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Enzymatic Synthesis of Phosphoroselenoate DNA Using Thymidine 5′-(α-P-seleno)triphosphate and DNA Polymerase for X-ray Crystallography via MAD

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X-ray crystallography is the most direct and powerful tool for 3-D structure determination of biomacromolecules, such as ribozymes, viral RNA, and RNA−protein and DNA−protein complexes. This technology has significantly facilitated studies of macromolecular functions and mechanisms at the atomic level.[1] Besides the difficulties related to crystallization, however, heavy atom derivatization for phase determination, a limiting factor in nucleic acid X-ray crystallography, has slowed determination of new structures. To facilitate phase and structure determination, the multiwavelength anomalous dispersion (MAD) technique has been developed for proteins via replacement of sulfur with selenium in methionine residues,[2] which approximately accounts for two-thirds of all new protein structures.[3] Derivatization with bromine on position 5 of pyrimidines and the RNA-binding protein U1A complex have also been used in MAD phasing of DNAs and RNAs.[1c,d,4] In comparison to those for protein, however, derivatization tools in X-ray crystallography for nucleic acids are quite limited.

We have recently demonstrated the successful strategy of oxygen replacement with selenium in nucleic acids for X-ray crystal structure determination using MAD[5,6] on the basis that oxygen and selenium are in the same family, VIA, in the periodic table. In the work by Huang, Egli, and co-workers,[5−8] selenium has been introduced at the 5′ and 2′ positions and phosphodiester backbones for MAD phasing. In the case of selenium substitution on the phosphodiester backbone, selenium can be directly introduced via selenization of hydrogen phosphonate[9] or phosphite[8] although a separation is needed to isolate the formed phosphoroselenoate (PSe) diastereomers for X-ray crystallography. This chemical approach is well suited with short oligonucleotides containing one PSe group.[8] For the preparation of longer nucleic acids, especially those containing multiple PSe groups, new approaches, such as enzymatic methods, would be ideal for obtaining X-ray crystallography-quality samples.

To develop the enzymatic approach, we have synthesized the nucleoside triphosphate analogues containing α-nonbridging selenium, such as α-Se-TTP (**1**, Figure 1A), analogous to the synthesis of the nucleoside 5′-(α-P-thio)triphosphates.[10] The formed Sp and Rp diastereomers of **1** were easily separated by HPLC (Figure 2). As the polymerases conduct the polymerization reaction stereoselectively,[11,12] this enzymatic approach will generate diastereomerically pure PSe-nucleic acids, thus rendering unnecessary separation of PSe diastereomers. This is an advantage of the enzymatic approach over the chemical synthesis.[8,9] We describe here the first study of the enzymatic synthesis of PSe DNAs using DNA polymerization.

To develop the polymerase-catalyzed DNA polymerization using the selenotriphosphate (**1**), we designed a DNA primer (21 nt) and a DNA template (55 nt) that allows incorporation of three "T"s complementary to the underlined "A"s (Figure 3A). Two of the Ts are close to each other in the extended 5′ region. The other T, four bases away from the 3′ terminus, was placed to probe the initial binding and digestion of exonuclease III.[13]

The results of a time-course experiment show that the full-length DNAs in quantitative yield were obtained from all primer extension

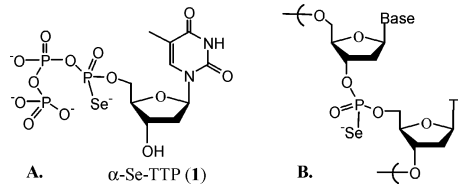

Figure 1. (A) Structure of thymidine 5′-(α-P-seleno)triphosphate. (B) Structure of phosphoroselenoate (PSe) DNA.

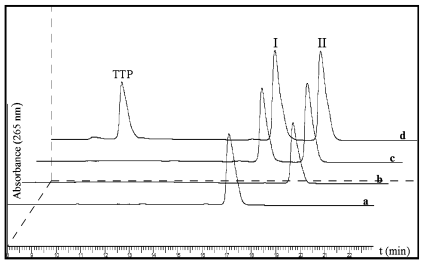

Figure 2. HPLC analysis of purified α-Se-TTP diastereomers on C18 column (4.6 mm × 25 cm). Samples were eluted (2 mL/min) with a linear gradient from buffer A [10 mM triethylammonium acetate (TEAA), pH 6.5] to 20% buffer B (30% acetonitrile in water, 10 mM TEAAc, pH 6.5) in 20 min. **a**: the fast-moving isomer (I); **b**: the slow-moving isomer (II); **c**: co-injection of isolated I and II; **d**: co-injection of I, II, and TTP. The retention times for TTP, peak I, and peak II were 10.91, 17.18, and 19.05 min, respectively.

reactions, including those with both diastereomers of **1** (Figure 3B). No full-length DNA was observed in the control experiment where TTP was absent (Figure 3C). The 55-nt DNA products made from the polymerization using diastereomer Se-TTP I and II, and TTP refer to DNA I, II, and III, respectively. Unlike the cases of the nucleoside thiotriphosphates,[11,12] where the Sp diastereomer was effectively recognized by T4 DNA polymerase while the Rp diastereomer was poorly recognized, we observed that both α-Se-TTP diastereomers were equally well recognized by the Klenow fragment of DNA polymerase I and that the recognition was almost as good as that for TTP (Figure 3B). The polymerase recognition of both Se diastereomers allowed the individual synthesis of two PSe−DNA isomers. The precise stereochemistry (Sp or Rp) of Se-TTP I and II is currently under investigation. As HPLC and MS analyses showed that the Se-TTP could be oxidized to TTP by air (data not shown) to confirm that the formations of DNA I and II were not due to oxidation but due to the incorporations of both diastereomers, we decided to design and conduct exonuclease digestion experiments.

