Structure of Hepatitis C Virus Polymerase in Complex with Primer-Template RNA

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The replication of the hepatitis C viral (HCV) genome is accomplished by the NS5B RNA-dependent RNA polymerase (RdRp), for which mechanistic understanding and structure-guided drug design efforts have been hampered by its propensity to crystallize in a closed, polymerization-incompetent state. The removal of an autoinhibitory β-hairpin loop from genotype 2a HCV NS5B increases de novo RNA synthesis by >100-fold, promotes RNA binding, and facilitated the determination of the first crystallographic structures of HCV polymerase in complex with RNA primer-template pairs. These crystal structures demonstrate the structural realignment required for primer-template recognition and elongation, provide new insights into HCV RNA synthesis at the molecular level, and may prove useful in the structure-based design of novel antiviral compounds. Additionally, our approach for obtaining the RNA primer-template-bound structure of HCV polymerase may be generally applicable to solving RNA-bound complexes for other viral RdRps that contain similar regulatory β-hairpin loops, including bovine viral diarrhea virus, dengue virus, and West Nile virus.

A n estimated 180 million persons worldwide are infected with hepatitis C virus (HCV). Approximately 80% of these will develop chronic liver disease, and a significant subset will progress to cirrhosis of the liver and eventually death (20). HCV is a small, single-stranded, positive-sense RNA virus and, like dengue virus, bovine viral diarrhea virus, and West Nile virus, is a member of the Flaviviridae family of viruses. The nonstructural 5B (NS5B) protein, a 66-kDa protein of 591 amino acids found at the C terminus of the virally encoded HCV polyprotein, provides the requisite RNA-dependent RNA polymerase (RdRp) functionality (32). The polymerase produces positive RNA strands for encapsidation into viral particles by using an intermediate negative RNA strand, which it synthesizes from the initial positive-strand RNA template provided by the virus. GTP-dependent de novo initiation is likely the preferred mode of nucleotide polymerization in vivo (24). The essential nature of this enzyme to HCV replication makes it an important target for direct-acting antivirals (DAAs), including nucleoside-based (NI) and nonnucleoside allosteric (NNI) inhibitors (34,39).

Nearly 100 crystal structures of HCV NS5B have been reported, covering genotypes 1a, 1b, 2a, and 2b, although all structures lack the C-terminal membrane-anchoring tail (5). HCV NS5B exhibits the so-called right-hand shape common to many polymerases, along with readily recognized finger, palm, and thumb domains (1, 3, 21), that appears in a closed-fist conformation without sufficient space for the RNA primer-template (Fig. 1A). However, it has been suggested that the closed conformation is almost suitable for de novo initiation, with enough room to bind a single strand of template RNA and priming nucleotides (37). By analogy to a bacteriophage θ-β polymerase initiation complex with GTP and template (4), Tyr448 of a β-hairpin loop spanning residues 442 to 454 of the thumb domain may stack against the initiating GTP during de novo initiation. Intriguingly, other RdRps of the Flaviviridae family, such as bovine viral diarrhea virus (7), dengue virus (43), and West Nile virus (26), contain a similar thumb domain β-hairpin loop descending into the palm domain with an appropriately situated aromatic residue analogous to Tyr448 in HCV NS5B.

Extensive efforts to obtain a high-resolution crystal structure of wild-type HCV polymerase in complex with growing RNA primer-template pairs have proven unsuccessful, although a structure has been reported with a polyuridine template in an unproductive conformation (31). The superposition of NS5B and HIV-1 reverse transcriptase (RT) crystal structures (17) provided the earliest models for HCV elongation (1, 3, 21). However, the autoinhibitory β-hairpin loop and a C-terminal linker blocks the egress necessary for elongation, and as was observed with HIV-1 RT, the thumb domain has been predicted to move in the presence of RNA (1, 3). Insights from more recent RNA-bound complexes of RdRps from Norwalk virus (44), poliovirus (15), and foot-and-mouth disease virus (FMDV) (12), which lack an equivalent β-hairpin loop, prompted us to evaluate different HCV NS5B constructs in which this loop had been modified with the goal of obtaining a high-resolution crystal structure of the mutant HCV polymerase complexed with an RNA primer-template pair.

