Mechanism of flexibility control for ATP access of hepatitis C virus NS3 helicase

Mírko Palla a,b, Chien-Pin Chen a, Yuan Zhang a, Jingyuan Li d, Jingyue Ju b and Jung-Chi Liao a,c*

aDepartment of Mechanical Engineering, Columbia University, 220 Mudd Building, 500 W. 120th St, New York, NY 10027, USA; bDepartment of Chemical Engineering, Columbia University, 801 Mudd Building, 500 W. 120th St, New York, NY 10027, USA; cDepartment of Biomedical Engineering, Columbia University, 351 Engineering Terrace, 1210 Amsterdam Avenue, New York, NY 10027, USA; dChinese Academy of Sciences, Institute of High Energy Physics, 19B YuquanLu, Shijingshan District, Beijing, 100049, China

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Hepatitis C virus (HCV) NS3 helicase couples adenosine triphosphate (ATP) binding and hydrolysis to polynucleotide unwinding. Understanding the regulation mechanism of ATP binding will facilitate targeting of the ATP-binding site for potential therapeutic development for hepatitis C. T324, an amino acid residue connecting domains 1 and 2 of NS3 helicase, has been suggested as part of a flexible hinge for opening of the ATP-binding cleft, although the detailed mechanism remains largely unclear. We used computational simulation to examine the mutational effect of T324 on the dynamics of the ATP-binding site. A mutant model, T324A, of the NS3 helicase apo structure was created and energy was minimized. Molecular dynamics simulation was conducted for both wild type and the T324A mutant apo structures to compare their differences. For the mutant structure, histogram analysis of pairwise distances between residues in domains 1 and 2 (E291-Q460, K210-R464 and R467-T212) showed that separation between the two domains was reduced by ~10% and the standard deviation by ~33%. Root mean square fluctuation (RMSF) analysis demonstrated that residues in close proximity to residue 324 have at least 30% RMSF value reductions in the mutant structure. Solvent RMSF analysis showed that more water molecules were trapped near D290 and H293 in domain 1 to form an extensive interaction network constraining cleft opening. We also demonstrated that the T324A mutation established a new atomic interaction with V331, revealing that an atomic interaction cascade from T324 to residues in domains 1 and 2 controls the flexibility of the ATP-binding cleft.

Keywords: molecular dynamics simulation; ATP-binding cleft; pairwise distance analysis; RMSF analysis; hinge motion

Introduction

Hepatitis C virus (HCV) is the major causative agent of parentally transmitted nonA and nonB hepatitis (Choo et al., 1989) causing chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (Choo et al., 1989; De Francesco & Migliaccio, 2005). About 3% of the worldwide population is infected by HCV and currently no protective vaccine is available. Recent research efforts have targeted the replicative enzymes of HCV as potential and important therapeutic targets in drug design (Frick, 2003). A variety of adenosine triphosphate (ATP)-binding inhibitors are under development to block the HCV replication, including ones which have already been used as successful therapeutic agents for chronic myeloid leukemia and other serious diseases (Druker et al., 2001). Even though these findings suggest feasible strategies for developing specific inhibitors to block the action of this critical drug target, an accurate description of protein flexibility and its influence on ATP recognition is unavailable. In the past decade, many advances have been made in incorporating protein flexibility in drug discovery, taking multiple conformational states for ligand binding into consideration (Abrams & Vanden-Eijnden, 2010; Boehr, Nussinov, & Wright, 2009; Carlson & McCammon, 2000; Durrant & McCammon, 2010; Ivetac & McCammon, 2011, 2012; Wright & Dyson, 2009). Therefore, more effective research efforts are urgently needed to understand the ensembles of HCV protein structures representing global and local flexibility.

The HCV genome encodes a large polyprotein called nonstructural protein 3 (NS3), which is responsible for viral replication. NS3 is classified as a superfamily-2 (SF2) helicase, which has polynucleotide triggered dNTPase activity and can unwind both RNA and DNA in the 3′–5′ direction (Suzich et al., 1993). It is composed of three domains, domains 1 and 2 with conserved RecA-like motifs able to sandwich ATP (Liao, 2011); and the
polynucleotide resides orthogonally in a groove between domain 3 and domains 1 and 2 (Gorbalenya & Koonin, 1993). ATP can form an extensive hydrogen bond network (Liao, Sun, Chandler, & Oster, 2004), and its binding to the catalytic site and its hydrolysis cycle are tightly coupled to the polynucleotide translocation and affinity control. The mechanisms of this coupling have been proposed and examined (Soultanas, Dillingham, Velankar, & Wigley, 1999; Zheng, Liao, Brooks, & Doniach, 2007), although the atomic details of how the ATPase cycle can regulate the conformational changes remain unclear.

Kinetic studies demonstrated that ATP binding results in an almost 100% decrease in the association rate constant for the enzyme and nucleic acid (Preugschat, Averett, Clarke, & Porter, 1996). Several crystal structures of NS3 helicases showed that the helicase domain was complexed with single-stranded DNA (Kim et al., 1998). Based on these protein structures, a model was proposed detailing the molecular mechanism of single-stranded polynucleotide translocation. These findings revealed that ATP binding induced closure between RecA-like domains 1 and 2, which resulted in an associated unidirectional movement of the bound polynucleotide relative to the protein. This model was supported by an inchworm mechanism proposed subsequently for HCV NS3 helicase, which extended the previous hypothesis with an additional mechanism responsible for duplex unwinding tightly coupled to the DNA translocation process (Dumont et al., 2006). A comparative structural study (Korolev, Yao, Lohman, Weber, & Waksman, 1998) between HCV NS3 helicase and Rep helicase, an SF1 family member, demonstrated the importance of motif III (322–326), which contains residue T324, in the indirect transduction of allosteric effects of nucleotide binding and hydrolysis upon DNA binding.

