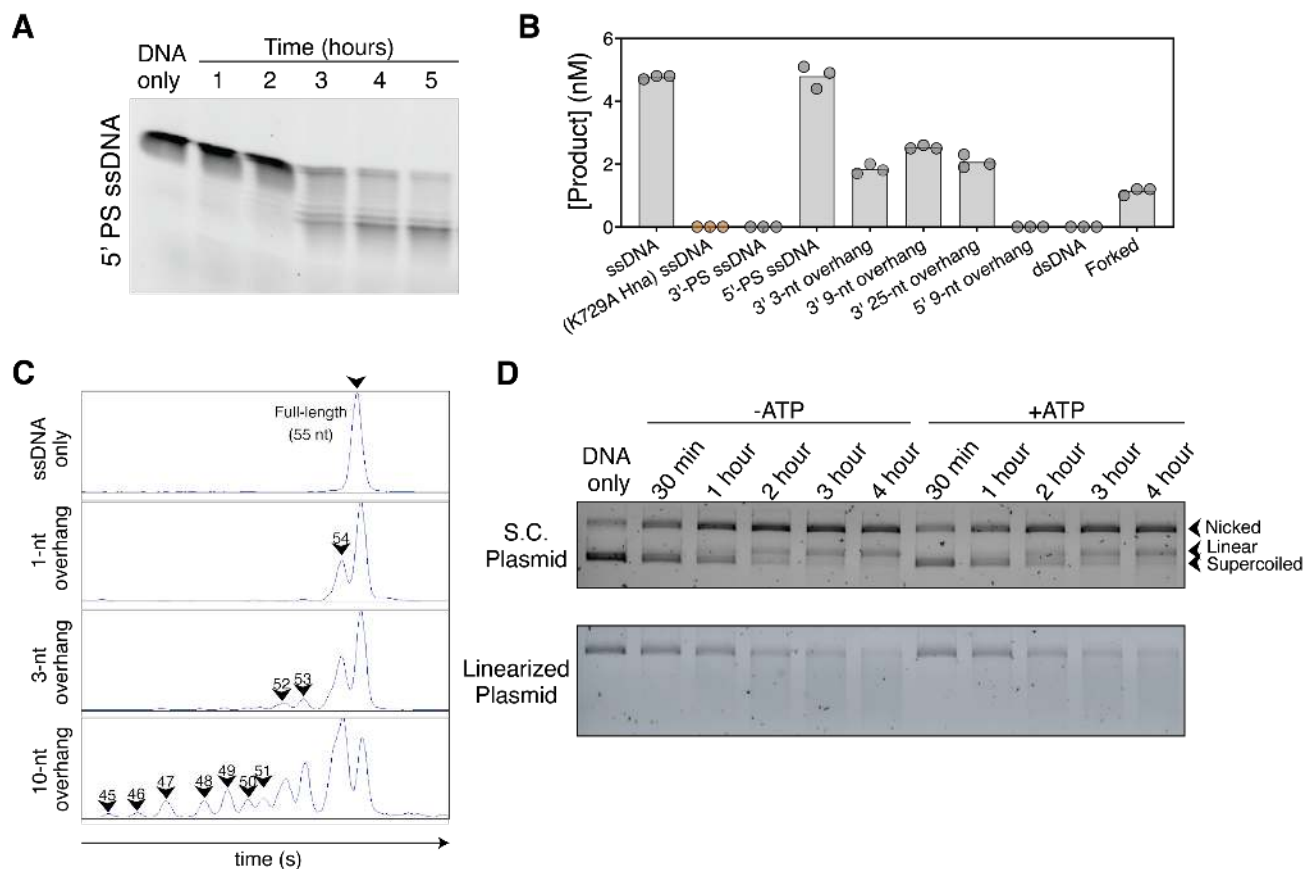
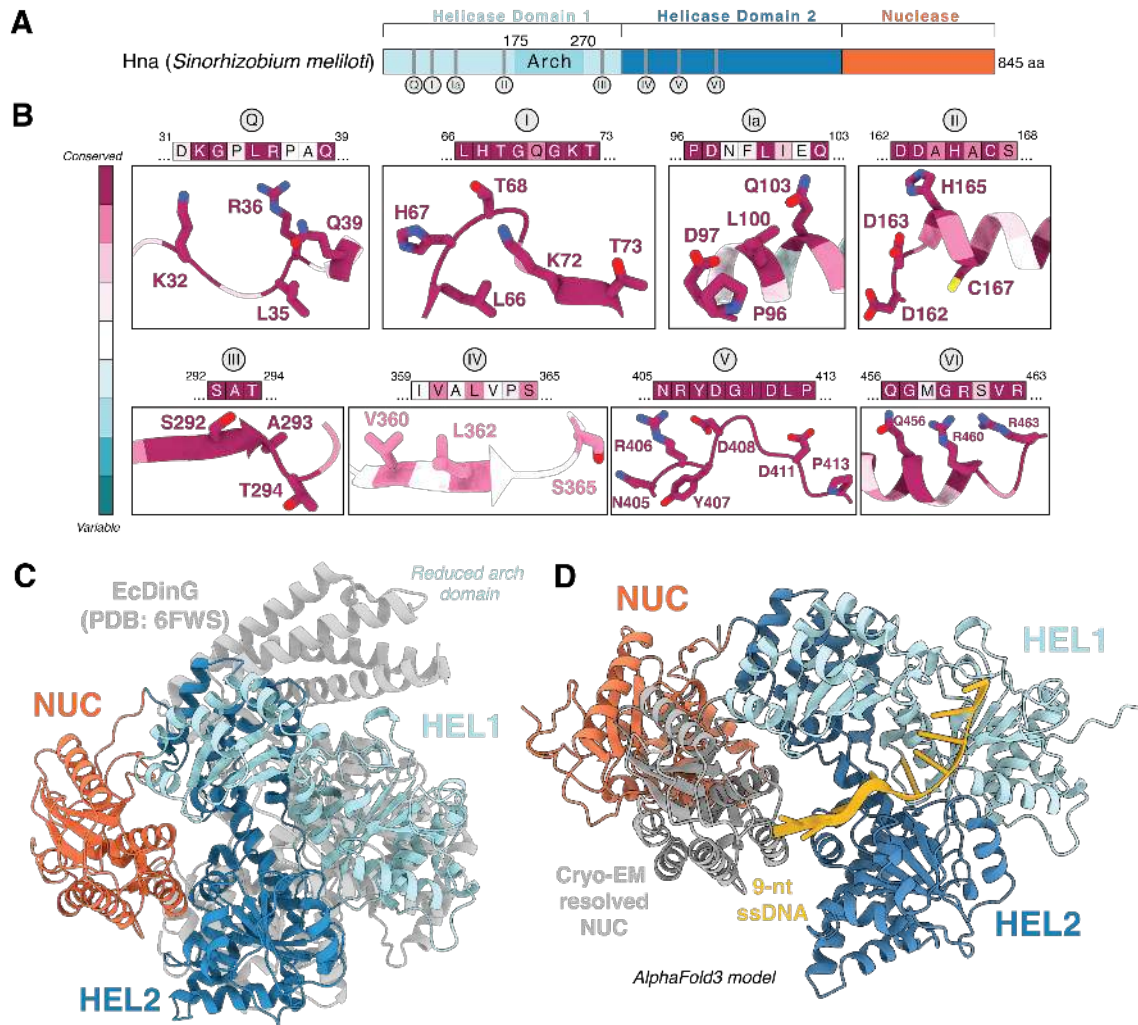
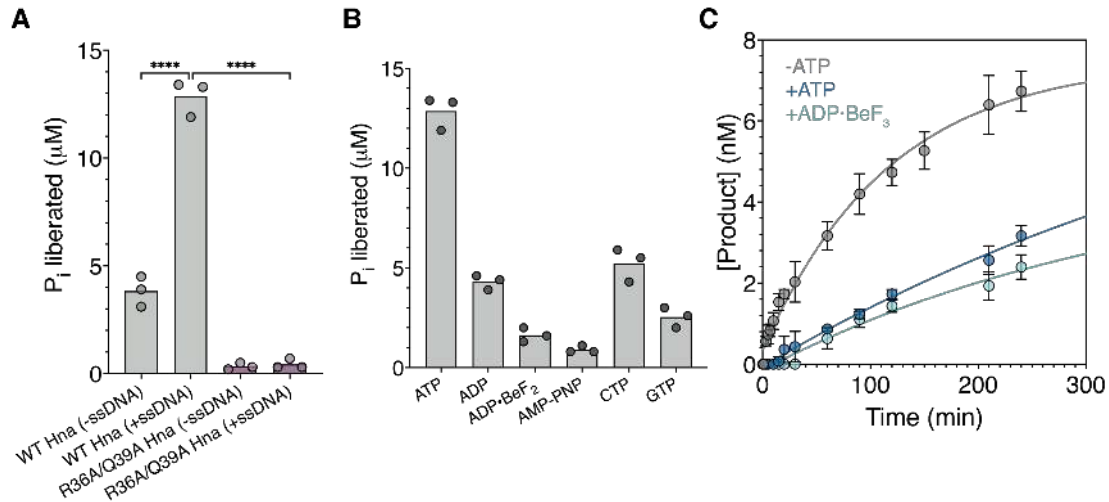




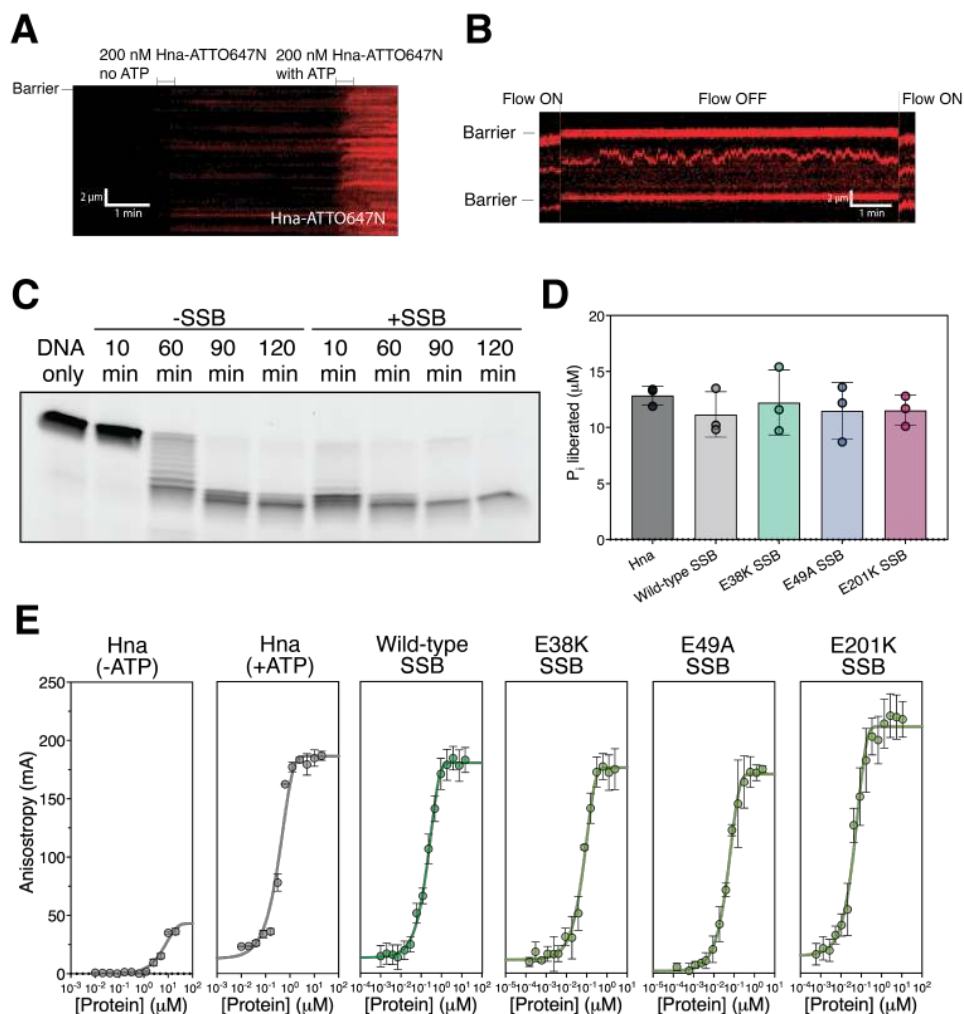
**Supplementary Fig. 1 | Hna degrades single-stranded and plasmid DNA.** **A**, Exonuclease activity over time using ssDNA with a series of 5'-phosphorothioate (PS) modifications showing unperturbed cleavage. Gel is representative of 3 independent experiments. **B**, Quantification of DNA cleavage by Hna in the presence of various substrates using capillary electrophoresis. Data represents product formation across three independent, biological replicates. **C**, Raw fluorescence peaks corresponding to differently sized fluorescently-labeled cleavage products. DNA only negative control (55 nucleotides) shown at the top. **D**, Time