Enzymatic properties of hepatitis C virus NS3-associated helicase

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The hepatitis C virus non-structural protein 3 (NS3) possesses a serine protease activity in the N-terminal one-third, whereas RNA-stimulated NTPase and helicase activities reside in the C-terminal portion. In this study, an N-terminal hexahistidine-tagged full-length NS3 polypeptide was expressed in Escherichia coli and purified to homogeneity by conventional chromatography. Detailed characterization of the helicase activity of NS3 is presented with regard to its binding and strand release activities on different RNA substrates. On RNA double-hybrid substrates, the enzyme was shown to perform unwinding activity starting from an internal ssRNA region of at least 3 nt and moving along the duplex in a 3' to 5' direction. In addition, data are presented suggesting that binding to ATP reduces the affinity of NS3 for ssRNA and increases its affinity for duplex RNA. Furthermore, we have ascertained the capacity of NS3 to specifically interact with and resolve the stem–loop RNA structure (SL I) within the 3'-terminal 46 bases of the viral genome. Finally, our analysis of NS3 processive unwinding under single cycle conditions by addition of heparin in both helicase and RNA-stimulated ATPase assays led to two conclusions: (i) NS3-associated helicase acts processively; (ii) most of the NS3 RNA-stimulated ATPase activity may not be directly coupled to translocation of the enzyme along the substrate RNA molecule.

Introduction

Hepatitis C virus (HCV) presents a major health problem. The global prevalence of chronic hepatitis C is estimated to average 3%. In industrialized countries, HCV accounts for 20% of cases of acute hepatitis, 70% of cases of chronic hepatitis, 40% of cases of end-stage cirrhosis, 60% of cases of hepatocellular carcinoma and 30% of liver transplants (reviewed by Houghton, 1996). HCV contains an approximately 9.6 kilobase positive-sense ssRNA genome and is classified in the Flaviviridae family of animal viruses (reviewed by Rice, 1996). Its genome consists of a conserved 5' non-translated sequence that serves as an internal ribosome entry site (5'-NTR), a single open reading frame that encodes a polypeptide of more than 3000 amino acids, and a 3' non-translated region that contains tracts of poly(U)n, followed by a conserved 98 nt sequence (3'-NTR). Proteolytic processing of the viral polyprotein by cellular and virus-encoded proteases generates mature core, envelope and non-structural (NS) proteins (C-E1-E2-p7-NS2-NS3A-NS4A-NS4B-NS5A-NS5B) (reviewed by Clarke, 1997; Lohmann et al., 1996). Non-structural protein 3 (NS3) is a bifunctional enzyme exhibiting a protease activity in the N-terminal one-third and an RNA helicase activity in the C-terminal portion. The NS3 protease belongs to the serine protease family and is responsible for processing the HCV polyprotein at NS3/NS4A, NS4A/NS4B, NS4B/NS5A and NS5A/NS5B junctions (reviewed by De Francesco et al., 1998; Kwong et al., 1998). The helicase activity, associated with a nucleic acid-stimulated NTPase activity (Gwack et al., 1995; Jin & Peterson, 1995; Kim et al., 1995; Porter, 1998; Preugschat et al., 1996), is presumed to be involved in the replication and/or translation of viral RNA. The minimal requirement for these latter functions lies in the C-terminal 465 amino acids of NS3, which represent a functionally and structurally separate domain (Kim et al., 1995). Recently, we have demonstrated that although the N-terminal protease domain has little if any effect on the ATPase and helicase activities of NS3 (Gallinari et al., 1998), NS4A-mediated stabilization of NS3 in the active protease conformation can negatively affect its ability to unwind dsRNA (Gallinari et al., 1999). Different three-dimensional structures of the isolated NS3 helicase domain have been determined (Cho et al., 1998; Kim...
et al., 1998; Yao et al., 1997). The enzyme comprises three domains termed 1, 2 and 3, with domains 1 and 2 being structurally similar. The ATP-binding site is situated in a cleft between domains 1 and 2 that is lined with conserved sequence motifs that are the hallmarks of helicases (Gorbalenya & Koonin, 1993). NS3 is a member of a large class of helicases that have a 3’ to 5’ directionality and share a number of structural features (Bird et al., 1998). Other members of this class include the Rep, PcrA and UvrD bacterial DNA helicases for which much structural data became available recently (Korolev et al., 1997; Subramanya et al., 1996). NS3 helicase domains 1 and 2 fold similar to domains 1A and 2A of the above-mentioned enzymes, but with a slightly different connectivity (Bird et al., 1998). The third domain has no structural similarity with Rep or PcrA but sits in a position approximately equivalent to that occupied by domain 1B in the Rep helicase (Korolev et al., 1998). The structure of a complex of the NS3 helicase domain with a (dU)n oligonucleotide (Kim et al., 1998) demonstrated the nucleic acid in a channel that separates domain 3 from domains 1 and 2, in a position equivalent to the binding site for ssDNA in the Rep (Korolev et al., 1997) and PcrA (Velankar et al., 1999) enzymes. Three different models for the mechanism of NS3 helicase have been proposed, one to accompany each of the three published crystal structures. To get more insight on the overall mechanism of unwinding, we have analysed the effect that binding of ATP (or non-hydrolysable ATP analogues) to the full-length (FL) enzyme exerts on its relative affinity for ssRNA and dsRNA.