In the work presented here, we show that the modified HCV NS5B polymerase has a significant fold increase in de novo RNA synthesis, remains susceptible to chain termination by known nucleotide inhibitors, and exhibits inhibitory patterns similar to those of the wild-type enzyme upon the introduction of the resistance-derived mutation, S282T. Additionally, we describe three crystal structures, including two different RNA primer and template strands complexed with the modified HCV poly-

Received 14 February 2012 Accepted 2 April 2012 Published ahead of print 11 April 2012

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doi:10.1128/JVI.00386-12
merase. These provide the first structure-based insights into how the enzyme interacts directly with RNA and, by extension, the nucleos(t)ide-derived medicines which terminate the growing RNA strand. This new information may make it possible to design even better nucleos(t)ide and nonnucleoside medicines than those being developed currently. Our approach to solving these complexes may extend to other structurally similar viral polymerases.

FIG 1 Structure of HCV NS5B polymerase and activity of an internal deletion mutant. (A) Crystal structure of genotype 2a HCV NS5B RdRp (37) with the finger, palm, thumb, and C-terminal linker domains numbered and colored according to convention (21). The palm domain is the most well conserved domain across all of the known polymerases and contains the catalytic residues. The thumb domain has the most variability among polymerases and is significantly larger in HCV and other Flaviviridae RdRps. This region contains a relatively unique β-hairpin loop which descends toward the palm domain, partially blocking what is undoubtedly the exit path for the RNA product strand. This β-hairpin loop, colored in yellow, was deleted in the current study. dsRNA, double-stranded RNA. (B) De novo RNA synthesis activity of a genotype 2a JFH1 isolate, wild-type HCV NS5B (2a WT), and a construct in which the β-hairpin loop has been deleted and replaced with a Gly-Gly linker (2a Δβ), demonstrating >100-fold higher total activity for 2a Δβ than for the 2a WT. The time-dependent formation of the radiolabeled products is shown in the blot. At the right, the activity for both 2a WT and 2a Δβ were measured in the presence of the nucleotide triphosphate analog inhibitor PSI-352666, which resulted in an IC50 of 6.05 ± 0.82 μM for 2a WT and 6.41 ± 0.75 μM for 2a Δβ.

FIG 2 Chain termination of HCV NS5B 1b Δβ. (A) RNA synthesis and chain termination by PSI-352666 for the HCV NS5B polymerase 1b Δβ construct. GG primer (0.5 μM) was preincubated with 5 μM RNA template (3′-CCGGUGUAAUAUAUGUA-5′), 5 μM 1b Δβ, and 50 μM CTP for 15 min to form *GGC. One hundred μM GTP or PSI-352666 alone or in combination with 100 μM ATP then was added. The PSI-352666-terminated product (*GGCX) was not further elongated in the presence of the next correct incoming nucleotide (ATP). Lanes indicate 0-, 2-, 5-, 10-, 20-, 40-, and 60-min time courses after preincubation. (B) Chain termination of HCV polymerase 1b WT, 1b S282T, 1b Δβ, and 1b Δβ S282T with PSI-352666 or 2′-C-MeGTP. Assays were performed as described in Materials and Methods. The calculated IC50s show that the 1b Δβ construct replicates the previously observed resistance at position S282T relative to the wild-type 1b polymerase.
TABLE 1 Crystallographic statistics for HCV NS5B 2a JFH1 Δ8 apo and RNA-bound structures

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*MATERIALS AND METHODS*

**Protein expression and purification.** A construct of the wild-type HCV polymerase genotype 2a JFH-1 isolate was designed with the C-terminal 21 amino acids removed and replaced with a noncleavable hexahistidine tag, and it was cloned into a pET-28a-derived vector. Two surface solubilization mutations, E86Q and E87Q, were introduced via site-directed mutagenesis (designated 2a WT). The construct (designated 2a Δ8) in which the β-hairpin residues 444 to 453 were removed and replaced with a Gly-Gly linker (see Fig. 3A) was designed using GeneComposer (22, 35) and engineered using site-directed mutagenesis. Both mutated constructs were transformed into Rosetta (DE3) Escherichia coli cells (Novagen). Recombinant protein was expressed in the Overnight Expression Autoinduction system (Novagen) at 22°C overnight. Cells were harvested by centrifugation at 5,000 × g for 20 min, and the cell pellet was resuspended in 20 mM Tris, pH 8.0, 500 mM NaCl, 20% glycerol, 2 mM Tris(2-carboxyethyl)phosphine (TCEP), 10 mM imidazole. The lyate was stirred in the presence of benzonase and egg white lysozyme for 1 h at 4°C, followed by clarification by centrifugation at 18,000 rpm for 40 min. The protein was purified by nickel immobilized affinity chromatography, resulting in ~90% pure protein as determined by Bio-Rad Experion capillary gel electrophoresis and SDS-PAGE analysis. The protein was concentrated to 3.9 to 4.4 mg/ml (~60 µl) in 20 mM Tris, pH 8.0, 500 mM NaCl, 10% glycerol, 2 mM TCEP, and ~200 mM imidazole.

