly affect RNA global folding and perturb their structure and function. The method described here is an efficient synthetic approach for NTP α Se without any protection of the nucleosides, which is a one-pot strategy without multiple steps of purification. This procedure allows for the synthesis of natural ribonucleotide triphosphates, α -phosphoroselenoate nucleotide triphosphates, α -phosphorothioate nucleoside triphosphates, and α -borano nucleotide triphosphates by selecting the appropriate corresponding oxidizing agent. The NTP α Se analogs can be easily purified by boronate affinity column for RNA transcription. The Sp-NTP α Se analogs are efficient substrates for T7 RNA polymerase, while the Rp-NTP α Se analogs are neither inhibitors nor substrates. A full-length PSe-hammerhead ribozyme with multiple Se-modifications was transcribed and confirmed by MALDI-TOF. PSe-RNAs are stable under a PAGE gel purification condition, which allows convenient preparation of PSe-RNAs for studying ribozyme catalytic mechanism, RNA crystal structure and function, and RNA interference.

MATERIALS AND METHODS

The following procedures are normally performed under argon, and all organic solvents and chemicals used in the experiments were purchased as anhydrous compounds. Unless specifically mentioned, the reactions were performed at room temperature. All compounds

are commercially available, including 2-chloro-4H-1,3,2-benzodioxaphosphorin (from Sigma, <http://www.sigmaaldrich.com>; SeNA Research, <http://www.senaresearch.com>) and 3H-1,2-benzothiaselenol-3-one (BTSe) (Stawinski and Thelin 1994) (commercially available from SeNA Research).

NTP α Se synthesis

Individual nucleoside (1, 0.45 mmol, A [120 mg], C [109 mg], G [127 mg], or U [110 mg]) (Fig. 2), tributylammonium pyrophosphate (3, 426 mg, 0.9 mmol, 2 eq.), and 3H-1, 2-benzothiaselenol-3-one (BTSe, 195 mg, 0.9 mmol, 2 eq.) were dried in separate flasks under high vacuum for 1 h. To the dried tributylammonium pyrophosphate (3), DMF (0.6 mL) and tributyl amine (TBA, 1.2 mL) were injected to dissolve the pyrophosphate. Anhydrous 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one (2, 182 mg, 0.9 mmol, 2 eq.) dissolved in DMF (1.2 mL) was then injected into the pyrophosphate solution (3). This reaction mixture was stirred under argon at room temperature for 30 min and then injected into the nucleoside (1, 0.45 mmol) dissolved in DMF (0.45 mL; special note: adenosine was dissolved in a mixed solvent of 0.32 mL DMF and 0.13 mL DMSO, and guanosine was dissolved in a mixed solvent of 0.22 mL DMF and 0.23 mL DMSO). After the nucleoside phosphorylating reaction was stirred under argon at room temperature for 1 h, 3H-1,2-benzothiaselenol-3-one (BTSe) dissolved in dioxane (1.5 mL) was injected to the nucleoside reaction mixture. The BTSe oxidation reaction was finished at room temperature for 1 h. Then, 10 mL water (approximately twice the volume of the reaction solution) was added to the BTSe reaction mixture. After hydrolysis at room temperature for 2 h, an ethanol/NaCl precipitation (NaCl [1.7 mL, 3 M] and ethanol [50 mL] were sequentially added to the NTP α Se solution, followed by freezing at -80°C for 30 min and centrifugation at 14,000 rpm for 20 min) was performed in the presence of fresh DTT (2 mM). The pellet was redissolved in a small amount of water (200 μL), and the concentration of NTP α Se analog (6) was determined by UV-Vis spectrometry.

HPLC analysis of NTP α Se

The synthesized NTP α Se analogs (Fig. 2, 6) were analyzed by HPLC. ATP α Se, CTP α Se, and GTP α Se were eluted (1 mL/min) with a linear gradient from Buffer A (20 mM triethylammonium acetate [TEAAc] at pH 7.0) to 12.5% Buffer B (50% acetonitrile in water, 20 mM triethylammonium acetate [TEAAc] at pH 7.0) for >20 min. UTP α Se was eluted (1 mL/min) with a linear gradient from Buffer A (20 mM triethylammonium acetate [TEAAc] at pH 7.0) to 20% Buffer B (50% ethanol in water, 20 mM triethylammonium acetate [TEAAc] at pH 7.0) for >20 min.

FIGURE 4. HHM RNAs transcribed with the NTP α Se analogs (diastereomer mixture) purified by boronate column. The shorter fragments are self-cleaved products.

FIGURE 5. MALDI-TOF MS analysis of transcribed RNAs. (A) Native RNA42.1 (22 nt), molecular formula: $C_{209}H_{261}N_{82}O_{165}P_{24}$; observed $[M+H]^+ = 7305$ (calcd. $[M+H]^+ = 7303$). PSe-U RNA42.1, molecular formula: $C_{209}H_{261}N_{82}O_{159}P_{24}Se_6$; observed $[M+H]^+ = 7687$ (calcd. $[M+H]^+ = 7686$). Their mass difference of 382 ($7687 - 7305 = 382$) reflects incorporation of six Se-modified U units ($6Se-6O = 6 \times 80 - 6 \times 16 = 384$). (B) PSe-U HHM, molecular formula: $C_{657}H_{814}N_{264}O_{465}P_{68}Se_{15}$ (calcd $[M] = 23,144$); the matrix (2',4',6'-trihydroxyacetophenone, THAP), molecular formula: $C_8H_8O_4$, molecular weight: 168.0; observed mass of $[M + THAP]$: 23,314 (calcd. 23,312).