To gain a better understanding of the underlying atomic details of this model, ATPase activities of a series of helicase mutants were measured in the presence of poly(U) to simulate the ATP hydrolysis of HCV NS3 (Tai et al., 2001). Enzymatic activity was measured kinetically by varying the concentration of the ATP substrate. Compared to the wild-type protein, all mutants showed a reduced level of ATPase activity (Tai et al., 2001). One of the two mutated threonine residues was T324, which is thought to act as a hinge connecting domains 1 and 2 of the ATP-binding cleft (Figure 1). When substituting alanine for T324 or T322, ATPase activity was dramatically reduced to 30 and 16% compared to the wild-type protein, respectively. These results revealed the importance of hinge residue T324 of motif III in ATPase activity regulation.

Figure 1. The ATP-binding pocket of HCV NS3 helicase. Residues from motifs I, II, III, V, and VI are shown to interact with different parts of ATP. T324 near motif III is located in the loop connecting domains 1 and 2 and regarded as a hinge residue. ATP is located in the cleft between domains 1 and 2 represented as CPK and color coded according to elements. The hotspot residues involved in ATP coordination and active site rearrangement for catalysis are represented as Licorice and labeled with single letter codes, residue numbers, and motif codes.
tion due to its vital role in modulating the opening and closing of the ATP-binding cleft between the two domains. Thus, the detailed understanding of the opening/closing of domains 1 and 2 associated with the ATP-binding mechanism on the atomic level provides a promising approach for new therapeutic options, targeting the ATP-binding site for potential hepatitis C treatment.

Recently a set of crystal structures of NS3 helicase were obtained (Gu & Rice, 2010), in which ATP mimics (ADP·BeF$_3$ and ADP·AlF$_4^-$) were captured in the ground and transition states. The results indicated that NS3 domains 1 and 2 close in the presence of ADP·AlF$_4^-$, which binds in a pocket, formed by motifs I, II, III, V, and VI (Figure 1). These findings suggest that residues including D290, E291, Q460, T212, and K210, which reside prominently in the interdomain cleft, play a critical role in active site rearrangement to stabilize the ATP moiety for catalysis (Gu & Rice, 2010). These interdomain residues were examined by in vitro kinetic experiments of mutant NS3 helicases (Tai et al., 2001), and the results revealed that K210 in the Walker A nucleotide-binding motif and D290, E291 in the Walker B motif were crucial to ATPase activity, while Q460 acted as a gatekeeper playing an essential role influencing the microenvironment modulating enzymatic activities in that region. It was also shown that T212 plays a role in stabilizing the ATP interaction for catalysis based on conformational snapshots of NS3 helicase (Gu & Rice, 2010).

Our previous correlation analysis (Zheng et al., 2007), which perturbs individual residues and computes how that change affects the global fluctuation of a subset of spatially distant residues based on an elastic network model, showed that the hotspot residues of NS3 helicase responsible for ATP binding do not only cover the immediate cleft vicinity, but span an extensive atomic network, which could even reach the polynucleotide binding site. These hotspot residues are postulated to be involved in the interaction network controlling the ATP coordination and active site rearrangement for catalysis and can potentially be affected by the T324A mutation. Due to change in the microenvironment surrounding the hinge, a downward projecting atomic cascade can affect the opening and closing of the ATP-binding cleft between domains 1 and 2.

Gu et al. demonstrated that water molecules – among other residues located in both domains 1 and 2 of the ATP-binding cleft – were actively involved with ATP coordination and active site rearrangement for catalysis (Gu & Rice, 2010). It was shown that α- and β-phosphate groups were coordinated by two spatially restricted water molecules along with motif I main chain nitrogens (G207 to T212) and side chain atoms (K210 and T212). Furthermore, the γ-phosphate groups were coordinated by three positively charged side chains (K210, R464, and R467) and water-mediated interactions. Finally, the metal ion essential for ATP catalysis located in the octahedral opening of the ATP-binding cleft was coordinated by two water molecules and some motif I/II residues (S211 and E291). Thus, by analyzing the atomic interaction changes involving the spatially restricted water molecules and the hinge residues, one could describe the atomic details of the flexibility modulation of the ATP-binding cleft.

Molecular dynamics (MD) simulations have been successfully used for analyzing the dynamic behavior of biomolecular structures (Dodson & Verma, 2006; Grant, Gorfe, & McCammon, 2010; Karplus & Kuriyan, 2005; Li & Cui, 2004; Sotomayor & Schulten, 2007). Since the rational design of possible inhibitors requires a precise understanding of the mechnochemical behavior of their target structures, the MD simulation along with the experimental mutagenesis results might shed light into key regions which can be targeted for developing antiviral drugs.

For example, a previous MD study (paired with stochastic-dynamics descriptions) of DNA translocation of PcrA helicase demonstrated that ATP hydrolysis couples to conformational changes of the motor protein (Yu, Ha, & Schulten, 2006). Their results also revealed that an “arginine finger” residue is essential for stabilizing the reaction intermediate for ATP hydrolysis, thereby providing a means of coupling this to large-scale protein conformational changes. Additionally, mutational studies on NS3 helicase showed that if the highly conserved “arginine finger” residues, such as R461, R462, R464, and R467 in motif VI, are mutated; the polynucleotide unwinding and ATPase activities are severely impaired (Kim, Kim, Gwack, Han, & Choe, 1997; Kwong, Kim, & Lin, 2000; Min, Sung, Choi, & Ahn, 1999). The combination of in silico and mutational study results would provide a perspective of residue interactions in hotspot regions during nucleic acid binding and ATPase activity of NS3.

Up to now, no dynamic analysis has been reported evaluating the effects of hinge residue T324 involved in ATP active site coordination of NS3, and its tight coupling to nucleic acid binding. In the present work, we investigate the behavior of the ATP active site microenvironment, using MD simulations, starting from a set of protein structures. Our MD simulation study focusing on the T324 residue mutation reveals that this residue plays a critical role in the atomic coordination of flexibility control of domains 1 and 2 of the ATP-binding cleft through a series of cleft residues. The interaction of these residues with residue 324 dictates the entropic degrees of freedom of the ATP-binding cleft.