**HCV activity assay.** Activity assays were performed in a 200-µl mixture containing 1 µM the four natural ribonucleotides, [α-32P]UTP, 20 ng/µl of genotype 1b (−) 341-nucleotide internal ribosomal entry site (IRES) RNA template (36), 1 U/µl of Superase In (Ambion, Austin, TX), 40 ng/µl of NSSB, 5 mM MgCl₂, and 2 mM dithiothreitol (DTT) in 50 mM HEPES buffer (pH 7.5). Activity assays with genotype 1b NSSB-derived proteins were performed in a 120-µl mixture containing 5 µM the four natural ribonucleotides. The reaction mixture was incubated at 27°C, and 20-µl aliquots were taken at the desired time points and quenched by mixing in 80 µl of stop solution (12.5 mM EDTA, 2.25 M NaCl, and 225 mM sodium citrate). Inhibition assays were performed in a 20-µl mixture containing various concentrations of PSI-352666 or 2'M-(MeGTP), 5 µM the four natural ribonucleotides, [α-32P]UTP, 20 ng/µl of genotype 1b (−) 341-nucleotide IRES RNA template, 1 U/µl of Superase In (Ambion, Austin, TX), 40 ng/µl of NSSB, 5 mM MgCl₂, and 2 mM DTT in 50 mM HEPES buffer (pH 7.5). The reaction mixture was incubated at 27°C and quenched by adding 80 µl of stop solution after 60 min of incubation with 2a WT or 15 min of incubation with 2a Δ8. The radioactive RNA products were separated from unreacted substrates using a Hybond N+ membrane (GE Healthcare) as described previously (29). The products were visualized and quantified using a phosphorimager. The reaction rates and the 50% inhibitory concentrations (IC₅₀) were calculated using GraphFit (Erichars Software, Horley, Surrey, United Kingdom).

**Crystallization and structure determination.** Crystals were grown using the sitting-drop vapor diffusion method in 96-well-format Compact Junior crystallization plates (from Emerald BioSystems) using 0.4 µl of protein solution and an equal volume of precipitant equilibrated against 80 µl of precipitant at 16°C. Rod-shaped crystals (20 by 20 by 120 µm³) appeared within 3 to 5 days in several conditions from the JCSG+ (Emerald BioSystems) and the Index (Hampton Research) sparse matrix diffraction screens.
screens. The apo NS5B 2aΔ8 structure was obtained from a crystal grown in the presence of 30% pentaerithritol ethoxylate, 0.1 M bis-Tris, pH 6.5, and 50 mM ammonium sulfate (Index E9). Apo NS5B 2aΔ8 crystals grown in the presence of 25% polyethylene glycol 3350, 0.1 M bis-Tris, pH 5.5 to 6.5, and 0.2 M ammonium acetate (Index G6-G7) were soaked overnight at 16°C with 0.2 mM 5'-UACCG 3'-deoxyguanosine (3’dG) or 5'-CAUGGCDideoxyctosine (ddC) (Dharmacon), precipitant, and 15% ethylene glycol as a cryoprotectant. NS5B 2a Δ8 crystals grown or soaked in the presence of morpholineethanesulfonic acid (MES) buffer were incompatible with RNA binding. Crystals were harvested and flash-frozen in liquid nitrogen for cryocrystallography. The apo data set was collected at the Advanced Light Source (ALS 5.0.3), and the RNA-bound data sets were collected at the Advanced Photon Source (APS LS-CAT 21-ID-G). The data were reduced in XDS/XSCALE (18). The structures were solved by molecular replacement in PHASER (28) using a previously determined apo structure of a HCV NS5B 2a triple mutant (unpublished), which in turn was solved by molecular replacement using the wild-type HCV NS5B 2a structure (Protein Data Bank [PDB] code 2XXD). The final models were produced after numerous iterative rounds of refinement in REFMAC5 (30) and manual model building in COOT (11). Structures were assessed for correctness and validated using Molprobity (6). Figures were generated with PyMol (9).