course Hna cleavage using supercoiled (S.C.) and linearized plasmid in the presence or absence of ATP. Distinct species of DNA are denoted on the right. Gel is representative of 4 independent experiments. Source data are provided as a Source Data file.



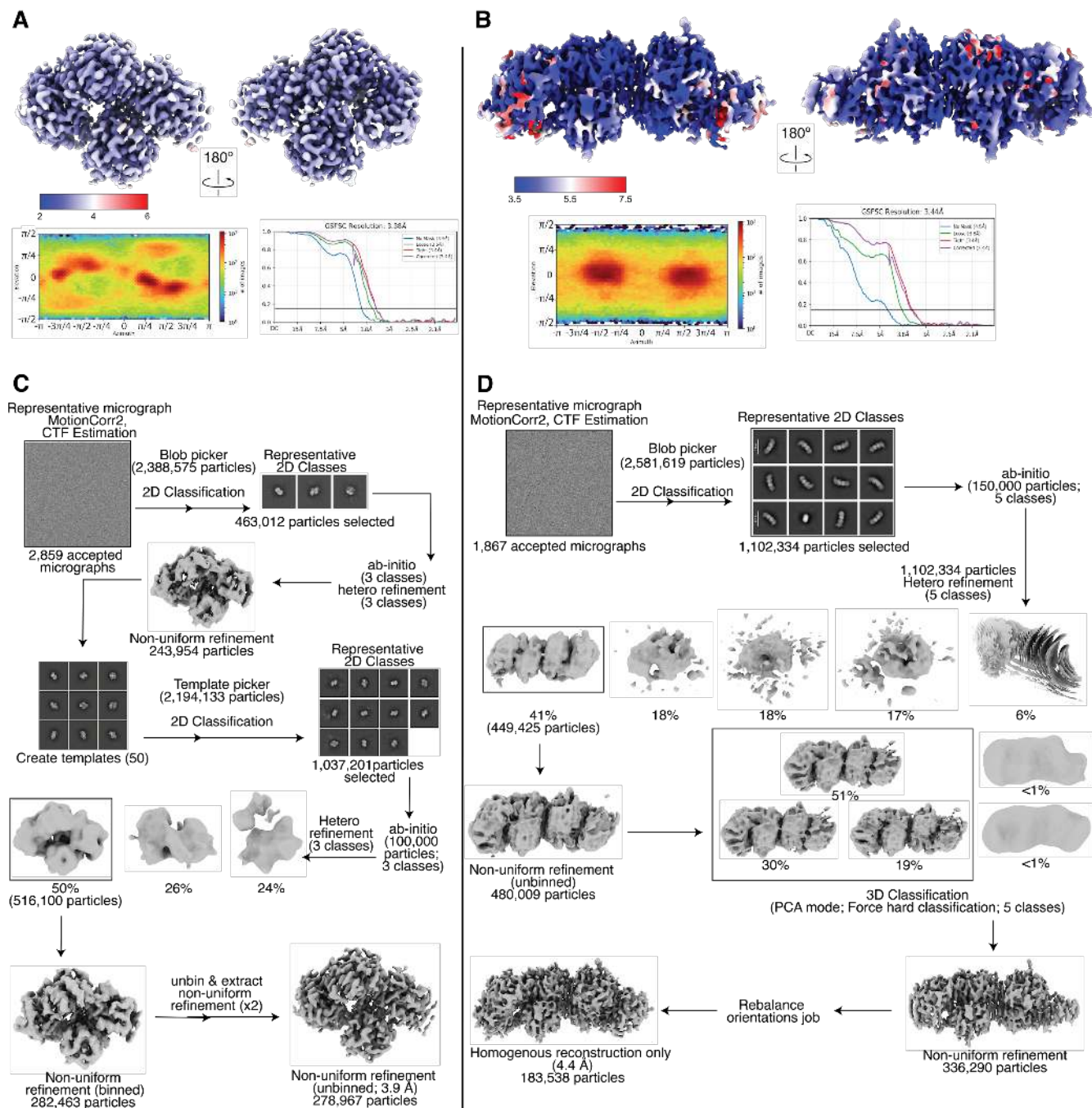
**Supplementary Fig. 2 | Hna structural motifs and conservation.** **A**, Domain organization of Hna from *S. meliloti*. The two helicase modules and C-terminal nuclease domain are shown in blue and orange, respectively. Canonical HEL1 and HEL2 functional motifs are labeled, as well as the HEL1 Arch domain. **B**, Magnified view of each identified SF2 helicase motif. Residues are colored by conservation with position and sequence listed above. **C**, Overlay of determined Hna monomer structure (colored) and structurally similar protein, *EcDinG* (PDB: 6FWS; gray). Hna and DinG helicase domains are highly similar with notably reduced Arch domain in Hna. **D**, AlphaFold3 prediction of Hna bound to a 9-nucleotide ssDNA substrate (colored) overlaid with the NUC domain of Hna monomer structure (gray). The predicted substrate fully occupies the Hna DNA binding site and promotes a unique NUC domain rearrangement.



**Supplementary Fig. 3 | Hna exhibits ssDNA-stimulated ATPase activity.** **A**, Malachite green ATPase assay using wild-type Hna or ATP-binding mutant (R36A/Q39A) with and without ssDNA present (n=3 independent, biological replicates). Phosphate concentration was determined after 30 minutes of incubation. Statistical significance is evaluated via one-way ANOVA; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001. **B**, Malachite green phosphate detection assay using wild-type Hna with ssDNA present and various nucleotide analogs. Phosphate concentration was determined after 30 minutes of incubation (n=3 independent, biological replicates). **C**, Quantification of ssDNA cleavage by Hna in the presence and absence of ATP or ADP·BeF<sub>3</sub> using capillary electrophoresis. Data shown are the mean ± standard deviation of three independent experiments for each condition. Source data are provided as a Source Data file.



**Supplementary Fig. 4 | Hna is an ATP-dependent DNA binding protein. A**, Representative kymograph showing ATP-dependent binding of ssDNA by Hna (red). ATP was injected at the time indicated on the kymograph promoting increased binding events. **B**, Representative kymograph showing passive diffusion of Hna (red) along ssDNA in the absence of buffer flow. **C**, Time course cleavage of ssDNA by Hna in the absence or presence of phage-encoded 5A SSB. **D**, Malachite green ATPase assay using wild-type Hna only (Hna) or combined with various SSB mutants with single-stranded DNA present. Phosphate concentration was determined after 30 minutes of incubation 37°C. Data shown are the mean  $\pm$  standard deviation of three independent, biological replicates for each condition. **E**, Hna and 5A SSB binding curves after incubation with fluorescently labeled ssDNA. Data shown are the mean  $\pm$  standard deviation of three independent, biological replicates fit to a one-phase decay function. Source data are provided as a Source Data file.