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 dissociating from the RNA substrate. Here we have analysed the FL-NS3 processive unwinding under single cycle conditions using heparin as a trapping molecule both in helicase and ATPase assays.

The helicase activity of NS3 was previously shown to require substrates with a free 3’ single-stranded tail (Gwack et al., 1996; Tai et al., 1996). In this study, we have measured NS3 unwinding activity on double-hybrid substrates designed to mimic stem-loop structures, containing internal ssRNA regions of different lengths. The ability of NS3 to interact preferentially with HCV genomic or antigenic RNA has not been addressed so far. We and others have previously shown that in the absence of ATP, NS3 binds ssRNA tightly with no particular sequence specificity and with efficiency depending only on the length of ssRNA (Gallinari et al., 1998; Gwack et al., 1996; Kanai et al., 1995; Tai et al., 1996). Therefore, it was of interest to assess whether NS3 shows a selective interaction with the 3’ ends of the viral genome and antigenome, the presumed initiation sites for negative- and positive-strand RNA synthesis, respectively. We have studied the interaction and the helicase function of FL-NS3 with a series of RNA oligonucleotides derived from the 46 nt stem-loop structure (SL I) at the 3’ terminus of HCV genomic positive-strand RNA (Blight & Rice, 1997; Kolykhlov et al., 1996; Tanaka et al., 1996). The stability and structural conservation of SL I suggested that it represents a recognition site for viral and/or cellular proteins involved in virus replication. While some of the protein–RNA interactions at the 3’-end of the genome have been recently elucidated (Cheng et al., 1999; Ito & Lai, 1997; Tsuschihara et al., 1997), our data represent the first evidence of the ability of HCV NS3 to recognize and resolve RNA secondary structures within the viral 3’-NTR.

Methods

Expression and purification of a histidine-tagged FL-NS3 protein. The cDNA fragment encoding FL-NS3 (residues 1027–1657 of the BK strain HCV polyprotein) was excised from pT7NS3-FL vector (Gallinari et al., 1998) and cloned between the NdeI and HindIII restriction sites of pET14b expression vector (Novagen). The recombinant protein containing an N-terminal hexahistidine tag was expressed in E. coli BL21 (DE3) cells (Studier et al., 1998) and purified as previously described (Gallinari et al., 1998), with the following modifications. After concentration by ammonium sulphate precipitation, soluble NS3 was dialysed against a buffer containing 25 mM HEPEs (pH 8), 20% glycerol, 0.5 M NaCl, 0.1% n-octyl-β-D-glucopyranoside (Calbiochem), and 10 mM β-mercaptoethanol and loaded on a 15 ml Ni2+-charged HiTrap metal-chelating column (Pharmacia) equilibrated in the same buffer. The protein was eluted with 3 column vols of the chromatographic buffer containing 200 mM imidazole and further purified on a Superdex 200 26/60 gel filtration column (Pharmacia) equilibrated with buffer A (Gallinari et al., 1998) containing 0.2 M NaCl. The peak fractions were then loaded on a poly(U)–Sepharose affinity column (Pharmacia) and pure NS3 was eluted in buffer A containing 0.5 M NaCl. Protein concentration was estimated by Bio-Rad assay and UV absorption spectroscopy at 280 nm by using a molar extinction coefficient of 64200 M-1 cm-1.

ATPase activity assays. ATPase activity was directly determined by monitoring [γ32P]ATP hydrolysis by thin-layer chromatography, as described by Gallinari et al. (1998). RNA titration assays were carried out by incubating 20 nM enzyme in the presence of increasing concentrations of ssRNA or partial dsRNA (0 to 10 µM) for 30 min at 37 °C in helicase activity buffer [25 mM MOPS–NaOH (pH 7), 2.5 mM DTT, 2.5 U RNasin (Promega), 100 µg/ml BSA, 5% glycerol, 3 mM MgCl2 containing 1 mM ATP and 2 µCi [γ32P]ATP (6000 Ci/mmol, 10 mCi/ml; Dupont NEN)] in a volume of 10 µl. For analysis under single processive cycle conditions, increasing amounts of heparin were added to the samples as described in the legend to Fig. 6. After termination with 5 mM EDTA, 0.5 µl aliquots were spotted onto polyethyleneimine cellulose sheets and developed by ascending chromatography in 150 mM LiCl, 150 mM formic acid (pH 3-0). The cellulose sheets were dried and released [32P]phosphoric acid was quantified with a PhosphorImager using ImageQuant software.