Materials and methods
MD simulation
Two HCV NS3 helicase structures (PDB: 3KQH and 3KQL) and two computationally mutated structures were
Table 1. Atomic and molecular details of the four HCV NS3 helicase systems studied.

| Crystallographic systems     | 3KQH  | 3KQL  | 3KQH-T324A | 3KQL-T324A |
|------------------------------|-------|-------|------------|------------|
| Total number of atoms in system | 39,078 | 35,709 | 39,068     | 35,699     |
| Total number of atoms in NS3 structure | 6705  | 6759  | 6701       | 6755       |
| H$_2$O                        | 10,764| 9625  | 10,762     | 9624       |
| Na$^+$                       | 46    | 42    | 44         | 40         |
| Cl$^-$                       | 35    | 31    | 35         | 31         |

Note: Designations for HCV NS3 helicase structures: 3KQH-T324A and 3KQL-T324A correspond to the T324A mutation of the two wild-type structures respectively.

used for MD simulation (Table 1). 3KQH was a structure in its apo state while 3KQL was a structure with ATP mimic (ADP·AlF$_4^-$) bound. The two mutant structures were created by replacing T324 of 3KQH and 3KQL with A324. Both of these structures were computationally mutated using Swiss-PdbViewer (Guex & Peitsch, 1997) and energy minimized with GROMACS, which allowed browsing of a rotamer library in order to change amino acid side chains.

MD simulations were carried out with GROMACS (Berendsen, 1995) using the AMBER-99 force field (Wang, Cieplak, & Kollman, 2000) in the Ewald-Berendsen type isobaric–isothermal thermodynamic ensemble at 1 ATM and 300 K. Each structure was neutralized and embedded in a periodic box containing SPC 3-point water model (Berendsen, Postma, Van Gunsteren, & Hermans, 1981) molecules (Table 1). Then, the energy minimization procedure was carried out using the steepest descent integration algorithm. After energy minimization, a 5000-step position restricted MD simulation was carried out keeping the complex and ions fixed using fast Particle Mesh Ewald (PME) summation for electrostatics. PME parameters were chosen with grid dimension of 1.2 Å, interpolation order of 4, and a 10 Å direct space cut-off value to further equilibrate the protein structures. For each construct, 3 × 20 ns MD simulations (three different seeds) were carried out with constraints applied on all bonds using the leapfrog algorithm (Hockney, 1970) for the integration of the equations of motion with a time step of 2 fs.

Details of the four systems studied including total number of atoms (whole system vs. structure only), solvent molecules and number of counter ions (Na$^+$, Cl$^-$) used to neutralize each system are given in Table 1.

Root mean square deviation analysis

For each case, root mean square positional deviations (RMSD) from the crystallographic structure of all backbone atoms were calculated by least-square fitting according to the equation:

$$\text{RMSD}(t, t_0) = \sqrt{\frac{1}{M} \sum_{i=1}^{N} m_i |r_i(t) - r_i(t_0)|^2}, \quad (1)$$

where $M$ is the total mass of the structure (sum of all atomic masses in the molecule); $N$ is the total number of residues in NS3 helicase (436); $m_i$ is the mass of atom $i$; and $r_i(t)$ is the position of atom $i$ at time $t$ and $t_0$ is the reference time for the structure or the initial time at the start of the simulation ($t=0$).

Pairwise distance analysis of hotspot residues

For all structures, the minimum distance was calculated between a hotspot residue group in domain 1 (E291, D290 and T212) and another selected residue group in domain 2 (Q460, R464, and R467) of the ATP-binding cleft, such that the distance could shed light onto the dynamic behavior of that particular region. The minimal distance between any pair of atoms from the respective groups was determined for both the wild-type and T324A mutant apo structures. Three such residue pairs, namely Q460-E291, R464-D290 and R467-T212, were selected based on results of “fluctuation-based correlation analysis” performed by Zheng et al. (2007), which probes the dynamic behavior of the top, middle, and bottom regions of the ATP-binding cleft, respectively. For every such hotspot residue, individual atomic groups were created containing three randomly selected backbone atoms belonging to each residue in the pair.

Identification of trapped water molecules

To understand the interaction cascade due to water molecules around the hinge region for both wild-type (PDB: 3KQH) and its mutant apo constructs, we performed an additional 3 × 500 ps simulations (three different seeds) after the original 20 ns MD run. We used the time after the 20 ns simulation instead of directly analyzing the data from the first 20 ns, because we wanted to make sure that the transient effects from the original structure for the localization of water molecules were subsided after 20 ns. The average root mean square fluctuation (RMSF) was calculated over time per solvent molecule. Then all water molecules were ordered with increasing RMSF values and 30 of those with the lowest RMSF values were identified. The hydrogen bonds between all possible donors (D) and acceptors (A) for these water molecules and residues within hydrogen bonding
distances were computed and analyzed using GROMACS. Hydrogen bonds were determined based on geometrical cutoff criteria for the angle (30°) A–D–H and the distance (3.5 Å) H–A.

**Solvent accessible area analysis**

For both wild-type (PDB: 3KQH) and its mutant apo constructs, GROMACS was utilized to calculate the average solvent accessible surface (SAS) area over time per residue. Then a custom MATLAB script was used to calculate the normalized SAS difference between the same residues of the wild-type and mutant protein structures with:

\[
\text{NSD}(S_1, S_2) = \frac{S_2 - S_1}{S_1},
\]

where NSD is the normalized SAS deviation of each residue for wild-type vs. mutant proteins; \(S_1\) is the SAS value (in nm/Å²/N) for residue \(x\) in the wild-type structure and \(S_2\) is the SAS value (in nm/Å²/N) for the same residue in the mutant structure, respectively. We selected the top 10% residues with the largest positive SAS deviation averaged over three different seeds to identify the locations with increased water access upon the hinge residue mutation.