Purification of NTP α Se analogs by boronate affinity column

Sample loading buffer (1 M triethylamine bicarbonate, TEABC) was prepared by bubbling CO_2 through a solution containing 28.2 mL triethylamine in 140 mL H_2O in an ice bath, until the pH dropped to 9.5. Then, the solution final volume was brought up to 200 mL by adding more deionized water. The prepared TEABC solution was stored at $4^\circ C$, and the pH was checked each time before the buffer was used. The elution buffer (1 L, 0.05% v/v acetic acid, pH 3.5) was prepared by dilution of concentrated acetic acid (0.5 mL) with 999.5 mL of deionized water. All working buffers were kept at $4^\circ C$ during the affinity purification.

Boronate affigel (0.20 g, 1.2 meq/g, Bio-Rad) was hydrated in 2 mL of TE buffer (10 mM Tris Hcl at pH 8.0, 1 mM EDTA) and packed in a column (1×4 cm). The column was equilibrated by 5 mL of 1 M TEABC (pH 9.5) for 1 h at $4^\circ C$. After draining the excess buffer, the NTPs (0.5 μ mol) or crude NTP α Se analogs (20 μ mol) dissolved in deionized water (100 μ L) were loaded onto the column and incubated for 1 h at $4^\circ C$. The column was then eluted with the loading buffer (1 M TEABC), and fractions (0.5 mL each) were collected. Each fraction was monitored by UV. We washed the column until the maximum absorbance dropped below 0.01 (the wavelengths monitored are the followings: ATP [259 nm], UTP [262 nm], GTP [252 nm], and CTP [271 nm]; special note: phosphorosele-noate modifications do not change UV absorbance of nucleotides). The NTP α Se on column was then eluted by the elution buffer (0.05% v/v acetic acid at pH 3.5), and fractions (0.5 mL each) were collected by tubes containing $Na-PO_4$ buffer (100 μ L, 1 M at pH 7.3). The neutralized individual fraction was analyzed by UV spectrometry. The fractions containing the eluted NTP α Se were combined and concentrated by lyophilization. The column was later washed by several column volumes of 1% acetic acid (pH 2.8) and water to regenerate it. HPLC analysis was done to confirm the quality of the affinity-purified NTP α Se analogs.

PSe-RNA transcription

Two types of hammerhead ribozymes were transcribed with either all native NTPs, or one of the NTPs was replaced with the

corresponding NTP α Se. The sequence of mutant hammerhead ribozyme (HHM) was 5'-GGGAGCCCUGUCACCGGAUGUGCUUCCGGUCUGAUGAGUCCGUGAGGACAAACAGGGCUCUCCGAAUU-3'. The sequence of wild-type hammerhead ribozyme (HHN) was 5'-GGGAGCCCUGUCACCGGAUGUGCUUCCGGUCUGAUGAGUCCGUGAGGACGAAACAGGGCUCUCCGAAUU-3'. The sequence of native RNA 42.1 was 5'-GGUCGCUUGUAAU GGGCGCCUU-3'. RNA transcribed with radioactive labeling was performed according to the protocol from the manufacturer (Epicentre T7 RNA polymerase, T7905K) for 1 h at $37^\circ C$. Each time one type of 10 mM NTP α Se was used to substitute for type of native 10 mM NTP solution in protocol (for example, in ATP α Se I transcription mixture, equal amounts of ATP α Se I, CTP, GTP, and UTP were added). RNAs were labeled by using radioactive NTPs, such as ATP [α - ^{32}P]. A typical reaction (10 μ L) was performed under the final conditions of 50 ng/ μ L plasmid or 5 ng/ μ L dsDNA template prepared by PCR, 0.50 mM NTP or NTP α Se analog (diastereomerically pure), 10 mM DTT, 1X Epicentre T7 RNA transcription buffer, 1 μ L of Epicentre T7 RNA polymerase (50 U), and RNase-free water to bring the volume to 10 μ L. For transcription with the affinity-purified NTP α Se analogs (diastereomer

FIGURE 6. Secondary structure of wild-type hammerhead ribozyme (HHN) derived from the satellite RNA of tobacco ringspot virus. Conserved bases are labeled in gray. The mutant hammerhead ribozyme (HHM) has a G→A mutation.

FIGURE 7. Transcription of the hammerhead ribozymes. (M) Mutant HHM; (N) wild-type HHN; (NTP) all native NTPs; (A) ATP α Se I and other natives; (C) CTP α Se I and other natives; (G) GTP α Se I and other natives; (U) UTP α Se I and other natives. One band from each cleavage is shown here, since the other band (12 nt) ran off the gel.

mixture), the NTP α Se I concentration was kept at 0.5 mM in the final reaction solution. The transcription reaction was quenched by adding an equal volume of loading dye containing EDTA (100 mM). The transcriptions were monitored by a 15% denature PAGE gel and autoradiography.

Stability study of phosphoroselenoate nucleotides

ATP α Se I was synthesized and purified by HPLC as described above. ATP or ATP α Se (10 μ L, 10 mM) was loaded on a denaturing polyacrylamide gel (12%) and recovered from the gel after gel electrophoresis. An ATP α Se band on the gel was visualized by UV shadowing and cut from the gel, then soaked in water overnight. The soaking solution was centrifuged the next day to remove gel fragments. The supernatant was collected and filtered with a 0.2- μ m syringe filter. The filtered solutions were analyzed by HPLC to compare ATP α Se with the native ATP recovered in the same manner.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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