Helicase assays. Helicase partial double-stranded substrates were obtained by annealing the corresponding complementary RNA (Genset) or DNA (Primm) synthetic oligonucleotides described in the legends to Figs 2, 4 and 5. Gel-purification and annealing were performed as described (Gallinari et al., 1998). The release strand was 5’ end-labelled with [γ32P]ATP by T4 polynucleotide kinase (Pharmacia) prior to the annealing reaction. The assays were performed in 20 µl of helicase
activity buffer (see above) containing 1·25 nM 32P-labelled RNA substrate and the indicated enzyme concentrations. After pre-incubation for 15 min at 23 °C, 5 mM ATP was added to start the helicase reaction. Unless otherwise specified, this was carried out at 37 °C for 30 min and then stopped by adding 5 µl of termination buffer (0·1 M Tris pH 7·5, 20 mM EDTA, 0·5% SDS, 0·1% NP-40, 0·1% bromphenol blue, 0·1% xylene cyanol). For analysis under single processive cycle conditions, heparin was used as trapping molecule as detailed in the legend to Fig. 6.

Aliquots (8 µl) were analysed on native 8–12% polyacrylamide gels containing 0·5% SDS, 0·1 M Tris–borate–EDTA, or UV-irradiated as described in the legend to Fig. 4. Bands corresponding to the protein-bound and unbound probe were visualized by autoradiography and quantification of the radioactivity was performed as above. The efficiency of the binding reaction was calculated as the ratio between the release strand-associated radioactivity and total radioactivity of both the unwound substrate and the release strand.

**Results**

**Purification of the N-terminal histidine-tagged FL-NS3 protein**

We have expressed in *E. coli* and purified to homogeneity the FL-NS3 67 kDa protein containing an N-terminal hexa-histidine tag to facilitate purification (Fig. 1). We have used a protocol successfully devised for the production of the soluble FL-NS3 untagged protein (Gallinari *et al.*, 1998), with the modifications described in Methods. A comparative analysis of the ATPase, helicase and NS4A-stimulated protease activities of the pure histidine-tagged protein proved it to be functionally indistinguishable from the corresponding untagged enzyme (not shown).

### NS3 helicase activity does not require a free 3′-end on the template strand

Earlier evidence showed that NS3 helicase unwinds substrates possessing a 3′ non-base-paired region on the template strand (Gwack *et al.*, 1996; Tai *et al.*, 1996). To investigate the absolute requirement for a free 3′-end, we constructed an RNA substrate containing two duplex regions each of 15 nt, separated by a 15 nt single-stranded region on the template strand (Fig. 2A; 15 1/3′-containing substrate). As shown in Fig. 2B (lanes 1 to 5), the 15 1/5′ oligonucleotide was efficiently released from the double-hybrid substrate to a level comparable to that seen with a standard single-hybrid substrate (Gallinari *et al.*, 1998), indicating that the enzyme is able to perform unwinding activity starting from an internal ssRNA region. Furthermore, NS3 helicase activity showed a marked 3′ to 5′ directionality, since the release of 15 1/5′ was significantly more effective than that of 15 1/3′ (Fig. 2B; lanes 7 to 11). To determine the minimal single-stranded region required for activity, we constructed a series of substrates containing internal ssRNA regions of decreasing length (Fig. 2A). The quantification of the NS3 unwinding efficiency on these double-hybrid substrates (Fig. 2C) indicated that strand-release was not affected by shortening the non-base-paired region from 15 down to 9 nt. Further reduction of the ssRNA length to 6 and 3 nt caused a decrease of NS3 unwinding efficiency to 50% and 25% of maximal activity, respectively. The binding affinity of NS3 to these substrates, analysed by gel retardation assay, showed the same ssRNA length-dependent inverse correlation demonstrated for unwinding (Fig. 2D). These observations are in agreement with published data (Preugschat *et al.*, 1996) showing, by fluorimetric measurements, that the affinity of NS3 helicase domain for single-stranded oligonucleotides is maximal with more than 10 nt.