**RMSF analysis**

For both wild-type (PDB: 3KQH) and its mutant apo constructs, the RMSF or the averaged standard deviation of atomic positions in the trajectory was calculated for each residue according to equation:

\[
\text{RMSF}(r) = \sqrt{\frac{1}{T} \sum_{t=1}^{T} (r_t - \mu)^2},
\]

where \(\mu = \frac{1}{T} \sum_{t=1}^{T} r_t\),

where \(T\) is the total number of frames (or time steps) during the MD simulation; \(r_t\) is the averaged standard deviation of atomic positions in the trajectory of a particular residue at time step \(t\), and \(\mu\) is the mean RMSF value for the same residue. Then a custom MATLAB script was used to calculate the normalized RMSF difference between the same residues of the wild-type and mutant protein structures using equation:

\[
\text{NRD}(R_1, R_2) = \frac{R_1 - R_2}{R_1},
\]

where NRD is the normalized RMSF deviation of each residue for wild-type vs. mutant structures; \(R_1\) is the RMSF value (nm) for residue \(x\) in the wild-type structure and \(R_2\) is the RMSF value (nm) for the same residue in the mutant structure. We selected the top 10% residues with the greatest positive RMSF deviation averaged over three different seeds, representing residues of the most significant decrease in protein flexibility upon mutation.

**Results**

**ATP-binding cleft of HCV NS3 helicase became less flexible with T324A mutation**

MD simulation was conducted for two wild-type HCV NS3 helicase structures with and without ATP, and two computationally mutated NS3 structures (T324A) with and without ATP. Structural relaxation was monitored by analyzing the time evolution of the RMSD of the frames with respect to the initial structure. In all four cases, the RMSD showed convergence of the simulation within 20 ns (Figure 2). The structures remain faithful to their starting configuration although an average RMSD calculated over all backbone atoms reached about 1.5 Å. This means that the initial crystal structure was well maintained during the time steps, thus providing proof of good quality of the MD simulations.

Figure 2 demonstrates that for all structures, the RMSD remained stable around an average value of 1.5 Å over a considerable time period (10 ns) of the later part of the trajectory. For this reason, 20 ns simulation length is believed to be a sufficient time period to sample the large scale domain dynamics caused by the T324A hinge residue mutation.

Based on the hotspot residues we identified earlier (Zheng et al., 2007) and the locations of these residues in the ATP-binding cleft, we selected pairs from residue subgroups located in domains 1 and 2 (Q460-E291, R464-D290, and R467-T212) to probe relative motion of domains 1 and 2 (Figure 3(A)). Pairwise distance analysis of these residues showed a change in flexibility of the ATP-binding cleft after T324A mutation. For example, D290 of domain 1 and R464 of domain 2, which probes the dynamic behavior of the middle portion of the ATP-binding cleft, showed that HCV NS3 structures without bound ATP have a significant distance of separation between them in the range of 15–24 Å. Both wild-type and mutant structures demonstrated high-fluctuation amplitudes, implying that the RecA-like domains 1 and 2 form a relatively dynamic active site when ATP was absent (Figure 3(B)). Histogram analysis of this residue pair showed that the mean of the minimum distance was reduced by 10%, while the standard deviation decreased by over 27% on average when comparing the wild-type and T324A mutant structures (Figure 3(C)). Similar results were reported when probing the distances between other domain 1 and 2 hotspot residue pairs.
Thus, when the hinge residue T324 was mutated to alanine and ATP was not present, the mutation made domains 1 and 2 more rigid restricting flexibility of that region.

When comparing the wild-type and mutant helicase structures with bound ATP for this hotspot residue pair, we observed that the fluctuation amplitude in the range of 15–18 Å was moderate compared to the structures without ATP. Both cases demonstrated a stable trend over time; the wild-type structure was especially stable around an approximate mean value of 16 Å. The distances were smaller than those when ATP was absent, indicated that domains 1 and 2 were more compacted when ATP was present. The wild-type and T324A mutant helicase constructs without ATP were further examined to understand the mutational effects on atomic interactions in the binding cleft.

Additional trapped water molecules were found in the mutant structure

To understand the interaction network due to water molecules around the hinge region, we analyzed the dynamics of water molecules for a 500 ps MD simulation after the original 20 ns MD run. The RMSF of each water molecule was calculated to identify a set of water molecules with the smallest RMSF values, i.e. the water molecules spatially restrained to a particular location. Comparing trapped water molecules of wild-type and mutant NS3 helicase apo structures for all three seeds, two water molecules (W1 and W2) were localized close to the hinge residue of A324 in the mutant structure, absent in the wild-type counterpart in all cases (Figure 4). Since W1 and W2 were localized close to the hinge for all seeds, this method provided high reproducibility for

![Figure 2](image-url)
pinpointing trapped water molecules. We identified a group of five residues (Figure 4(B)) in the vicinity of these trapped water molecules (within 3 Å distance), which could be possible interaction members in the atomic network of domain 1 and 2 flexibility modulation. These residues included S483 and G484 located in the kinked region behind the hinge; D454 and V456 resided in the top groove of α-helix of domain 2 and the

Figure 3. The effects of T324A mutation of NS3 helicase on ATP-binding cleft width and fluctuation. (A) Hotspot residue locations in the interdomain region of the ATP-binding cleft for pairwise distance calculations. Hotspot residue pair Q460-E291 monitors the distance at the top, R464-D290 in the middle, and R467-T212 at the bottom section of the two domains. (B) Trajectories of minimum distances between hotspot residues R464 and D290 for the wild-type structure (red) and the T324A mutant structure (blue). (C) Histograms of minimum distance trajectories in (B). There is a 10% reduction of the mean, and ~27% decrease in the standard deviation for mutant (blue) relative to wild-type (red) structures.

