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Supplementary Material

Novel Complex MAD Phasing and RNase H Structural Insights by Selenium Oligonucleotides

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MATERIALS AND METHODS

Equipment and reagents:

All chemistry reagents or buffers were purchased from commercial manufacturers or made in the laboratory using standard protocols unless otherwise stated. Commercially available starting reagents were used without further purification. All cell cultures and protein expression reagents were purchased from Invitrogen. Protein purifications were carried out by FPLC, and the protein purification columns were purchased from Amersham (GE healthcare life sciences). Expressed proteins were monitored at 259 nm and collected by FPLC purification.

Oligonucleotide synthesis:

Native or modified DNA or RNA oligonucleotides were synthesized in the laboratory or purchased from commercial manufacturers. Native DNA template (DNA-N), selenium-modified DNA template (DNA-Se; Ref. 1), and sulfur-modified DNA template (DNA-S) were synthesized in the laboratory according to the methods as described (Ref. 1). DNA-Se1: 5'-AT-^{Se}G-TCGp-3'; DNA-Se2: 5'-AT-^{Se}G-TC-^{Se}Gp-3'; DNA-S1: 5'-AT-^SG-TCGp-3'; DNA-S2: 5'-AT-^SG-TC-^SGp-3'. The native RNA substrate (5'-UCGACA-3') and sulfur-modified RNA substrate (RNA-S: 5'-UCGA-S-CA-3', Sp and Rp) were purchased from Integrated DNA Technology (IDT, USA). The native DNA (5'-TGTCGTGTCG-3'), sulfur-DNA (5'-TGTCGT-^SG-

TCG-3'), and selenium-DNA (5'-TGTCGT-^{Se}G-TCG-3') for T_m study were synthesized in the laboratory. 6-Se-2'-deoxyguanosine (^{Se}G) was synthesized in the laboratory and 6-S-2'-deoxyguanosine (^SG) was purchased from Glen Research.

Polynucleotide kinase reaction:

A reaction solution (10 μ L), containing a mixture of RNA substrate (1 μ L, 1 μ M), 1 μ L 10X PNK buffer (70 mM Tris-HCl, 10 mM MgCl₂, 5 mM DTT, pH 7.6), γ -³²P-ATP (1 μ L, 3,000 Ci/mmol, 5 mCi/ml), T4 polynucleotide kinase (1 unit, 1 μ L), and water (6 μ L), was incubated in a water bath for 1 hr at 37 °C (Ref. 2-3). The reaction was heated at 68 °C for 10 min to inactivate the enzyme. Ethanol precipitation was then performed to recover the ³²P-labeled RNA by adding 3 M NaCl (1.1 μ L, final concentration 0.3 M) and 100% ethyl alcohol (33.3 μ L), followed by cooling at -80 °C for 15 min and centrifugation (14,000 rpm). Supernatant was discarded and pellet was washed 3 times with 70% cold ethanol. After air drying the pellet, H₂O (10 μ L) was added to dissolve the ³²P-labeled RNA.

(A) Wild-type RNase H (WT: 1-196):

MAKSKYYVWNGRKRKPGIYTSWSACEAQVKG YTGAKFKSYPSKEEAEEAAFRG
EEATPKLAKEEIIWESLSVDVGSQGNPGIVEYKGVDTKTGEVLFEREPIPIGTNN
MGEFLAIVHGLR YLKERN SRKPIYS **D**SQTAIKWVKDKKAKSTLVRNEETALIW
KLVDEAE EWLNTHTYETPILKWQTDKWGEIKADYGRK

(B) Truncated RNase H (TR RNase H: 59-196):

AKEEIIWESLSVDVGSQGNPGIVEYKGVDTKTGEVLFEREPIPIGTNNMGEFLAI
VHGLR YLKERN SRKPIYS **D**SQTAIKWVKDKKAKSTLVRNEETALIWKLVDEAE
EWLNTHTYETPILKWQTDKWGEIKADYGRK

(C) Truncated RNase H inactive mutant (D132N: 59-196):

AKEEIIWESLSVDVGSQGNPGIVEYKGVDTKTGEVLFEREPIPIGTNNMGEFLAI
VHGLR YLKERN SRKPIYS **N**SQTAIKWVKDKKAKSTLVRNEETALIWKLVDEAE
EWLNTHTYETPILKWQTDKWGEIKADYGRK