### Effect of nucleotides on the affinity of NS3 for ssRNA and dsRNA

Although we have shown that NS3 is able to form stable complexes with double-hybrid substrates containing single-stranded regions down to 3 nt in length, no direct binding was observed on ssRNA molecules shorter than 18 nt in the same experimental conditions (unpublished results). Since NS3 bound ssRNA molecules with lower affinity than partial dsRNA molecules containing ssRNA regions of equal size, a contribution to the overall binding affinity of NS3 for these latter substrates could be given by additional interactions with the dsRNA region. We were interested in evaluating the effect...
that binding of ATP to NS3 might exert on its relative affinity for ssRNA and dsRNA. Previously we demonstrated that formation of a stable complex of NS3 with a ssRNA oligonucleotide was severely impaired by the addition of 5 mM ATP (Gallinari et al., 1998). Here we compared the binding affinity of the enzyme for the helicase double-hybrid substrate in the presence or absence of 5 mM ATP (or its non-hydrolysable analogue AMP-PCP) (Fig. 3). In the experiment shown in Fig. 3 (A, B) addition of ATP to the reaction mixture prior to addition of the enzyme determined a significant decrease of the binding efficiency, which probably reflected a diminished affinity for the ssRNA region of the probe. Moreover, in these conditions, strand release was also inhibited (not shown), presumably as a consequence of a reduction in the number of pre-formed enzyme–substrate complexes. Substituting AMP-PCP for ATP produced a similar decrease in the binding efficiency (not shown), thus suggesting that binding to the nucleotide and not hydrolysis was responsible for the weakened enzyme–RNA interaction. When ATP was added after pre-incubation of the enzyme with the labelled substrate, no retarded band was visible and release of the labelled strand was observed, as expected (not shown). However, addition of AMP-PCP, which does not support NS3 unwinding activity (Gallinari et al., 1998), after pre-incubating the enzyme with the substrate caused a marked increase in the binding efficiency (Fig. 3C, D), which probably reflected an increased affinity for the dsRNA region of the probe. In conclusion, the effect of the order of addition of ATP or AMP-PCP on the level of binding
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Fig. 3. Effect of ATP and AMP-PCP on the NS3 binding affinity for ssRNA and dsRNA. (A) Increasing enzyme concentrations [25 nM (lanes 1, 4), 50 nM (lanes 2, 5) and 100 nM (lanes 3, 6)] were added in a standard gel retardation reaction mixture using as a probe 45u RNA annealed with 32P-labelled 15 I/5' and unlabelled 15 I/3' RNAs (see Fig. 2) at a concentration of 1-25 nM. Lane 7, no enzyme added. Reactions were performed in 3 mM MgCl2 and either in the absence or in the presence of 5 mM ATP. Where present, ATP was always added before the enzyme and after 15 min of pre-incubation at 23 °C, the binding reactions were continued for an additional 30 min at 37 °C. Aliquots (8 µl) were analysed on a native 6% polyacrylamide/0.25 TBE gel using autoradiography. B, Protein-bound probe; F, free probe. (B) Quantification of the radioactive bands in the experiment shown in (A) was performed as described in Methods. (C) Gel retardation reaction mixtures were as in (A), but in lanes 2, 4 and 6 the non-hydrolysable ATP analogue AMP-PCP was added at the end of a 15 min pre-incubation of the enzyme with the probe at 23 °C. In lanes 1, 3 and 5, no AMP-PCP was added. The binding reactions were carried out for an additional 30 min at 37 °C and samples were analysed as above. Lane 7, no enzyme added. (D) Quantification of the radioactive bands in the experiment shown in (C) was performed as described in Methods.

activity to the partial dsRNA substrate suggests that the enzyme–nucleotide complex has a lower affinity for ssRNA and a higher affinity for duplex RNA than does the non-complexed protein.

**NS3 interacts with the stem–loop RNA structure (SL I) within the 3’-terminal 46 nt of the HCV genome**

Analysis of the secondary structure of the X region at the 3’-end of the HCV genomic positive-strand (Blight & Rice, 1997) revealed a stable stem–loop structure (SL I) within the 3’-terminal 46 bases containing a 6 nt single-stranded loop and 2 nt representing bulges (Fig. 4 A). A common role envisaged for viral helicases is that of causing RNA strand separation during genome replication. We assessed the ability of FL-NS3 to bind a 46 nt RNA oligonucleotide representing SL I. Gel retardation and UV-cross-linking experiments (Fig. 4 B, C, respectively) indicated that NS3 is able to bind tightly and in an ATP-sensitive manner to the stable stem–loop structure, presumably interacting with the 6 ribonucleotide single-stranded loop. Furthermore, the competition experiment shown in Fig. 4 (D, E) indicated that a linear dsRNA containing a 3’ ssRNA tail of 6 nt only partially inhibited the complex formation between NS3 and SL I RNA at concentrations at which the unlabelled SL I RNA did compete efficiently. This observation suggests that the preferential binding observed might depend not only on the presence of the 6 nt loop but also on the presence of RNA secondary structure.