**Protein structure accession numbers.** Atomic coordinates and structure factors have been deposited in the Protein Data Bank under accession codes 4E76 [2aΔ8 apo], 4E78 [2aΔ8 with RNA1, 5'-UACCG(3’-dG)], and 4E7A [2aΔ8 with RNA2, 5'-CAUGG(ddC)].

**RESULTS AND DISCUSSION**

Based on the in silico superposition of the more recent RNA-bound complexes of RdRps from Norwalk virus (44), poliovirus (15), and foot-and-mouth disease virus (FMDV) (12), which lack an equivalent β-hairpin loop, we synthesized an initial HCV NS5B 1b BK construct in which residues 442 to 456 had been excised. While this 1b Δ14 polymerase had reduced activity (data not shown), it provided the foundation for our interest in a report that the replacement of residues 444 to 453 (Δ8) in this β-hairpin loop with a Tyr-Gly linker in HCV NS5B genotype 1b (designated 1b Δ8) resulted in a 17-fold increase in primer extension activity above that of the wild type, and the ability to bind primer-template RNA (16, 25). We observed a >10-fold increase in de novo RNA synthesis for 1b Δ8 with a Gly-Gly linker (data not shown), as well as evidence for RNA binding via thermofluor analysis (8, 27, and data not shown). We found that this construct was susceptible to chain termination (Fig. 2), and similar IC_{50}s were obtained for the GTP analogues PSI-352666 (4 μM) and 2’-C-MeGTP (7 to 13 μM) for both wild-type 1b (1b WT) and 1b Δ8 (Fig. 2). Furthermore, upon the introduction of the most commonly identified resistance mutation, S282T, similar inhibitory patterns for either the 1b WT or the 1b Δ8 construct were noted for these same compounds (IC_{50} for PSI-352666, 9 to 14 μM, an ~2.5-fold increase; IC_{50} for 2’-C-MeGTP, >100 μM, a >10-fold increase) (Fig. 2) (14). Although the biological characterization demonstrated 1b Δ8 was functionally similar to 1b WT, we were unable to obtain a crystal structure of apo 1b Δ8 or of it complexed with RNA.

The JFH1 isolate of genotype 2a is the only HCV strain capable of efficient replication in cell culture as well as in vivo (37). A construct of 2a JFH1 NS5B with the β-hairpin loop (residues 444 to 543) replaced with a Gly-Gly linker (designated 2a Δ8) was observed to be >100-fold more active than wild-type 2a in de novo RNA synthesis assays (Fig. 1B), capable of binding RNA in thermofluor analysis (data not shown), and resulted in a similar IC_{50} for chain termination with PSI-352666 (6 μM) relative to wild-type 2a (Fig. 1B). We obtained a 2.5-Å resolution apo crystal structure of 2a Δ8 (Table 1) which revealed substantial structural changes relative to previously determined 2a NS5B structures (2, 37, 38), with an overall root mean square deviation (RMSD) value...
of 1.8 Å (Fig. 3A). The 2a Δ8 Gly444-Gly445 linker was ordered, and Phe551 was the last ordered residue, indicating that an additional 27 amino acids of the C terminus, including the affinity tag, were disordered. The alignment of the palm and finger domains of a closed apo 2a structure (37) with the apo 2a Δ8 structure shows an overall ~20° movement of the thumb domain (Fig. 3A). The lack of the β-hairpin loop, the disorder of the C-terminal linker region, and the movement of the thumb domain combine to generate a large cavity in the center of the polymerase. The thumb domain movement is accompanied by the significant reordering of residues 397 to 412, which connect the primer grip helix with the primer buttress helix (Fig. 3). In particular, Ile405, which is a valine in other HCV strains and was previously detailed to be important for de novo initiation across all genotypes (37), moved more than 12 Å away from the β-hairpin loop in the closed wild-type structure to extend the primer buttress helix and pack on top.
of the highly conserved Trp408 (Fig. 3). Trp408 stacked on top of the nearly invariant Phe429 in the closed wild-type structure, and both residues adopt different rotamer conformations in the 2a Δ8 structure. In addition, the highly conserved Pro404, which contacts His95 of the finger domain in the closed apo structure (Fig. 3B), forms a key turn in the loop while packing on top of the main chain of Trp397 in the apo 2a Δ8 structure (Fig. 3C). This loop reordering may be critical to the transition from de novo initiation with GTP to the elongation of the growing primer-template RNA. Intriguingly, this same loop reordering was observed in the 2.5-Å structure of an apo 2a JFH1 isolate HCV NS5B resistance-derived triple mutant (S15G/C223H/V321I) (19) in which the β-hairpin loop is extant (unpublished data). The comparison of the apo 2a Δ8 structure to other RdRp ternary complexes (12, 15, 44) suggested that in this open conformation, HCV NS5B is able to bind primer-template RNA.