Figure 4. Recruitment of two stable water molecules close to residue 324 due to the T324A mutation of NS3 helicase. Comparison of the structure around residue 324 for wild-type (A) and T324A mutant (B) illustrates newly trapped water molecules W1 and W2 for the mutant structure. These water molecules form possible atomic interaction partners, including residues S483 (orange), G484 (green), H293 (cyan), D454 (yellow), and V456 (magenta). (C) Interactions with the water molecules potentially decreasing the flexibility of the ATP-binding cleft. The newly trapped water molecules W1 and W2 potentially interact with H293 in domain 1 and D454 in domain 2 to reduce the flexibility of the ATP-binding cleft.
gatekeeper residue H293 at the bottom of interdomain cleft (Table 2). We determined that both H293 in domain 1 and D454 in the α-helix of domain 2 contained the arginine finger form two hydrogen bonds (H-bonds) with W1 and W2 (Table S-I), while W1 and W2 were also hydrogen bonded to each other forming a D454–W1–W2–H293 “hand-cuff” (Figure 4(C)). Similarly to a previous MD study using water bridge analysis (Roy & Thakur, 2010), we have found all of these H-bonds to be statistically significant based on the total time of hydrogen bond occurrence and the distance distribution of all hydrogen bonds. These results along with the low RMSF values (1.423 and 1.758 Å) corresponding to W1 and W2, demonstrated that this polar “hand-cuff” effect could possibly play a role in preventing the flexible movement of the ATP-binding cleft between domains 1 and 2 upon the hinge residue mutation.

Solvent accessible area differences were observed close to the hinge residue

In addition to identifying trapped water molecules, we have also examined SAS area and determined the locations with large changes in SAS upon mutation of the hinge residue. The SAS analysis showed that there is an obvious atomic rearrangement (Figure 5) of the microenvironment surrounding hinge residue T324 after mutation with respect to water accessibility. By averaging over three different seeds, we found three groups of residues close to the hinge residue with large SAS changes: (1) residues close to the Walker B motif, including D290, E291, C292, and H293 (~26% SAS increase); (2) residues in the immediate neighborhood of the hinge residue 324, from A321 to S328 (~20% SAS increase); and (3) a kinked region in domain 2 facing the hinge, including P482, S483, and G484 (Table 2).

The T324A mutation opened up a new 3-dimensional (3D) pocket formed by a tetrahedron with residue vertexes H293, T324, S483, and D454, where there was drastic increase in SAS and the two new water molecules (W1 and W2) localized. Our results showed not only that the 3D structural opening helped to recruit these solvent molecules, but also that the surrounding residues with increased SAS were responsible for their atomic positioning into this water pocket. We also found that the increased SAS of H293 and S483 directly influence the establishment of the hydrogen bonds formed between these residues with polar side chains and the trapped water molecules (W1 and W2) supporting our model of water molecule interactions bridging two domains. It was highly possible that the increased SAS of P326, G327, S328, and P482 indirectly promoted water molecule positioning by opening up more SAS in this newly formed water pocket.

Large normalized RMSF was identified in the mutant structure

In addition to identifying regions of increased SAS, we have also examined the RMSF of each residue and determined the locations with significant changes in RMSF after mutating the hinge residue. In the RMSF analysis, we observed that there was an atomic rearrangement (Figure 6) of the microenvironment causing a flexibility decrease in regions of the protein surrounding the hinge residue after T324A mutation. By averaging over three different seeds, we found five groups of residues close to the hinge residue with large RMSF decrease: (1) residues in the immediate neighborhood of hinge residue 324,
residues close to the Walker B motif (290–magenta), the α-helix of domain 2 (462–467, cyan), and the residues close to the Walker B motif (290–293, yellow).

Next, we zoomed down to the atomic level to identify the unique pair of participating atoms, where the true minimum distance occurred. Our results demonstrated a direct hydrogen bond with one of the trapped water molecules (W2). It was possible that this hydrogen bond restricted the atomic fluctuation of H293 (and its neighborhood by 35% projecting down to D290, E291), as they form the right-hand pillar of the multi-atomic bridge connecting the gatekeeper region with the top section of the α-helix of domain 2 of the ATP-binding cleft (Figure 4(C)). Our results also indicated that residue group 454–457, which formed the upper portion of the α-helix of domain 2, undergoes about 20% RMSF reduction, containing some of the strategically selected hotspot residues in the previously performed pairwise distance analysis. It also supported our model for water molecule interactions in the polar “hand-cuff”, since when the other trapped water molecule (W1) established a possible hydrogen bond with residue D454, the D454–W1–W2–H293 bridge restricted the flexibility of the ATP-binding cleft by a long-range atomic cascade originating at the hinge mutation site. It is interesting to note that the ~25% SAS increase in the immediate vicinity of T324 and H293 shown in the “Solvent Accessible Area Differences” section further explains the model developed here, since the two proposed residue pillars connecting to W1 and W2 may open up more area for water accessibility.

**Atomic interactions in the hinge region were altered by mutation**

To demonstrate that T324A mutation directly affected other residues in its immediate vicinity by forming chemical bonds or molecular interactions with particular atoms of the hinge, we defined a group of residues in the proximate neighborhood of the hinge within 3.25 Å comparing the neighboring residues of the wild-type and T324A mutant NS3 helicase apo structures.

By comparing three different seeds, we found two new residues unique to the T324A mutation in this group, namely residues H203 and V331 (Table 2), which were not present in the wild-type structure. To decide which one of the two was the best candidate to establish a direct atomic interaction with the hinge, we measured minimum distances for residue pairs 324-H203 and 324-V331 (Figure 7) based on the whole amino acids. We found that residue V331 demonstrated a more drastic decrease in the minimum distance measurements, i.e. it is the closest to the hinge (on average) compared to residue H203. When we next assessed the minimum distance between all the atoms of residue 324 and all the atoms of residue V331, our results indicated that it reduced from about 5 to 2.5 Å, which was close enough to form an atomic interaction between these two residues (Figure 8).