**NS3 can resolve an SL I-containing stem–loop RNA structure**

Next we addressed whether the helicase activity of FL-NS3 was able to resolve the SL I RNA structure by unwinding the
Fig. 4. Binding of NS3 to SL I. (A) Structure of SL I. A 46-mer RNA oligonucleotide with the sequence 5’ GCAUGACUGAGAGUCUGAUACUGCCUCUCUCAGAUAUGU 3’ was 32P 5'-end-labelled, denatured and renatured under the conditions for oligonucleotide annealing described in Methods. An RNA binding experiment was performed exactly as described in Fig. 3 (A), using labelled SL I as a probe. At the end of the binding reaction, 8 µl aliquots were analysed by native PAGE (B) and the remaining 12 µl aliquots were UV-irradiated (0–12 J, 260 nM) for 15 min at 4 °C using a Stratalinker 2400 apparatus (Stratagene). Samples were analysed by SDS–PAGE (C). (D) Binding reactions were performed in the presence of 1–25 nM labelled SL I probe, 20 nM enzyme and the indicated fold-excess concentrations of unlabelled specific (SL I) and non-specific competitor RNAs. The non-specific competitor was a partial dsRNA of unrelated sequence containing a 20 nt duplex region and a 6 nt single-stranded 3’-end tail. It was obtained by annealing the 26-mer oligonucleotide (5’ GUUGAGAGAGAGAGAGUUUGAGAG 3’) with the 32P-labelled 20-mer oligonucleotide (5’ CAAACUCUCUCUCUCAAC 3’). (E) Quantification of the radioactive bands in the experiment shown in (D) was performed as described in Methods. ●, Specific competitor RNA; ■, non-specific competitor RNA.

stem region upon binding to the 6 nt loop. First attempts to devise an unwinding assay using optimized NS3 helicase conditions and the 46-mer SL I RNA oligonucleotide as a substrate failed to reveal any reaction product co-migrating with the corresponding linearized form in a native gel (not shown). A possible explanation resides in the extreme thermodynamic stability of SL I [AG = −26.5 kcal/mol with a melting temperature of 85 °C; Blight & Rice, 1997], which probably favours re-annealing of the base-paired region, thus preventing the isolation of enzymatically melted RNA molecules. Therefore, by annealing oligonucleotides 1 and 2 schematized in Fig. 5 (A), we created a new stem–loop substrate in which we extended both the 5’- and 3’-ends of SL I stem with complementary tails of 10 nt and introduced a nick in the middle of the original base-paired region. This new stem–loop substrate (SL I-l, Fig. 5 A) allowed us to monitor strand release rather than a melting product, with the assumption that the two unwinding reactions were equivalent in all other respects.

To verify that the presence of the nick in the stem structure would not destabilize the base-paired region by creating an artificial single-stranded tail which could trigger unwinding, we designed two negative control substrates. By annealing oligonucleotide 1 either with oligonucleotides 3 and 4 or 3a and 4a, blunt-ended linear dsDNA molecules were constructed.
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Fig. 5. Unwinding activity on SL I-derived substrates. (A) Structures of the SL I-derived DNA substrates. SL I-l was obtained by annealing the 20-mer oligonucleotide 1 (5′-TTGAGAGAGAGCATGACTGC-3′) with the 46-mer oligonucleotide 2 (5′-AGAGAGTGCCGATACTGGCCTCTCTGCAGATCATGCTCTCTCTCAA-3′). C1 was obtained by annealing to the 36-mer oligonucleotide 3 (5′-CCCACTGGCCTCTCTGCAGATCATGCTCTCTCTCAA-3′) both the oligonucleotide 1 and the 16-mer oligonucleotide 4 (5′-AGAGAGTGCCAGTGGG-3′). C2 was obtained by annealing to the 35-mer oligonucleotide 3a (5′-CCCACTGGCCTCTCTGCAGTCATGCTCTCTCTCAA-3′) both the oligonucleotide 1 and the 15-mer oligonucleotide 4a (5′-AGAGAGGCCAGTGGG-3′). In all substrates, oligonucleotide 1 was 5′ end-labelled with 32P (release strand). (B) Unwinding activity on SL I-derived substrates. Increasing enzyme concentrations [6–25 nM (lanes 1, 6, 11), 12.5–5 nM (lanes 2, 7, 12), 25 nM (lanes 3, 8, 13), 50 nM (lanes 4, 9, 14)] were added in a standard strand-displacement assay using as a substrate SL I-l (lanes 1–5), C1 (lanes 6–10) or C2 (lanes 11–15). Lanes 5, 10, 15, no enzyme added to SL I-l, C1 or C2–containing samples, respectively. Substrate concentration was 1–25 nM. (C) Quantification of radioactive bands in the experiment shown in (B) was performed as described in Methods and the efficiency of unwinding versus NS3 concentration determined as in Fig. 2(C). ●, SL I-l substrate; □, C1 substrate; ▲, C2 substrate. (D) Binding activity on SL I-derived substrates. Enzyme (6.25–50 nM) was added in standard gel retardation mixtures containing either SL I-l, C1 or C2 in the absence of MgCl2. Lane assignment was as in (B).