Figure S1. Wild-type and mutant RNase H proteins (*Bacillus halodurans*). *Bacillus halodurans* RNase H (*rhnA*) was used for the study. **A:** The wild-type enzyme is a single polypeptide (1-196 amino acids) and contains two domains: a RNA-DNA hybrid binding domain (1-58 aa, in black) and a catalytic domain (59-196 aa, in blue). **B:** The truncated RNase H (59-196 aa) was generated genetically and retains the RNA hydrolytic activity on RNA/DNA duplex (Ref. 4-5). **C:** Truncated RNase H inactive mutant was created, for crystal structure study, by mutating the aspartate residue (D132) of the truncated RNase H to asparagine (D132N). This mutation abolishes the metal ion coordination (B site) capability of the enzyme and makes the enzyme inactive catalytically.

Expression of RNase H protein:

RNase H wild-type (WT, Figure S1), truncated (TR), and truncated mutant (D132N) constructs (pET15, pET 42, and pET13, respectively) were kindly given by Dr. Wei Yang lab at NIH as a gift. Protein expressions were carried out in BL21 (DE3; pLys E. coli; purchased from Invitrogen). Transformation was accomplished by heat shock method. One picomol of plasmid carrying the gene of interest was added in a vial (50 μ L) of competent cells and swirled gently. The vial was placed on ice for 30 min and heat shock was then performed by placing the vial in a water bath (42 °C) for 40 sec. Placed it again on ice for 5 min. SOC medium (150 μ L, purchased from Invitrogen) was added in a vial and the vial was shaken for 1 hr at 37 °C (220 rpm). The bacterial suspension (50 μ L) was spread on a LB-agar plate [containing ampicillin (100 mg/L) and chloramphenicol (35 mg/L)] and the plate was incubated at 37 °C overnight. A

single colony was picked up and added to LB-ampicillin-chloramphenicol broth (300 mL). The culture was shaken (220 rpm) overnight at 37 °C. Two liters LB-ampicillin-chloramphenicol broth was prepared and the overnight culture (30 mL) was added to inoculate it. The inoculated broth was incubated for 2-3 hrs by shaking (220 rpm) at 37 °C. When the OD₆₀₀ reached at 0.6 OD, protein expression was then induced by adding IPTG (20 mL, 100 mM; final concentration 1 mM) and the culture was allowed to grow for 4 hrs more. Cells were harvested by centrifugation, washed twice with buffer A (75 mM NaCl, 40 mM NaH₂PO₄, pH 7.0, 0.1 mM EDTA, 1 mM DTT, and 5% glycerol), and the pellet was suspended in the same buffer. The cells were then lysed by sonication (12 x 10 sec duration) with a Branson digital sonifier (Fisher Scientific; microtip, 45% amplitude) with intervening cooling time. The cell lysate was centrifuged at 16,000 rpm for 30 min and the supernatant was collected for FPLC purification.

Purification of RNase H by FPLC:

Before the supernatant loading, the Ni-affinity column on FPLC was washed with 10 column volumes (CV) of buffer B (300 mM NaCl, 40 mM NaH₂PO₄, pH 7.0, 1 mM DTT, 5% glycerol, and 500 mM imidazole) to remove impurities and the column was equilibrated with 10 CV of buffer A. After loading the supernatant, the column was eluted with buffer A for 10 min and then with 15% buffer B for 10 min with a flow rate of 1.5 mL/min to remove impurities and unbound proteins. Subsequently, the protein was eluted with buffer B (15-100%) with a flow rate 1 mL/min over 100 mL, the RNase H elution peak was observed at 40-45% buffer B, and the protein was collected. The buffer of the protein was exchanged with buffer C (40 mM NaH₂PO₄, pH 7.0, 150 mM NaCl, 5% glycerol, 2 mM DTT, and 0.5 mM EDTA) by dialysis in 3-kDa cut-off membranes at 4 °C overnight. His-tag of the protein was removed by thrombin digestion, which was performed by addition of 10 units of thrombin for every 40 mg of protein (determined by UV at 280 nm) and incubation at room temperature for 1 hr. Digested protein solution was mixed with an equal volume of buffer D (75 mM NaCl, 40 mM NaH₂PO₄, pH 7, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, and 4 M ammonium sulfate). The tag-free protein was further purified by Phenyl Sepharose column (hydrophobic column). The Phenyl Sepharose column was prepared by washing with buffer F (75 mM NaCl, 40 mM NaH₂PO₄, pH 7.0, 0.1 mM EDTA, 1 mM DTT, and 5% glycerol) and then equilibrated with buffer E (75 mM NaCl, 40 mM NaH₂PO₄, pH 7.0, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, and 2 M ammonium sulfate) before loading the sample. The tag-free protein was loaded on the column with a flow rate of 0.8 mL/min and washed with 10 CV of buffer E to remove short peptides and impurities. Protein was further eluted by 0-100% buffer F with the same flow rate over 100 mL, and the RNase H elution peak was observed between 45-50% buffer F. Eluted protein was exchanged with buffer G (75 mM NaCl, 20 mM HEPES, pH 7.0, 5% glycerol, 0.5 mM EDTA, and 2 mM DTT) by dialysis in 3-kDa cut-off membranes at 4 °C overnight and stored at -20 °C. Protein concentration was determined by UV analysis at 280 nm using a calculated ϵ_{280} value (58900 M⁻¹ cm⁻¹) for the wild-type and a ϵ_{280} value (40450 M⁻¹ cm⁻¹) for the truncated RNase H (Ref. 6).

Hydrolysis of RNase H substrates:

Each RNase H hydrolysis reaction (volume 5 μ L) contains DNA template (150 nM final concentration; DNA-N, DNA-S, or DNA-Se) and ³²P-labeled RNA substrate (native or S-modified RNAs; mixture of cold and hot RNAs; 150 nM final concentration). To each hydrolysis reaction, WT or TR RNase H enzyme (10 nM, final) and the reaction buffer (final conditions: 75 mM KCl, 50 mM Tris-HCl, pH 7.8, 3 mM MgCl₂, and 1 mM diborane) were added. The reactions were incubated for 30 min at 37 °C, unless otherwise mentioned. After incubation, the reactions were quenched by immediately adding gel-loading dye (contained 7 M urea and 1 mM EDTA) and placing them on dry ice. The reactions were analyzed by 21% w/v urea-polyacrylamide (19:1 acrylamide:bisacrylamide) gel electrophoresis. The gels were run for 1 h at 500 volts, and the gels were fixed by fixing buffer (10% acetic acid in methanol) and dried. The gel images were taken by exposing on X-ray films. The bands were also quantified by phosphorimager and

image-quantifying software. For the time-course analysis, a reaction solution (20 μ L) was prepared for each DNA template. All experimental conditions were as same as the hydrolysis reaction described above. Before adding the enzyme, aliquot was transferred and referred to as 'zero' min. The same amount (3 μ L) of aliquots was removed at 0.25, 0.5, 1, 3, 5, 7, and 10 min time point and used for gel analysis.

Hydrolysis of RNase H substrates in the presence of Mg^{2+} or Mn^{2+}

The RNA hydrolysis and the rescue reactions used the similar conditions above. The divalent cation (Mg^{2+}) was used or replaced with Mn^{2+} in the buffer. Native or Sp- or Rp-sulfur-modified RNAs were used as the substrates. The final reaction buffer conditions (Ref. 9) are 75 mM KCl, 50 mM Tris-HCl, pH 7.8, 3 mM $MnCl_2$ (or $MgCl_2$) and 1 mM diborane. The reaction time is either 5 min (Figure 5A-C) or indicated (Figure 5D).