**Supplementary Fig. 5 | Structural analysis of Hna. A**, Unsharpened maps colored by local resolution (top) for Hna monomer and **B**, Hna dimer. Accompanying gold-standard FSC curves for cryo-EM reconstructions (left) with Euler diagrams showing orientation distributions of cryo-EM reconstructions (right). Resolutions were estimated at FSC=0.143. **C**, Data processing pipelines for Hna monomer and **D**, Hna dimer.

**Supplementary Table 1 | List of DNA sequences used in this study.**

| <b>Oligo Name</b>              | <b>Sequence (5' to 3')</b>                              |
|--------------------------------|---------------------------------------------------------|
| ssDNA (55-nt)                  | agctgacgtttgtatgtctgctgtcatctttatgcgtcagcagagatttctgct  |
| dsDNA complement               | agcagaaatctctgctgacgcataaagatgagacgcagacatacaaacgtcagct |
| 3' 1-base overhang complement  | gcagaaatctctgctgacgcataaagatgagacgcagacatacaaacgtcagct  |
| 3' 3-base overhang complement  | agaaatctctgctgacgcataaagatgagacgcagacatacaaacgtcagct    |
| 3' 9-base overhang complement  | ctctgctgacgcataaagatgagacgcagacatacaaacgtcagct          |
| 3' 10-base overhang complement | tctgctgacgcataaagatgagacgcagacatacaaacgtcagct           |
| 3' 25-base overhang complement | agatgagacgcagacatacaaacgtcagct                          |
| 5' 9-base overhang complement  | agcagaaatctctgctgacgcataaagatgagacgcagacatacaa          |
| Forked complement              | tgagacgcagacatacaaacgtcagcttgagacgcagacatacaaacgtcagct  |

**Supplementary Table 2 | Cryo-EM data collection, refinement, and validations statistics.**

|                                           | <b>Hna Monomer (EMD-<br/>75470; PDB 10UJ)</b> | <b>Hna Dimer (EMD-<br/>73047; PDB 9YKJ)</b> |
|-------------------------------------------|-----------------------------------------------|---------------------------------------------|
| <b>Data Collection and Processing</b>     |                                               |                                             |
| Voltage (kV)                              | 200                                           | 200                                         |
| Electron exposure (e-/Å <sup>2</sup> )    | 49                                            | 49                                          |
| Defocus range (µm)                        | -1.5 to -2.5                                  | -1.5 to -2.5                                |
| Pixel size (Å)                            | 0.94                                          | 0.94                                        |
| Symmetry imposed                          | C1                                            | C1                                          |
| Initial particle images (no.)             | 2,194,133                                     | 2,581,619                                   |
| Final particle images (no.)               | 278,967                                       | 185,538                                     |
| Map resolution (Å)                        | 3.9                                           | 4.4                                         |
| FSC threshold                             | 0.143                                         | 0.143                                       |
| <b>Refinement</b>                         |                                               |                                             |
| Initial model used (PDB code)             | AlphaFold3                                    | AlphaFold3                                  |
| Model resolution (Å)                      | 3.8                                           | 3.7                                         |
| FSC threshold                             | 0.5                                           | 0.5                                         |
| Map sharpening B factor (Å <sup>2</sup> ) | 167.3                                         | 121.9                                       |
| <b>Model composition</b>                  |                                               |                                             |
| Non-hydrogen atoms                        | 6490                                          | 5952                                        |
| Protein residues                          | 821                                           | 1205                                        |
| Nucleotides                               | 0                                             | 0                                           |
| <b>R.m.s deviations</b>                   |                                               |                                             |
| Bond lengths (Å)                          | 0.004                                         | 0.003                                       |
| Bond angles (°)                           | 0.808                                         | 0.819                                       |
| <b>Validation</b>                         |                                               |                                             |
| MolProbity score                          | 1.2                                           | 1.18                                        |
| Clashscore                                | 3.93                                          | 0.57                                        |
| Poor rotamers (%)                         | 0                                             | 0                                           |
| <b>Ramachandran plot</b>                  |                                               |                                             |
| Favored (%)                               | 97.92                                         | 92.03                                       |
| Allowed (%)                               | 2.08                                          | 7.29                                        |
| Disallowed (%)                            | 0                                             | 0                                           |