which no longer included the single-stranded loop but retained the nick-containing extended stem with (C1) or without (C2) bulges, respectively (Fig. 5 A). As shown in Fig. 5 (B, C), the labelled oligonucleotide 1 was efficiently released from the SL I-derived stem–loop structure by the NS3-associated helicase activity and the enzyme showed a significant preference for this substrate compared with the negative controls. This difference was reflected in a higher binding affinity for the stem–loop than for the blunt-ended substrates (Fig. 5 D), thus confirming that NS3 was able to interact with the 6 nt single-stranded loop and to unwind the double-stranded SL I stem starting from it. The presence of the two bulges did not cause any significant additional destabilization of the base-paired region as judged by the two control curves in Fig. 5 (C).

Effect of heparin on ATP hydrolysis and helicase activities

NS3 helicase has a large intrinsic ATPase activity ($k_{cat} = 3 \text{s}^{-1}$) which is stimulated up to 30-fold by ssRNA or DNA (Preugschat et al., 1996). We have compared the stimulation of NS3 ATPase activity in the presence of increasing concentrations of either a 20 nt ssRNA or a dsRNA substrate containing a 10 nt 3′-tail (Gallinari et al., 1998). The two titration curves shown in Fig. 6 (A) were very similar, indicating that the degree of stimulation of the ATPase activity observed with the ssRNA and the tailed dsRNA was identical (approximately 10-fold). The dissociation constant values for the two activator RNAs were also very similar (2 and 2.1 µM, respectively). This result suggests that the presence of a duplex region does not influence the efficiency of the RNA-mediated activation of ATP hydrolysis. Although it is known that NS3 unwinding activity is ATP-dependent (Tai et al., 1996), the mechanism of coupling ATP hydrolysis to unwinding of the duplex is not completely understood. We reasoned that if ATPase activity in the presence of a 3′-tailed dsRNA was effectively coupled with the unwinding reaction, we should be able to determine the enzyme processivity by measuring the hydrolysis of ATP under single processive cycle conditions. To this aim, we added increasing concentrations of heparin in a standard ATPase reaction stimulated by the addition of a saturating amount of 3′-tailed dsRNA (Fig. 6B). In parallel, we performed the same heparin titration experiment adding the trapping molecule in a standard helicase assay (Fig. 6C). In
Fig. 6. ATPase and helicase activities under single processive cycle conditions. (A) ATPase activity in the presence of increasing concentrations of either ssRNA or partial dsRNA. RNA titration experiments were performed by incubating 20 nM NS3 in the presence of 2, 5, or 10 µM of either a 20 nt ssRNA (square) or a dsRNA substrate containing a 10 nt 3′-tail and a 20 base-paired region (circle). Reaction conditions were as described in Methods. The dissociation constants of the NS3–ssRNA and NS3–partial dsRNA complexes were calculated from non-linear least-squares fit to the equation 
\[ V = V_0 + \frac{V_{\text{max}}[\text{RNA}]}{K_d + [\text{RNA}]} \]. (B) Effect of heparin on the partial dsRNA-stimulated ATPase activity. FL-NS3 (20 nM) was preincubated with 10 µM 3′-tailed dsRNA in ATPase standard conditions and the reaction started by the addition of 1 mM cold/hot ATP mix. Increasing amounts of heparin (0, 3–125, 6–25, 12–5, 5, 10 µg/ml) were added either before the enzyme (squares) or together with ATP after pre-incubation of the protein with the 3′-tailed dsRNA (triangles). In (A) and (B) curves were obtained by subtracting from each experimental data-point the ADP produced by the basal ATPase activity. (C) Effect of heparin on duplex unwinding activity. The same heparin increasing concentrations were added in a standard helicase assay either before the enzyme (squares) or together with 5 mM ATP after pre-incubation of the protein with 1:25 nM of labelled 3′-tailed dsDNA (triangles). The helicase substrate was obtained by annealing the unlabelled 3a oligonucleotide (35 nt, template strand) with the 5′-32P-labelled 4a oligonucleotide (15 nt, release strand) described in Fig. 5. Final NS3 concentration was 12–5 nM. Each unlabelled oligonucleotide 1 and 4a (see Fig. 5) (18–75 nM) was added together with ATP to prevent product re-annealing during the reaction in the presence of the trapping molecule. (D) Time-course of duplex unwinding under single processive cycle conditions. Reaction mixtures with and without heparin were as in (C) and contained 6:25 nM NS3 and 1:25 nM labelled 3′-tailed dsDNA substrate. After a pre-incubation of 15 min at 23 °C, the reactions were started by addition of ATP as in (C) and carried out at 23 °C. At the indicated time-points, aliquots were withdrawn, mixed with the stop solution and analysed by native PAGE.