Crystals of apo 2a Δ8 were soaked with self-annealing RNA, yielding symmetrical primer-template RNA with 5' overhangs, and structures were determined at 2.9- and 3.0-Å resolution (Fig. 4 and Table 1). A-form RNA was readily apparent in the resulting electron density maps (Fig. 5), clearly showing the differences in the purine/pyrimidine pairings of the two RNA sequences. Both symmetrical primer-template RNA pairs were designed as obligate chain terminators with either 3'-dG (RNA1) or 2',3'-ddC (RNA2), and thus, unsurprisingly, a product state, pretranslocation, was observed in both complexes (Fig. 4). In both structures, four bases are paired in the primer-template RNA dimer with a 5' 1-base overhang observed within the electron density map. None of the nucleobase hydrogen bond acceptors or donors is recognized by the polymerase, indicating sequence-independent recognition by the polymerase. The interactions between the polymerase and the nucleic acids include packing with the pairing nucleotide of the template strand (residue + 1 by convention) and residue +1 of the primer strand in the current, product-state assembly (equivalent to the incoming nucleoside triphosphate [NTP]), as well as numerous interactions with the phosphodiester backbone. The sugar and phosphate of the +2 residue of the template strand head down the template entrance tunnel. Template-strand phosphates are recognized by the backbone amide nitrogen atoms of Arg98 (+2) and Ala97 (+1) and the side chains of Arg168 (+0), Lys172 (-1), and Gln180 (-2) (Fig. 6). The 2'-hydroxyl of the pairing nucleotide (+1) is recognized by the backbone oxygen of strictly conserved Gly283, while the other 2'-hydroxyls of the template strand are recognized by the backbone oxygen of Val284 (+0), the side chain of Ser288 (-1), and possibly the backbone oxygen of Phe193 (-2) (Fig. 6B), demonstrating the importance of an RNA template for HCV. The phosphates of the primer strand are recognized by the side chains of Arg158 (+1) of the finger domain, Arg586 and Arg394 (+0), Arg394 (-1), and His402 (-2) from the primer grip helix in the thumb domain (Fig. 6C). The primer buttress helix, spanning residues 405 to 414, has moved away from the central cavity relative to the closed apo wild-type structure, allowing for strictly conserved residue Gly410 to come within 3.3 Å of the 2'-hydroxyl of primer-strand residue -2, demonstrating that any other amino acid at this position would likely clash with the primer. The 2'-hydroxyl of primer residue +1 of the product, pretranslocation state, which resides at the same position as the incoming NTP in the substrate registry, is recognized by the side chain of Asp225 (Fig. 6A). Since both structures contain a 3'-deoxygen terminal residue, the other carboxylate oxygen of Asp225 is free to hydrogen bond with Asn291. The equivalent residue (Asp238) of the poliovirus RdRp was shown to adopt different conformations depending on the incoming NTP, translocation state, and presence of divalent metal ions (15). None of the other 2'-hydroxyls of the primer strand are recognized by NS5B, which is consistent with reduced activity with DNA primers (42). Despite low sequence identity outside the catalytic residues, the overall primer-template RNA recognition strategy of HCV is essentially identical to that observed for Norwalk virus (44), poliovirus (15), and FMDV (12) (Fig. 6D).