Exonuclease III removes mononucleotides of duplex DNAs in a 3′-to-5′ direction processively,[14] and it has also been reported that modification of the nucleotide backbone can prevent this enzyme digestion.[12,15] Our time-course experiments showed that both DNA I and II, synthesized quantitatively and individually from the Sp and Rp diastereomers, were resistant to the exonuclease digestion (Figure 3C). Interestingly, the digestion resistance patterns of these two DNAs were different. DNA I showed stronger resistance at

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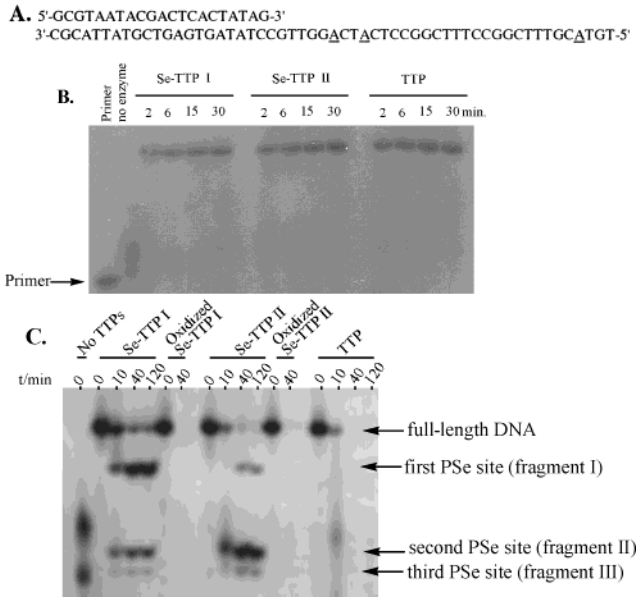

Figure 3. (A) Primer and template sequences. (B) Time-course primer extension using α-Se-TTP I and II (Sp and Rp) on 19% polyacrylamide gel. Primer extensions were performed with 5′-^{32}P-primer/template (5 μM), Klenow (0.02 units/μL), and dNTPs (0.4 mM each). (C) Exonuclease III digestion after primer extension (30 min.). The digestions were performed with *Escherichia coli* exonuclease III (0.6 units/μL).

the first PSe site than DNA II. This stronger resistance of DNA I suggests that its first PSe stereocenter, which is three phosphate groups away from its 3′ terminus, may interfere with the enzyme binding and digestion.[13] On the other hand, DNA II did not show much resistance to the enzyme at the same PSe position, and it only showed strong resistance at the second PSe position, which is 24 phosphate groups away from its 3′-terminal end. The different resistances between the first and second PSe sites of DNA II suggest that the first several nucleotides may play a role in initial enzyme binding and digestion and also suggest that the enzymatic digestion mechanism at the initial phase may be quite different from that at the stationary phase. In addition, we observed in digestion of both DNA I and II that the ratio of fragment I and II (or III) after 40 min (or 120 min) was larger than that after 10 min, which suggests that the enzyme molecule is probably more sensitive to the modification in its later-stage turnover. The different resistances to digestion of DNA I and II are expected from these PSe centers due to their different stereochemistries (Sp or Rp).

To further confirm that the exonuclease resistance was only due to the PSe functionality, we conducted the DNA polymerization and exonuclease digestion after oxidizing both diastereomers to natural TTP by bubbling air overnight. As expected, the DNAs made from oxidized α-Se-TTP I and II completely lost the nuclease resistance (Figure 3C). DNA III, made from natural TTP as a control, did not show any resistance to exonuclease digestion.

In conclusion, we have successfully demonstrated here the enzymatic synthesis of two PSe DNA isomers by using the two α-Se-TTP diastereomers (Sp and Rp). The experimental results indicate that Klenow equally recognizes the two individual diastereomers at the same level as natural TTP, suggesting its broader substrate specificity. As the polymerase reaction is stereospecific, with an inversion of configuration at the phosphorus center,[11,12] the PSe DNA made from each diastereomer ought to be diastereomerically pure. The precise stereochemistry of the PSe centers in the DNAs will be subject to further investigation via X-ray crystallographic studies. The incorporations of the PSe groups at the expected sites have been confirmed by the digestion resistance to exonuclease III. Unlike chemical synthesis, which is limited to short DNAs and where the separation of the PSe DNA diastereomers is necessary, this enzymatic method can be used to prepare longer DNAs without diastereomer separation. The PSe functionality is relatively stable in air,[9] and selenium of the PSe functionality has been used to determine crystal structures of oligonucleotides.[8] In addition, large-scale preparation (such as 6 mg for dsDNA with 100 bp) can be conveniently achieved using a larger volume (5 mL) and higher primer/template concentration (20 μM). Therefore, this quantitative enzymatic approach is particular valuable for synthesis of longer DNAs with multiple PSe groups in large scale for X-ray crystal structure determination of DNAs and DNA−protein complexes by the MAD phasing technique. The selenium Sp and Rp diastereomers of the nucleotides can also be used to study the structure and biochemistry of nucleotide-binding enzymes, including polymerases, nucleases (e.g. DNA damage repair enzymes),[13] phosphatases, and kinases, which are often involved in regulation and signal transduction.[16]

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Note Added after ASAP: The version published on the Web 12/18/2003 was missing Figure 3a. The version published 12/22/2003 is correct.

Supporting Information Available: The synthesis and HR-MS of α-Se-TTP diastereomers (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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