Crystallization of DNA/RNA/RNase H ternary complexes:

Protocol-1: The DNA portion of the DNA/RNA hybrid (5'-ATGTCG-3'/5'-UCGACA-3'; one-base overhang at both ends) was derivatized. Prior to co-crystallization with RNase H, the purified Se-DNA (5'-AT-^{Se}G-TC-^{Se}G-3') and RNA (5'-UCGACA-3') were annealed at 1:1 molar ratio by first heating the mixture to 90°C for 1 min, and then allowing it to cool slowly down to 25°C. The resulting Se-DNA/RNA duplex was mixed with the protein (final concentration: 8 mg/mL) at 1:1 molar ratio in the presence of 5 mM $MgCl_2$. Co-crystallization of Se-DNA/RNA hybrid with RNase H was achieved by screening with the QIAGEN Classics Suite Kit (www.qiagen.com). By using the sitting-drop vapor diffusion method at 25°C, the crystals were readily obtained from the mixture #96 of the crystallization screen [Buffer: 0.1 M MES, pH 6.5; precipitant: 12% (w/v), PEG 20000].

Protocol-2: The DNA portion of the DNA/RNA hybrid possesses a phosphate group at its 3'-end. In the duplex, the 5'-ends of both DNA and RNA remains one-base overhang (5'-ATGTCG-3'/5'-UC-^{Se}G-ACA-3'). Prior to co-crystallization with RNase H, the purified DNA (5'-ATGTCG-3') and RNA (5'-UC-^{Se}G-ACA-3') were annealed at 1:1 molar ratio. The duplex mixture was heated to 90°C for 1 min and allowed to cool down slowly to 25°C. The resulting DNA/RNA duplex was mixed with the protein (final concentration in the complex: 8 mg/mL) at 1:1.5 ratio (protein:DNA-p-3'/RNA duplex) in the presence of 5 mM $MgCl_2$. Co-crystallization of DNA-p-3'/RNA hybrid with RNase H was achieved by screening with Index screening Kit (HR2-144 Reagent Formulation, Hampton research, USA). By using the sitting-drop vapor diffusion method at 22°C, the diffractable crystals were obtained in 0.04 M Magnesium chloride, 0.05 M Sodium cacodylate, pH 6.0, 5% v/v 2-Methyl-2,4-pentanediol.

MAD Data Collection and Phasing

Crystal diffraction data of the Se-DNA/RNA/RNase H complex were collected at beamline X25 and X29 in the National Synchrotron Light Source (NSLS) of Brookhaven National Laboratory. A number of crystals were scanned to find the one with strong anomalous scattering at the K-edge absorption of selenium. 25% glycerol was used as cryoprotectant while X-ray data were collected under the liquid nitrogen stream at 99 °K. The selected wavelengths for selenium MAD data are listed in Table S1. Each crystal was exposed for 15 seconds per image with one degree rotation, and a total of 180 images were taken for each data set. Two crystals were used to collect the MAD/SAD data sets. The figure of merit of the individual SAD phasing data was relatively low, which could not produce a good electron density map for the model. We used one SAD data set as a reference for the MAD phasing of the other diffraction data set. The overall figures of merit (FOM) of the initial phases were 0.630, which produced an interpretable electron density map. All data (Table S1) were processed using HKL2000 and DENZO/SCALEPACK (Ref. 7 and 8). The structure was solved by MAD method using program Solve/Resolve. The resulted model was refined using Refmac5 within CCP4i. The DNA/RNA duplex was modeled into the structure using Coot. Metal ions and water molecules were added either automatically or manually using Coot. The

comparisons between the native and Se-modified structures are presented in Figure 2. The unique cleavage site of the RNA/DNA substrate is supported by the structure study.

Table S1. The table of B-factor comparison between the crystal structures of RNase H complexed with the native and Se-modified DNA/RNA duplexes.

| PDB ID | 3TWH | 2G8U | 1ZBI |
|-------------------------------------|-----------|-----------|-----------|
| Resolution (Å) | 1.80 | 2.70 | 1.85 |
| B-factor (Å) ² | | | |
| Overall | 24.0 | 49.0 | 29.4 |
| Protein | 22.3 | 50.8 | 29.4 |
| RNA/DNA duplex | 27.2 | 42.4 | different |
| RNA | 25.2 | 44.4 | RNA and |
| DNA | 29.1 | 40.3 | DNA |
| rC5/d ^{Se} G3 (or rC5/dG3) | 19.2/32.3 | 40.8/32.7 | sequences |
| (base pair at the cleaving site) | | | |
| Mg ²⁺ -A | 23.8 | 26.9 | 26.64 |
| Mg ²⁺ -B | 32.3 | 35.0 | 29.73 |

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