Crystal structure of RNA helicase domain from genotype 1b hepatitis C virus has been determined at 2.3 Å resolution by the multiple isomorphous replacement method. The structure consists of three domains that form a Y-shaped molecule. One is a NTPase domain containing two highly conserved NTP binding motifs. Another is an RNA binding domain containing a conserved RNA binding motif. The third is a helical domain that contains no β-strand. The RNA binding domain of the molecule is distinctively separated from the other two domains forming an interdomain cleft into which single stranded RNA can be modeled. A channel is found between a pair of symmetry-related molecules which exhibit the most extensive crystal packing interactions. A stretch of single stranded RNA can be modeled with electrostatic complementarity into the interdomain cleft and continuously through the channel. These observations suggest that some form of this dimer is likely to be the functional form that unwinds double stranded RNA processively by passing one strand of RNA through the channel and passing the other strand outside of the dimer. A “descending molecular see-saw” model is proposed that is consistent with directionality of unwinding and other physicochemical properties of RNA helicases.

Helicases are enzymes catalyzing strand separation of double stranded DNA (dsDNA) or dsRNA coupled with hydrolysis of NTP. They are required for many cellular events including transcription, RNA processing, translation, and DNA or RNA replication (1–3). Since the first discovery of DNA helicase activity more than 20 years ago (4, 5), many different helicases have been identified with preferences for unwinding duplexes of DNA or RNA. In in vitro experiments, nearly all helicases require a single stranded region; some require a 3’ overhang region (3’ to 5’ helicases), whereas others require a 5’ overhang region (5’ to 3’ helicases). This single stranded region is proposed to provide an initiation site for unwinding duplex nucleic acids (6). *Escherichia coli* Rep DNA helicase, an extensively studied helicase, exhibited highly processive unwinding of replicative form of phage DNA in an in vitro experiment (7). In order for a helicase to unwind duplex nucleic acids in a processive manner, the enzyme should destabilize the hydrogen bonds between the base pairs, translocate to the next base paired region, and repeat the cycle without fully dissociating (6, 8). Recently, oligomeric forms, generally dimers or hexamers, were observed for some DNA helicases (8, 9). These oligomers are believed to provide the helicases with multiple nucleic acid binding sites necessary for the helicase function (6). Although Rep DNA helicase, for example, is a stable monomer in solution in the absence of DNA, a dimeric form of Rep is induced in the presence of DNA which is known as the functional form (6, 8).

Hepatitis C viruses (HCVs) are the major etiologic agents of non-A, non-B hepatitis that are estimated to have infected about 1% of the population worldwide. HCV belongs to flaviviridae, the positive-strand RNA virus family (10). Its genome consists of about 9400 nucleotides with the gene order of N-E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-C (10) encoding a viral polyprotein of about 3010 residues (11). The polyprotein is processed into functional proteins by host- and virus-encoded proteases. Among the processed proteins, NS3 is best characterized. The N-terminal one-third of NS3 is a serine protease domain (12) which is known to cleave the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions (13–15). The C-terminal two-thirds of NS3 is an RNA helicase domain exhibiting nucleotide triphosphatase/RNA helicase activity (16, 17). The domain was shown to unwind not only dsRNA but also RNA/DNA heteroduplex and dsDNA (18). For its function the helicase domain strictly requires a 3’ overhang region, and it unwinds double stranded nucleic acids only in the 3’ to 5’ direction (18).

Sequence alignment of many RNA helicases revealed four highly conserved sequence motifs, and in HCV RNA helicase they are conserved as G207SGKST, D285ECH, T322AT, and Q460RRGRTGRGGR sequences. The G207SGKST sequence, known as Walker A motif, is found in nearly all NTP hydrolyzing enzymes and is responsible for NTP binding. The D285ECH sequence is a variant of Walker B motif (19). Biochemical and mutational analyses showed that the T322AT sequence is important in unwinding of RNA, whereas the Q460RRGRTGRGGR sequence is important in the RNA binding and the unwinding of RNA (20, 21).