Although the nucleobase hydrogen bond acceptors or donors are not recognized by the polymerase, it does appear that the polymerase is able to differentiate between a purine and pyrimidine base at the +1 position in the RNA template strand, as seen in these two complexes of 2a HCV NS5B Δ8 with RNA1 and RNA2. The nucleobase of the pairing nucleotide of the template strand (residue +1) stacks on top of the strictly conserved Ile160, as predicted (3), while the sugar stacks on top of Tyr162, which is conserved as Tyr or Phe (Fig. 7). In the RNA1 complex, the Cys2 of the Ile160 side chain appears to interact with the face of the C3 base, the +1 position of the template RNA, while the G61 of the side chain extends beyond the C3 base to interact with the nucleobase of G6C, the +1 position of the primer RNA (Fig. 7A). Conversely, in the RNA2 complex, both Cys2 and C61 of Ile160 appear to interact only with the bulkier nucleobase of G4, the +1 position of the template strand, and do not seem capable of interacting directly with the necessarily more distant ddC7, the +1 position in the RNA2 primer strand (Fig. 7B). To our knowledge, this is the first crystallographic evidence for such differentiation in an RdRp-RNA complex: in the poliovirus RdRp-RNA complexes (15), which have the analogous Ile176, the +1 RNA template base is always a purine; in the norovirus RdRp-RNA complexes (44), with the analogous Leu184, the +1 RNA template base is also always a purine. Only in the FMDV-RNA complexes (13), with the analogous Val181, which is too small to extend beyond the pairing

FIG 5 Relaxed-eye stereo view of omit electron density map [Fcalc−|Fobs|] into which the symmetrical primer-template RNA1 5'-UACCG(3'-dG) model was built (shown in green mesh) contoured at 3.0 σ (A), and the final 2|Fcalc−|Fobs| electron density map is shown in blue mesh contoured at 1.0 σ (B).
nucleobase, does the +1 RNA template base vary between purine and pyrimidine. If these same observations are made in additional HCV RdRp-RNA complexes, it will be intriguing to speculate how such a differentiation is evidenced in the efficiency of the enzyme as it relates to the incorporation of natural nucleotides or various NIs during initiation or elongation or when perturbed by nearby mutations, such as S282T, as has been previously documented (10).

In addition to demonstrating the molecular basis for primer-template recognition and elongation by HCV polymerase, these structures may provide insight into the structural basis by which resistance-derived mutations permit the polymerase to continue to function while diminishing the impact of the inhibitor, be it an NI or NNI. For example, two of the most frequently arising resistance mutations for NIs in HCV are S96T and S282T. In our structures, Ser96 hydrogen bonds with Arg168, which directly positions the pairing nucleotide phosphate (Fig. 6B). Mutation to threonine likely repositions the template strand toward the thumb domain, decreasing the space available and thereby increasing the preference for the incoming NTP over the typically bulkier NI.

With regard to the S282T mutant, the threonine has been speculated to directly impede the incoming 2’-C-MeNTP as its mode of action (10). However, proximity to two nearby residues, the aforementioned Ile160 and Gly283, which interacts with the 2’-hydroxyl of template RNA ribose, suggests a more complicated picture for S282T-derived resistance. One can also imagine a scenario in which resistance-derived mutations are modeled into these new structures, potentially providing insights into which mutated residues block access to an inhibitory binding cavity and which allow the protein to compensate for concomitantly reduced efficacy.

The more open nature of the primer-template-bound complexes described here also offers a more complete glimpse into the
mode of action for thumb site II NNIs. Clearly, these structures provide a valuable crystallization platform for structure-guided drug design, in particular for nucleotide analog inhibitors or NNIs that target the ternary complex. Finally, the methodology of deleting elements of the β-hairpin loop to afford the primer-template-bound complex may prove similarly useful through iterative application to other viral RdRps with this structural feature.

ACKNOWLEDGMENTS

We thank K. Potts for assistance with molecular biology, S. Moen for fermentation, J. Bullen and M. Namekata for protein purification, R. Baydo for thermolflou experiments, M. Clifton for structure peer review, C. Lugo and H. Bao for assisting in biochemical characterization, and N. Dave at ALS and J. Brunzelle at APS LS-CAT for assistance during data collection.


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