Both experiments heparin was added either before the enzyme or together with ATP after pre-incubation of the protein with the helicase substrate. Heparin completely inhibited both NS3 enzymatic activities when present in the reaction before the enzyme was added (Fig. 6B, C). In the helicase experiment (Fig. 6C), residual activity was observed when heparin was added together with ATP. In this latter case, the strand release measured (about 0:02 nM product/nM enzyme) was the result of a single processive cycle of unwinding and represents therefore an index of helicase processivity. In contrast, the RNA-stimulated ATPase activity was completely inhibited by heparin in these conditions (Fig. 6B), suggesting that most of the observed RNA-stimulated ATP hydrolysis is not directly coupled with the translocation of the enzyme on the dsRNA substrate. On the contrary, binding of the protein to ssRNA appears the only event important for the stimulation of ATPase activity. Interestingly, the residual RNA-stimulated activity following heparin addition was equal to the intrinsic ATPase, confirming that the observed effect depended on the competition of the trapping molecule for binding to RNA rather than on a non-specific inhibitory interaction with the enzyme.

Time-courses of duplex unwinding (Fig. 6D) revealed that NS3 reached a plateau in the reaction after only 10 min, both in the absence and in the presence of heparin. Under single processive cycle conditions, the maximal concentration of strand released was 0:3 nM, about 40% of that measured in the absence of the trapping molecule. In our time-course experi-
mements, we were not able to measure with accuracy the amplitude of the rapid phase of the reaction. This value depends also on the length of the duplex region that is unwound by the enzyme in a single cycle, 15 base pairs in our substrate, and further experiments on much longer duplex substrates are needed to more precisely define the intrinsic rate of unwinding by NS3.

Discussion

Recent mechanistic studies on E. coli Rep helicase form the basis for a detailed understanding of the kinetic mechanism for DNA unwinding by a helicase (Lohman & Bjornson, 1996). The alternative mechanisms proposed for the action of helicase have been classified as ‘passive’ or ‘active’ (reviewed by Bird et al., 1998). In the passive-type mechanism, the enzyme binds preferentially to ssDNA and unwinds dsDNA by interacting with the ssDNA that is formed transiently from the duplex as the result of thermal fluctuations. In contrast, the active rolling model implies the binding of helicase alternatively to ssDNA and dsDNA. During catalysis, the alternating affinity for ssDNA and dsDNA is coupled to ATP binding and hydrolysis as the enzyme rolls along the duplex. Although the original active model applied only to oligomeric enzymes with multiple DNA-binding sites on different subunits (Wong & Lohman, 1992), a more general proposal was recently put forward from the comparison of two different structures of the monomeric PcrA DNA helicase complexed with a single-strand-tailed DNA duplex in the presence or absence of a non-hydrolysable ATP analogue (Velankar et al., 1999). In this latter model, the conformational changes that occur on binding ATP not only destabilize the interaction with ssDNA, allowing the sliding of one of the helicase domains along it, but also set up the protein surface to bind duplex DNA, hence creating strain at the base pairing at the fork. Biochemical and structural data showed NS3 to be monomeric under a range of different conditions (Gallinar et al., 1998; Kim et al., 1998; Porter et al., 1998; Yao et al., 1997). It has been demonstrated previously that ATP decreases the affinity of the NS3 helicase domain for a (dU)$_{18}$ by 95% (Preugschat et al., 1996). Our data indicate that the FL enzyme in complex with ATP or an ATP non-hydrolysable analogue has a lower affinity for ssRNA and a higher affinity for duplex DNA than does the non-complexed protein. These observations are consistent with the ‘inchworm’ active mechanism proposed for the monomeric PcrA helicase discussed above, in which the free energy of hydrolysis of ATP is utilized for both unidirectional translocation and strand separation. Additional crystallographic structures of NS3 complexed with its substrates are, however, needed to shed more light on the mechanistic details of its unwinding function.