Here we report the crystal structure of the genotype 1b HCV RNA helicase domain and discuss in detail the structural features of the conserved motifs. Based on a modeling experiment we propose a mechanism of processive unwinding of the duplex RNA consistent with previously observed physicochemical properties of the enzyme.
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**MATERIALS AND METHODS**

**Protein Purification and Crystallization**—The HCV RNA helicase domain was isolated from an overexpressing E. coli strain (BL21(DE3)) and purified using a Ni-NTA-agarose column (Qiagen) and a poly(U)-Sepharose column (Amersham Pharmacia Biotech) successively as described previously (22). Crystals were grown at 4 °C from a precipitant solution containing 30% polyethylene glycol 4000, 0.1 M sodium cacodylate (pH 6.5), and 0.2 M ammonium acetate on micro batch plates under Al’s oil (Hampton Research). The crystals belong to the space group P3121 with the unit cell dimensions of a = b = 93.3 Å, c = 140.6 Å. The crystals contain one molecule of the enzyme in the asymmetric unit.

**Data Collection, Structure Determination, and Refinement**—All data were measured from flash-frozen crystals on a DI2020 area detector system with graphite monochromated CuKα radiation. The crystals contain one molecule of the enzyme in the asymmetric unit. The crystals belong to the space group P3121 with the unit cell dimensions of a = b = 93.3 Å, c = 140.6 Å. The crystals contain one molecule of the enzyme in the asymmetric unit. The crystals contain one molecule of the enzyme in the asymmetric unit.

**RESULTS AND DISCUSSION**

**Protein Fold and Structural Features**—The structure of HCV RNA helicase consists of three nearly equal-sized domains that form a Y-shaped molecule (Fig. 1). The N-terminal one-third of the enzyme is a NTPase domain consisting of a typical central core of pleated sheet surrounded by helices (27). The active site cleft for the NTP hydrolysis can be readily identified at the periphery of the domain by the G207SGKST sequence, the NTP binding motif. The second domain is an RNA binding domain containing the highly conserved G460RRGRGRGRGRG sequence identified as an RNA binding motif (Fig. 1). The folding pattern of the RNA binding domain is similar to that of the NTPase domain, but it contains fewer α-helices. The C-terminal one-third of the enzyme is a helical domain composed of five α-helices and loops. The NTPase and the helical domain are more or less continuously linked with a shallow groove between the two, but the RNA binding domain is distinctly separated from the other two domains forming a deep interdomain cleft as shown in Fig. 1. The size of the interdomain cleft is adequate for binding ssRNA (or ssDNA) but too narrow for binding double stranded nucleic acids. The NTPase and the RNA binding domain are connected by two random coils (Fig. 1). In contrast, two antiparallel β-strands, unusually protruding from the RNA binding domain, are inserted into the helical domain like an anchor linking the two domains (Fig. 1). The turn region of the antiparallel β-strands is rich in hydrophobic amino acids and interact extensively with apolar residues of the helical domain.

It remains a question why NS3 containing two completely different activities, protease and helicase activities, is cleaved into two polypeptides in the processing of the viral polyprotein. In the crystal structure presented here, the electron density for the first 39 amino acid is not visible, indicating that the protease domain and the helicase domain of NS3 should be linked to each other by a highly flexible loop region. The visible N terminus of the helicase is at the back side of the molecule (opposite the interdomain cleft), and thus the enzyme activities of the protease and the helicase domain appear independent of each other (see below).

**NTP Binding Site**—The NTP binding site is located at the periphery of the NTPase domain. The G207SGKST and the D296ECH sequences are close to each other, lining part of the active site cavity (Fig. 2). The side chain of Asp296 is involved in an ionic interaction with Lys210 and a hydrogen bond with the side chain of Ser291. The D296ECH sequence is on an unusual loop structure which orients the side chains of Asp296, Glu291, and His293 toward the cavity and that of Cys392 in the opposite direction (Fig. 3). The functional roles of Asp296 and Glu291 can be inferred from the structures of other NTPases. The crystal structures of Bacillus stearothermophilus PrCα (28) and E. coli Rep DNA helicases (29) were determined as complexes with ADP. It has been proposed that in the major domain of RecA, which exhibits similarity to the NTPase domains of the DNA helicases, the magnesium ion of Mg-ATP is coordinated by Asp290 and Glu291 and thus the protease domain and the helicase domain of NS3 should be linked to each other by a highly flexible loop region. The visible N terminus of the helicase is at the back side of the molecule (opposite the interdomain cleft), and thus the enzyme activities of the protease and the helicase domain appear independent of each other (see below).