**Table 2**

| Residue | Distance (Å) |
|---------|--------------|
| H203    | 3.25         |
| V331    | 2.50         |
| H203    | 5.00         |
| V331    | 2.50         |
| H203    | 5.00         |
| V331    | 2.50         |

From A323 to S328 (~42% RMSF decrease, t-test p-value<0.01); (2) a kinked region in domain 2 facing the hinge, including P482, S483, and G484 (~40% RMSF decrease, p<0.01); (3) a residue cluster, from D454 to Q460, which connects the hinge to the α-helix of domain 2 (~30% RMSF decrease, p=0.012); (4) the α-helix of domain 2, which forms the left-hand region of the ATP-binding cleft, including R464 and R467 (~26% decrease, p=0.213); and (5) residues close to the Walker B motif, including D290, E291, C292, and H293 (~35% RMSF decrease, p<0.01) (Table 2).
that a particular atom pair of a hydrophobic interaction (A324:HB3–V331:HG12) was the pair having the minimum distance through most of the trajectory for the mutant structure (Figure 9). So, we not only demonstrated the minimum distance decrease from 5 to 2.5 Å between these two newly identified interaction partners, but also pinpointed the exact atomic interaction connections of A324 and V331, which only appeared with the hinge mutation. Based on these findings, residue V331 may contribute to the flexibility modulation of the ATP-binding cleft by directly interacting with the hinge.

Discussion

In this study, we have investigated the dynamic mechanism of HCV NS3 helicase ATP-binding cleft flexibility modulation. In our histogram analysis of pairwise distances between residues in domains 1 and 2, we showed a drastically reduced standard deviation of separation between the two domains in the mutant structure. This indicated that mutation of the hinge residue T324 to A triggers a flexibility decrease in the ATP-binding cleft. We showed that two new water molecules were recruited in the hinge vicinity, which directly interact with residues H293 and D454 via hydrogen bonds. We further hypothesized that these two residues and the trapped water molecules form an atomic bridge connecting the hinge region to the α-helix of domain 2, and thus restrict the flexibility of the ATP-binding cleft by a “hand-cuffing” effect. Our analysis identified a small set of residues, including residue H293 and S483, with significantly increased SAS when the hinge residue was mutated. We also found a set of residue groups with large RMSF decrease, in the immediate neighborhood of the hinge including A323 to S328; residues such as S483, which probably coordinated the immobilization of the two new water molecules; residues involved in the atomic connection between the hinge and the α-helix of domain 2; the left-hand arm of ATP-binding cleft, including the arginine finger R467; and residues close to the Walker B motif, including residue H293. Both of our SAS and RMSF results supported our water interaction model of a multiatomic bridge, thus providing a better understanding of the underlying molecular mechanism of the ATP-binding cleft flexibility modulation. Finally, we
pinpointed a direct atomic interaction between hinge residue A324 and V331 that provided suggestions for future experiments to decipher the atomic interaction cascade of flexibility control of the ATP-binding cleft.

The dynamic flexibility change of the ATP-binding cleft can possibly be explained by the following water molecule interactions. When T324 was mutated to A, it loses an OH group. This 3D structural change around the hinge residue may open up more space and allow the immobilization of two water molecules (W1 and W2) into a newly formed pocket, which was in close proximity to T324. During this atomic rearrangement, the gatekeeper residue H293 may establish a hydrogen bond (1.70 Å) with W2, while D454—located at the top region of the same α-helix on which the domain 2 hot-spot residues of the ATP-binding cleft reside—can also form a hydrogen bond (1.65 Å) with W1. Furthermore, W1 and W2 were also connected by a possible hydrogen bond (1.71 Å) completing an atomic bridge containing four molecules (D454–W1–W2–H293) linking the hinge region to the α-helix of domain 2, which was part of the highly conserved arginine finger critical for catalysis. We proposed that this intricate atomic cascade caused a “hand-cuffing” polar effect, such that the dynamic nature of hinge mechanism becomes more rigid, restricting flexibility of domains 1 and 2 for opening and closing of the ATP-binding cleft.

Even though residue S483 was not close enough (2.49 Å) to form a direct hydrogen bond with W2, we hypothesized that it can guide the two water molecules into the newly formed trapping pocket by polar–polar interaction of its side chain. It is also interesting to note that nonpolar residues G484, A455, and V456 do not possess a dipole moment, because side chains of such residues are insoluble in water. These hydrophobic residues do not form hydrogen bonds with water molecules (W1 and W2), but can force them into a rigid cage of hydrogen-bonded residues around it. Water molecules were normally in constant motion, and the formation of such cages restricts their motion. The synergistic effect of these polar (“hand-cuff”) and nonpolar (“water-cage”) atomic interactions may be responsible for water molecule immobilization close to the hinge region.

This proposed water molecule interaction was supported by the results of our RMSF analysis, since we observed a drastic 57% RMSF drop of residue S483 with an averaged 40% decrease in the residue cluster that is claimed to be responsible for the 3D guidance of water molecules into this pocket. Our results indicated that the polar–polar interaction between W1 and S483 restricted the atomic fluctuation of this residue, which also influenced the RMSF of the neighboring residues P482 and G484 as shown in a previous section. Similarly, we found a significant 30 and 16% RMSF decrease in residues G327 and S328, respectively, with an averaged 33% decrease in the residue group that may be responsible for the atomic rearrangement to form the water trapping pocket. P326 was a member of this group and may indirectly promote water molecule positioning by opening up more SAS. Its RMSF decrease clearly demonstrated that this nonpolar region can form one of the sides of the rigid water box.