It is reasonable to assume that following HCV infection, the initiation of negative-strand RNA synthesis depends on an initial recognition and specific binding of the replicative complex to the 3'-end of the viral genomic RNA. NS5B polymerase has been recently shown to specifically interact with conserved stem–loop structures in the 3' coding region of the HCV genomic RNA (Cheng et al., 1999). Since the 98 nt X region at the 3' terminus of the HCV genome is highly conserved in sequence and has a stable secondary structure (Blight & Rice, 1997; Ito & Lai, 1997; Kolykhlov et al., 1996; Tanaka et al., 1996), it has been proposed to be involved in viral RNA synthesis and in multiple protein–RNA interactions. It has been recently suggested that NS5B polymerase can use this region as a cis-acting sequence to initiate HCV RNA synthesis in vitro (Oh et al., 1999), although previous studies failed to demonstrate a specific interaction between NS5B and a viral RNA containing the 98 nt sequence (Cheng et al., 1999; Lohmann et al., 1997). Furthermore, cellular proteins including polypyrimidine tract-binding protein have been demonstrated to bind specifically to the SL 2 and SL 3 stem–loop structures of the conserved X region (Ito & Lai, 1997; Tsuchihara et al., 1997). For efficient transcription initiation at the 3'-end of the HCV positive- and negative-strands, NS3-associated unwinding activity might be required to remove the secondary structure on the template RNA. Our analysis of NS3 unwinding activity on double-hybrid substrates indicates that the minimal internal non-base-paired region for optimal strand displacement lies between 9 and 6 nt, while shortening this region down to 3 nt causes a decrease to 25% of maximal activity. This would suggest that NS3 might require three or more non-base-paired ribonucleotides to trigger its unwinding activity and resolve the secondary structure elements on the template RNA. NS3 protein from HCV-related dengue virus has been recently demonstrated to interact with stem–loop structures in the 3' non-coding region of the genomic RNA that plays an important role in the initiation of the negative-strand RNA synthesis (Chen et al., 1997; Cui et al., 1998). Our data indicate that HCV NS3 protein is able to bind tightly and with some specificity to the stable stem–loop structure SL I formed by the 3'-terminal 46 bases of HCV positive-strand RNA (Blight & Rice, 1997). Furthermore, NS3-associated helicase activity is able to resolve this kind of structure in a standard unwinding assay, presumably through the initial binding to the 6 nt loop followed by the ATP-dependent translocation of the enzyme along the base-paired stem. The specificity of the interaction might depend only on the presence of RNA secondary structure and not on the primary sequence. Indeed all base changes identified within SL I in different HCV genotypes occur either in the single-stranded loop or, when they arise in the double-stranded stem, compensatory mutations are always present which preserve the secondary structure (Blight & Rice, 1997). It would be of interest to assess whether NS3 could interact with other stem–loop structures within the ends of the genomic and anti-genomic RNA, and the degree of binding selectivity in the absence or presence of other replication factors.

Processivity of the NS3 helicase was inferred by its capacity to unwind a tailed RNA substrate containing a 15 nt duplex
region under single processive cycle conditions. We included heparin to trap the enzyme not bound to RNA. Heparin has been used as a nucleic acid analogue in studies of a number of enzymes including DNA helicases (Korangy & Julin, 1992, 1993) and affinity chromatography on a heparin column was used in the purification protocol of native FL NS3 (Gallinari et al., 1998). The effect exhibited by the order of addition of the trapping molecule on the amount of strand released by NS3 helicase activity is consistent with a processive action of the enzyme. This implies that heparin does not bind to the enzyme while unwinding its substrate. A similar inhibitory effect on the helicase activity of NS3 was observed by including excess amounts of oligo(U)$_{18}$ in the assay (not shown). Assuming that the enzyme progresses on the duplex RNA by two base pairs for every molecule of ATP hydrolysed (Porter et al., 1998), theoretically 200 nM ATP (half of the amount of base pairs contained in 20 nM of 20 nt dsRNA) should be hydrolysed in the presence of heparin by 20 nM enzyme during a complete single cycle of unwinding (assuming that all the enzyme is catalytically active). On the contrary, the ATPase activity stimulated by partial dsRNA was completely inhibited by heparin, independent of the order of addition. This would suggest that the extent of ATP hydrolysis measured in this assay is not coupled, for the most part, with the translocation of the enzyme on duplex RNA but is only reflecting NS3 binding to the ssRNA tail. This is consistent with the unanticipated features of a K1235E mutant in motif I of NS3 helicase (Kim et al., 1997). This mutation almost completely abolished both the intrinsic ATPase and helicase activities of the isolated helicase domain, although the RNA-stimulated ATPase activity was only partially reduced. Similar results were also reported for a different mutation at the same K residue in FL-NS3 (Wardell et al., 1999). It has been reported that, under single cycle conditions, the processivity of the NS3 helicase domain is low (Porter et al., 1998). The evidence presented here with FL-NS3 does not support or disagree with this observation and additional experiments are required to establish whether the isolated helicase domain and the FL enzyme are equally processive in vitro. Furthermore, the association to other cellular and/or viral proteins might increase the processivity of NS3 in vivo as has been shown for several viral and cellular replication helicases (Boehmer, 1998; Dong et al., 1996; Phillips et al., 1997).

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References