**TABLE I**

| Crystal | Resolution (Å) | Completeness (%) | Soaking concentration | Soaking time (h) | R_{sym} (%) | R_{free} (%) | Phasing power (acent/cent) | R_{c2l} (%) | Figure of merit |
|---------|----------------|------------------|-----------------------|-----------------|------------|-------------|-------------------|-----------|---------------|
| Native  | 2.30           | 65               | 98.8                  | 0.5             | 21.7       | 4           | (1.0/1.4)         | (0.86/0.82) | 0.55          |
| Thimerosal | 2.80         | 5.5              | 85.2                  | 10              | 6          | 13.4        | (0.7/1.0)         | (0.78/0.71) |               |
| KIrCl₆   | 2.80           | 4.4              | 89.4                  | 6               | 6          | 19.3        | (0.5/0.7)         | (0.88/0.86) | 0.55          |
| KAu(CN)₂ | 4.0            | 7.0              | 89.6                  | 6               |            |             |                   |           |               |

**Stereochemical quality**

- R_{med} bond lengths (Å): 0.011
- R_{med} bond angles (degree): 1.649
- Ramachandran plot (%): Most favored regions 89.1
- Additional allowed regions 10.9
- Disallowed regions 0.0

**Results**

The visible N terminus of the helicase is at the back side of the molecule (opposite the interdomain cleft), and thus the enzyme activities of the protease and the helicase domain appear independent of each other (see below).
hydrolysis of ATP. When the NTPase domains of HCV RNA helicase and PcrA DNA helicase were superposed, Asp<sup>290</sup> and Glu<sup>291</sup> of HCV RNA helicase occupy relatively the same space as do Asp<sup>223</sup> and Glu<sup>224</sup> of the DNA helicase (data not shown), although overall structures of the two enzymes are quite different. This observation implies that the two residues probably share the same functions in the hydrolysis of NTP with the corresponding residues in the DNA helicases and RecA. Consistently, studies of the related DEXH helicase from vaccinia virus demonstrated that substitution of Asp<sup>296</sup> or Glu<sup>297</sup> in the DE<sub>WX</sub>XH motif with alanine abolishes the NTPase activity and the helicase activity without affecting the RNA binding affinity (31). The electron density for the NTP binding site is very strong showing detailed features including many bound water molecules (Fig. 2). However, it was not possible to predict a correct binding mode of NTP by simple model building due to severe steric clashes. Some conformational change in the active site cavity is expected to occur upon NTP binding.

Flexible Hinge—One of the two loops connecting the NTPase and the RNA binding domain contains the invariant T<sup>322</sup>AT. In the structure presented here, the hydroxyl group of Thr<sup>322</sup>, located at the beginning of the loop (Fig. 1, 3), is involved in a hydrogen bond with the imidazole ring nitrogen of His<sup>293</sup>. In contrast, the hydroxyl group of Thr<sup>324</sup>, located toward the middle of the loop and about 4 Å apart from His<sup>292</sup>, is just exposed to the bulk solvent (Fig. 3). It is generally believed based on mutational studies that the T<sup>322</sup>AT sequence couples the NTP hydrolysis and the duplex unwinding by the enzyme (20). In other experiments, H<sub>293</sub>A mutation in HCV RNA helicase and the corresponding mutation in vaccinia virus RNA helicase were shown to affect severely the duplex unwinding activity without affecting the NTP hydrolysis activity (31, 32). Thus, His<sup>293</sup>, Thr<sup>322</sup>, and Thr<sup>324</sup> may function as a triad in coupling the NTP hydrolysis and the helicase activity. It is possible that His<sup>293</sup> could switch its hydrogen bond to and from Thr<sup>322</sup> and Thr<sup>324</sup> during the helicase function, which should require a small flexible hinge motion of the connecting loops considering the proximity of the three residues. As supporting evidence, in the crystal structure of highly homologous genotype 1a HCV RNA helicase which was determined very recently, two molecules in the asymmetric unit displaying 3–4° rigid body rotation of the RNA binding domain with respect to each other undergo a small hinge bending motion of the two loops (33). The domain movement appears intrinsically small due to the presence of the two antiparallel β-strands which link...
the RNA binding and the helical domain. The "structured" strands, interacting heavily with the helical domain, are unlikely to undergo an appreciable conformational change. In the crystal structure of genotype 1a HCV RNA helicase, the β-strands of the two molecules in the asymmetric unit show a negligible twist with respect to each other. Consistently, de-
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spite the completely different crystal packing, the RNA helicase structure presented here (in the space group P3_121) does not exhibit any noticeable closure or opening of the interdomain cleft compared with the structure of genotype 1a HCV RNA helicase (in the space group P2_121).