In a previous study (Caruthers & McKay, 2002) that examined functions of conserved helicase motifs and their mechanistic role in ATP-dependent duplex oligonucleotide separation, it was shown that residues within Walker B motifs of SF-2 helicases, residue H293 in particular for HCV, interacted with the conserved glutamine of motif VI in domain 2. It was also pointed out that significant conformational rearrangement is required for the participating arginine residues to ligate ATP, which is only plausible because of the high interdomain flexibility of the protein. Our RMSF analysis was consistent with this observation, since the α-helix of domain 2 involved

![Figure 9. Possible hydrogen group interactions in the hinge region upon T324A mutation. (A) Trajectories of minimum distances between hydrogen groups of T324 and V331 for the wild-type (black) and the T324A mutant structure (green). (B) Histograms of minimum distance trajectories in (A).](image-url)
in the coupling of ATPase to helicase undergoes a drastic 33% RMSF decrease. This domain contained one of the strategically selected hotspot residues in our pairwise distance analysis, namely residue Q460 with 35% RMSF drop compared to the wild-type NS3 helicase apo structure. Furthermore, the α-helix of domain 2, which forms the left-hand region of ATP-binding cleft and contains R464 and R467 hotspot residues used in the pairwise distance analysis, undergoes a significant 25% RMSF decrease. The average distance between the mutated hinge residue A324 and the spatially distant residues (R458, S459, Q460, R464, and R467) was found to be in the range of 10–17 Å with a mean distance of 13.37 Å. These results indicated that the T324A mutation did not only influence the immediate neighborhood of the hinge by new water recruitment, but other spatially distant residues were also affected, demonstrating a long-range coupling mechanism that can transmit signals between distant sites resulting in the blocking of ATPase activity by restricting interdomain flexibility.

Consistent with our previous SAS and RMSF analysis, we found that residue V331 established a possible hydrophobic interaction with A324, and thus forms a loop containing residue cluster 325–328 (Figure 7). We proposed a mechanism in which residue A324 grabs onto V331 and pulls that protein region close to the hinge after mutation, thus promoting a regional flexibility decrease which is reflected by the 30 and 16% RMSF reductions of residues G327 and S328, respectively, with an averaged 33% decrease in the residue group that organized the water trapping pocket formation. This atomic linkage can be further explained by the RMSF reduction in the residue group which possibly forms one of the sides of the hydrophobic water box caging W1 and W2 into the newly formed pocket.

This analysis predicted a long-range atomic network effecting residues located in domain 1 of the ATP-binding cleft, which have not been explored with computational studies thus far. This provided targets for future MD simulations to evaluate the changes in this microenvironment. Also, our hypothesis that residue A324 grabs onto V331 and pulls that protein region close to the hinge after mutation, opens up further possibilities for domain 2 and residue V331 to interact. We suspected that the RMSF decrease in residues 454–457 was not only due to the water bridge, but also the atomic interaction between domain 2 and V331. This hypothesis calls for future evaluation to elucidate the exact relationship between ATPase activity decrease and 3D structural changes.

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Supplementary material

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References

Abrams, C.F., & Vanden-Eijnden, E. (2010). Large-scale conformational sampling of proteins using temperature-accelerated molecular dynamics. Proceedings of the National Academy of Sciences USA, 107, 4961–4966. doi: 10.1073/pnas.0914540107

Berendsen, H. (1995). GROMACS: A message-passing parallel molecular dynamics implementation. Computer Physics Communications, 91, 43–56. doi: 10.1016/0010-4655(95)00042-E

Berendsen, H.J.C., Postma, J.P.M., Van Gunsteren, W.F., & Hermans, J. (1981). Interaction models for water in relation to protein hydration. Intermolecular Forces, 11, 331–338. doi: 10.1111/j.1574-695X.1996.tb00128.x

Boehr, D.D., Nussinov, R., & Wright, P.E. (2009). The role of dynamic conformational ensembles in biomolecular recognition. Nature Chemical Biology, 5, 789–796. doi: 10.1038/ncb1822

Carlson, H.A., & McCammon, J.A. (2000). Accommodating protein flexibility in computational drug design. Molecular Pharmacology, 57, 213–218. Retrieved from http://molpharm.aspetjournals.org/content/57/2/213.full

Caruthers, J.M., & McKay, D.B. (2002). Helicase structure and mechanism. Current Opinion in Structural Biology, 12, 123–133. doi: 10.1016/S0959-440X(02)00298-1

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., & Houghton, M. (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science, 244, 359–362. doi: 10.1126/science.2523562

De Francesco, R., & Migliazzo, G. (2005). Challenges and successes in developing new therapies for hepatitis C. Nature, 436, 953–960. doi: 10.1038/nature04080

Dodson, G., & Verma, C.S. (2006). Protein flexibility: Its role in structure and mechanism revealed by molecular simulations. Cellular and Molecular Life Sciences, 63, 207–219. doi: 10.1007/s00018-005-5256-7

Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., ... Sawyers, C.L. (2001). Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. The New England Journal of Medicine, 344, 1031–1037. doi: 10.1056/NEJM200104053441401

Dumont, S., Cheng, W., Serebrov, V., Beran, R.K., Tinoco, I., Jr., Pyle, A.M., & Bustamante, C. (2006). RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. Nature, 439, 105–108. doi: 10.1038/nature04331

Durrant, J.D., & McCammon, J.A. (2010). Computer-aided drug-discovery techniques that account for receptor flexibility. Current Opinion in Pharmacology, 10, 770–774. doi: 10.1016/j.coph.2010.09.001

Frick, D.N. (2003). Helicases as antiviral drug targets. Drug News Perspect, 16, 355–362.