**RNA Binding Motif**—The RNA binding motif, Q^{460}RRGR^T^GRRGG sequence, shows unusually high occurrence of glycine and arginine. The first three residues constitute the end of an α-helix, and the rest of the residues forms a loop structure (Fig. 1, 4). The high occurrence of glycine on the loop structure strongly suggest that the sequence can easily undergo a conformational change necessary for the alignment of the arginine residues on the loop structure in favorable contacts with the phosphate backbone of RNA. It was noted that the side chains of the most conserved residues, Gln^{460}, Arg^{461}, Arg^{464}, and Arg^{467}, point to the interdomain cleft with an ~7 Å spacing (Fig. 4).

**Putative Functional Dimeric Model**—Entrapment of substrates within protein structures has been observed as a common theme for several proteins which possess a processivity in the interaction with nucleic acids, including β subunit of E. coli DNA polymerase III (34) and 1-exonuclease (35). A functional oligomeric state of HCV helicase is not known but has been proposed equivocally as a monomer or a dimer (36). We examined crystal packing interfaces in the crystal structure and found that a symmetry-related monomer-monomer interaction could reflect interfaces of a functional form of the RNA helicase. The interfaces are formed by the interactions between the NTPase and the RNA binding domains of the two molecules and represent the most extensive crystal packing interactions.

Despite the completely different crystal packing, the RNA helicase structure presented here (in the space group P3_121) does not exhibit any noticeable closure or opening of the interdomain cleft compared with the structure of genotype 1a HCV RNA helicase (in the space group P2_121).

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ssRNA at the interdomain cleft can be considered as an activated molecule, whereas the RNA helicase without a bound ssRNA can be considered as a resting molecule. It is not known whether the RNA and the NTP binding to the enzyme are sequential or random. Our modeling experiment, which shows that the bound ssRNA dose not block the NTP binding site, cannot distinguish the two. Whether it is sequential or random, ATP hydrolysis would occur mainly on the activated molecule.

**Fig. 7.** A side view of “descending molecular see-saw” model for the translocation of HCV RNA helicase along ssRNA. The dimer (left) translocates along the ssRNA (right) by a rotation of about 60° (with respect to the axis of the RNA double helix) along an axis passing the front part of RNA binding motif. The right figure represents a translocation of the dimer by a half-turn of RNA with respect to the location of the dimer on ssRNA in the left figure. For clear presentation of the translational motion, the two figures are shown with orientations different by 180° with respect to each step. Figs. 1, 3, and 5 were produced using the program MOLSCRIPT, Fig. 2 using the program O, Fig. 4 using the program GRASP, and Figs. 6 and 7 using the program QUANTA.

**Fig. 8.** Schematic drawings for the proposed mechanism of processive duplex unwinding by HCV RNA helicase. A, a helicase molecule (α) binds the single stranded portion of RNA. B, subsequently, a functional dimer is formed by the binding of another helicase molecule (β). The ssRNA bound to the interdomain cleft of α is proposed to induce a small conformational change which increases the NTP hydrolysis activity. The dimer is stabilized by the interaction of ssRNA with the RNA binding motifs of α and β. C, the NTP hydrolysis by α in B results in the detachment of the ssRNA and a rigid body rotation of the dimer along an axis at the RNA binding motif of α. As a result, the dimer translocates along the ssRNA in the 5’ direction, and the interdomain cleft of β binds the other portion of the ssRNA. D, the dimer reaches the junction of ssRNA and dsRNA by repeated cycles of the translocation. E, in the same manner, the dimer translocates along the same strand of RNA. Energy required for the disruption of base pairings can be supplied by favorable interactions between the interdomain cleft and the ssRNA. One strand passing through the channel at the dimer interface is separated from the other strand hanging out of the dimer.
symmetric dimer composed of the two resting molecules presented here. The dimer interface in the crystal structure does not involve any specific interaction such as ion pairs between charged amino acids. Thus, the slight rotation of the RNA binding domain at the dimer interface could easily occur upon the induced conformational change by the ssRNA binding.