Gorbaleny, A.E., & Koonin, E.V. (1993). Helicases: Amino acid sequence comparisons and structure–function relationships. Current Opinion in Structural Biology, 3, 419–429. doi: 10.1016/S0959-440X(05)80116-2
Grant, B.J., Gorfe, A.A., & McCammon, J.A. (2010). Large conformational changes in proteins: Signaling and other functions. *Current Opinion in Structural Biology*, 20, 142–147. doi: 10.1016/j.sbi.2009.12.004

Gu, M., & Rice, C.M. (2010). Three conformational snapshots of the hepatitis C virus NS3 helicase reveal a ratchet translational mechanism. *Proceedings of the National Academy of Sciences USA*, 107, 521–528. doi: 10.1073/pnas.0913380107

Guex, N., & Peitsch, M.C. (1997). SWISS-MODEL and the Swiss-PdbViewer: An environment for comparative protein modeling. *Electrophoresis*, 18, 2714–2723. doi: 10.1002/elps.1150181505

Hockney, R.W. (1970). The potential calculation and some applications. *Methods in Computational Physics*, 9, 136–211.

Ivetac, A., & McCammon, J.A. (2011). Molecular recognition in the case of flexible targets. *Current Pharmaceutical Design*, 17, 1663–1671.

Ivetac, A., & McCammon, J.A. (2012). A molecular dynamics ensemble-based approach for the mapping of druggable binding sites. *Methods in Molecular Biology*, 819, 3–12. doi: 10.1007/978-1-61779-299-5_1

Karplus, M., & Kuriyan, J. (2005). Molecular dynamics and protein function. *Proceedings of the National Academy of Sciences USA*, 102, 6679–6685. doi: 10.1073/pnas.0408930102

Kim, D.W., Kim, J., Gwack, Y., Han, J.H., & Choe, J. (1997). Mutational analysis of the hepatitis C virus RNA helicase. *Journal of Virology*, 71, 9400–9409.

Kim, J.L., Morgenstern, K.A., Griffith, J.P., Dwyer, M.D., Thomson, J.A., Murcko, M.A., … Caron, P.R. (1998). Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: The crystal structure provides insights into the mode of unwinding. *Structure*, 6, 89–100. doi: 10.1016/S0969-2126(98)00010-0

Korolov, S., Yao, N., Lohman, T.M., Weber, P.C., & Waksman, G. (1998). Comparisons between the structures of HCV and Rep helicases reveal structural similarities between SF1 and SF2 super-families of helicases. *Protein Science*, 7, 605–610. doi: 10.1002/pro.5560070309

Kwong, A.D., Kim, J.L., & Lin, C. (2000). Structure and function of hepatitis C virus NS3 helicase. *Current Topics in Microbiology and Immunology*, 242, 171–196.

Li, G., & Cui, Q. (2004). Mechanochemical coupling in myosin: A Theoretical analysis with molecular dynamics and combined QM/MM reaction path calculations. *The Journal of Physical Chemistry B*, 108, 3342–3357. doi: 10.1021/jp037183

Liao, J.C. (2011). Mechanical transduction mechanisms of RecA-like molecular motors. *Journal of Biomolecular Structure & Dynamics*, 29, 497–507.

Liao, J.C., Sun, S., Chandler, D., & Oster, G. (2004). The conformational states of Mg-ATP in water. *European Biophysics Journal*, 33, 29–37. doi: 10.1007/s00249-003-0339-2

Min, K.H., Sung, Y.C., Choi, S.Y., & Ahn, B.Y. (1999). Functional interactions between conserved motifs of the hepatitis C virus RNA helicase protein NS3. *Virus Genes*, 19, 33–43. doi: 10.1023/A:1008184522153

Preuschat, F., Averett, D.R., Clarke, B.E., & Porter, D.J. (1996). A steady-state and pre-steady-state kinetic analysis of the NTPase activity associated with the hepatitis C virus NS3 helicase domain. *Journal of Biological Chemistry*, 271, 24449–24457. doi: 10.1074/jbc.271.40.24449

Roy, S., & Thakur, A.R. (2010). 20ns molecular dynamics simulation of the antennapedia homeodomain-DNA complex: Water interaction and DNA structure analysis. *Journal of Biomolecular Structure & Dynamics*, 27, 443–456.

Sotomayor, M., & Schulten, K. (2007). Single-molecule experiments in vitro and in silico. *Science*, 316, 1144–1148. doi: 10.1126/science.1137591

Soulutanas, P., Dillingham, M.S., Velankar, S.S., & Wigley, D.B. (1999). DNA binding mediates conformational changes and metal ion coordination in the active site of PcrA helicase. *Journal of Molecular Biology*, 290, 137–148. doi: 10.1006/jmbi.1999.2873

Suzich, J.A., Tamura, J.K., Palmer-Hill, F., Warrener, P., Grakoui, A., Rice, C.M., Feinstone, S.M., & Collett, M.S. (1993). Hepatitis C virus NS3 protein polynucleotide-stimulated nucleoside triphosphatase and comparison with the related pestivirus and flavivirus enzymes. *Journal of Virology*, 67, 6152–6158.

Tai, C.L., Pan, W.C., Liaw, S.H., Yang, U.C., Hwang, L.H., & Chen, D.S. (2001). Structure-based mutational analysis of the hepatitis C virus NS3 helicase. *Journal of Virology*, 75, 8289–8297.

Wang, J., Cieplak, P., & Kollman, P.A. (2000). How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? *Journal of Computational Chemistry*, 21, 1049–1074. doi: 10.1002/1096-987X(200009)21:12<1049::AID-JCC3>3.3.CO;2-6

Wright, P.E., & Dyson, H.J. (2009). Linking folding and binding. *Current Opinion in Structural Biology*, 19, 31–38. doi: 10.1016/j.sbi.2008.12.003

Yu, J., Ha, T., & Schulten, K. (2006). Structure-based model of the stepping motor of PcrA helicase. *Biophysical Journal*, 91, 2097–2114. doi: 10.1529/biophysj.105.08203

Zheng, W., Liao, J.C., Brooks, B.R., & Doniach, S. (2007). Toward the mechanism of dynamical couplings and translocation in hepatitis C virus NS3 helicase using elastic network model. *Proteins*, 67, 886–896. doi: 10.1002/prot.21326