Mechanism of Duplex Unwinding—With this putative functional dimeric model, we propose a mechanism of processive unwinding of duplex RNA coupled with the NTP hydrolysis by HCV and related RNA helicases. ATP decreases the affinity of HCV RNA helicase for deoxyuracil 18 mer by 95% (36), indicating that the ATP hydrolysis results in the dissociation of RNA from the enzyme. Based on this observation, it can be hypothesized that the NTP hydrolysis causes a hinge bending motion which transforms the activated conformation of the enzyme to the resting conformation concomitant with the detachment of the bound ssRNA from the interdomain cleft. In the model presented here, the detached ssRNA can be closer to and bound by the interdomain cleft of the resting molecule of the putative functional dimer. This can be described as a rotation of the dimer relative to the bound ssRNA (Fig. 7). In order for the dimer to translocate on the ssRNA by the rotational motion, a rotation axis should be toward the 5′ end of the bound RNA relative to the “pseudo-” 2-fold symmetry axis of the functional dimer. In the structure of the dimer, the front part of the RNA binding motif of one molecule is below the 2-fold symmetry axis (Fig. 6). It was shown that individual alanine substitutions of the conserved arginine residues in the RNA binding motif in vaccinia virus RNA helicase cause severe defects in RNA unwinding with slight reduction in RNA binding affinity (21). This mutational study led to the conclusion that the motif must play an essential role in the helicase mechanism. The structural observation and the biochemical data suggest that a front part of the RNA binding motif serves as the pivoting region for the rotation in the context of the proposed model. About 60° rotation of the dimer along an axis passing the front part of the RNA binding motif of the activated molecule brings the resting molecule into contact with the ssRNA and results in a translocation of the dimer toward the 5′ direction as shown in Fig. 7. After this one cycle of the rotation and translation the conformations between the activated molecule and the resting molecule are exchanged, reproducing the same RNA binding mode of the functional dimer. Repeated cycles of the rotation and translation along the ssRNA containing the 3′ overhang can be described as “descending molecular see-saw” motion (Fig. 7). Since the size of the interdomain cleft is adequate only for ssRNA, duplex unwinding by the disruption of base pairings should occur at the ssRNA and dsRNA junction. Required energy can be provided by the favorable interaction between the interdomain cleft and the ssRNA. The translocation of the dimer along the ssRNA is the process of duplex unwinding because one strand of RNA passing through the channel is separated from the other strand hanging out of the dimer (Fig. 6, 7). The step size of duplex unwinding and translation along the ssRNA is about 5 nucleotides when the dimer rotates along an axis at the front part of the RNA binding motif (about Arg462). This coincides with the step size of UvrD helicase obtained by kinetic measurement (39).

Second, the functional dimer moves along the ssRNA containing 3′ overhang in the 3′ to 5′ direction as previously observed (40). It was not possible to insert ssRNA into the dimeric structure in the 5′ to 3′ direction simply due to severe steric clashes. Third, the model requires the pivoting motion around the RNA binding motif for the duplex unwinding. This explains that the RNA binding motif is necessary not only for the binding of RNA but also more importantly for the unwinding activity of the enzyme. Fourth, since the functional dimer translates along only one strand of nucleic acids in this model, the dimer is able to unwind dsRNA, dsDNA, and DNA/RNA duplex as previously known. Besides, since the dimer uses only the front side of the molecules for the duplex unwinding and the flexible N terminus is located at the back side of the molecule, the protease domain linked to the helicase domain in NS3 would not interfere the movement of NS3 along RNA substrates in vivo.

The proposed model is analogous in concept to the “rotary-type” mechanism of F1-ATPase. It is believed that a 120° rotation of γ subunit of F1-ATPase induces sequential conformational changes of the α, β subunits. Each subunit alternates among three conformations, ADP and P, bound, ATP bound, and none-bound (41). Like the γ subunit of F1-ATPase, the bound ssRNA results in the asymmetric functional dimer, each molecule of which alternates between the activated and the resting conformation. Interestingly, it was proposed recently that hexameric T7 DNA helicase encircles only one strand of DNA (42), as does the functional dimeric model proposed here. In conclusion, the “descending molecular see-saw” model is presented consistent with the previously observed biochemical data for the RNA helicases. The model provides a plausible framework explaining how the enzymes achieve the duplex unwinding and the translocation along nucleic acids in a processive manner.

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