Direct fluorometric measurement of hepatitis C virus helicase activity

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Abstract The non-structural protein 3 (NS3) of hepatitis C virus (HCV) is a highly promising target for anti-HCV therapy because of its multiple enzymatic activities, such as RNA-stimulated nucleoside triphosphatase, RNA helicase and serine protease. The helicase domain of NS3 as well as domain 2 of the helicase were expressed in a baculovirus system to obtain in high yield active proteins for prospective studies of complexes of the helicase with its inhibitors. A novel direct fluorometric test of helicase activity with a quenched DNA substrate, 3′ labeled with a Cy3 dye and 5′ labeled with a Black Hole Quencher, was developed and optimal reaction conditions established. This test based on fluorescence resonance energy transfer is simple and fast. It allows for direct measurements of enzyme activity, circumventing laborious and complicated radioactive techniques that are poorly reproducible. The results obtained encourage us to propose this new fluorescent assay as a method enabling high throughput screening of anti-helicase compounds.

Keywords: Hepatitis C virus; NS3 helicase; Fluorometric assay

1. Introduction

Hepatitis C virus (HCV) is a single-stranded (+) RNA virus of the Hepacivirus genus in the Flaviviridae family. Its genome of ~9600 nucleotides encodes a polyprotein of ~3000 amino acids that is co- and post-translationally cleaved into 10 mature proteins. Three have structural functions (C, E1, E2), and one (p7) is a membrane protein possibly involved in virion release and maturation [1,2]. The non-structural proteins include NS2 (metallo/thiol protease), non-structural protein 3 (NS3) (serine protease/RNA helicase), NS4A (serine protease cofactor), NS4B (unknown function), NS5A (role in replication and resistance to interferon-α), and NS5B (RNA-dependent RNA polymerase).

HCV represents a serious worldwide epidemiological threat, infecting up to 3% of the population. Chronic hepatitis resulting from HCV infection develops in most cases into cirrhosis and highly increases the risk of hepatocellular carcinoma [3]. No vaccine for HCV has been developed to date in spite of numerous attempts [4–6]. No efficient treatment exists; even the new dual therapy with pegylated interferon and ribavirin is effective only in up to 56% of the cases, depending on the genotype of the virus and duration of treatment [7].

The most promising target for anti-HCV therapy seems to be NS3 because of its multiple enzymatic activities. The helicase activity is indispensable for viral replication [8], presumably unwinding double-stranded replication intermediates and secondary structures, allowing RNA amplification [1]. Recent data demonstrate that NS3 can interact with NS4B and NS5B to form a complex modulating template recognition by NS5B [9]. NS3 may also influence the type of host response to HCV infection and could serve to prepare DNA vaccines against HCV [6].

The HCV helicase activity can be measured using different techniques, the most widely applied being a radioactive test, initially developed for the NS3 helicase by Kim et al. [10], and since then applied by others [11–14] who characterized the enzyme, establishing its 3′ to 5′ directionality, requirement for a 3′ overhang to start unwinding and dsDNA helicase activity. The enzyme has a higher affinity for dsDNA than for dsRNA and requires the NS4A cofactor for full activity on RNA [15].

In spite of its high sensitivity (the dsDNA template concentration may be as low as a few pM; [16]), the radioactive test has some disadvantages, especially the tedious preparation and instability of the radiolabeled substrate [13]. Moreover, it is not a simple direct measurement of enzyme activity since the reaction products must be separated by gel electrophoresis and then submitted to further analyses, i.e., visualization by autoradiography and quantification, that leads to a low reproducibility of final results. Therefore, considerable work was performed to establish high throughput methods. This resulted in three types of assays, two involving radioactivity and signal amplification by scintillation (scintillation proximity assay [17] and the Flashplate™ assay [18]), and one ELISA-based assay [19,20]. The latter test avoids radioactivity but is slow; in addition, neither this nor the former methods allow to follow directly the helicase activity.

Our objective was to develop a fast non-radioactive high throughput assay to easily and directly assess enzyme activity, and measure the inhibitory potential of different HCV helicase inhibitors in a microtitration plate-format test. We chose to

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Abbreviations: BHQ, Black Hole Quencher; fu, fluorescence units; HCV, hepatitis C virus; NS3, non-structural protein 3; NTPase, nucleoside triphosphatase

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make use of fluorescent dyes and the fluorescence resonance energy transfer (FRET) phenomenon ([21–23], J. George (Amersham Biosciences), personal communication) to create such a method involving a newly introduced fluorescence quenching molecule (Black Hole Quencher, BHQ, Biosearch Technologies).

Here, we describe the expression and purification of the HCV helicase and its domain 2, and the development and optimization of the new helicase assay.

2. Materials and methods

2.1. Cloning and expression of the HCV helicase and its domain 2

The Bac-to-Bac baculovirus expression system (Gibco BRL) was chosen to construct a recombinant baculovirus. The helicase cDNA (coding for amino acids 1193–1658 of the HCV-1 isolate (Acc. No. AAA45676)) [10] was cloned in the pFastBacHTb vector using the *Bam*HI and *Hin*dIII cloning sites. After sequencing, the termination codon from the original construct lost during cloning was restored by inserting a self-annealed oligonucleotide (AGCTCTC**TAG**AG) into the *Hin*dIII site. Transposition to a bacmid (bMON14272), transfection of SF21 insect cells and amplification of the recombinant virus were done according to the Gibco BRL manual. The HCV helicase, located downstream of the polyhedrin promotor of AcMNPV and carrying an N-terminal His-tag, was expressed in the High Five (HF) insect suspension cell culture.

Domain 2 (amino acids 1352–1509 of the HCV-1 isolate) was cloned in the pFastBacHTa vector by PCR amplification using the sense (TTGGAATTCCCGGGCTCCGTCACTG) and the antisense (AAAAAAGCTTGGGCGCTCCCCCGGTG) primers and the HCV-1 cDNA template, and subsequent cleavage with *Eco*RI and *Hin*dIII of the PCR product and the vector. After transposition of the domain 2 coding sequence to the bacmid, the recombinant virus was transfected into SF21 insect cells for amplification and the protein with an N-terminal His-tag was expressed in HF insect cells.

2.2. Purification of the HCV helicase

HF insect cells were infected with the recombinant baculoviruses at a multiplicity of infection of 1, they were propagated as a suspension cell culture in Express Five SFM (Invitrogen) medium, and harvested 44–48 h post-infection (h.p.i.).

The standard helicase and domain 2 purification protocol, calculated for 50 ml of infected insect cell culture (1×10^8 cells), included centrifugation for 10 min at $720 \times g$ at 4 °C and resuspension of the cells in 5 ml of buffer A (20 mM Tris–HCl, pH 7.0, 500 mM NaCl, and 5 mM imidazole) supplemented with protease inhibitor cocktail (Complete, EDTA-free, Roche). The cells were frozen in liquid nitrogen, brought to 37 °C and vortexed; freezing and thawing were repeated five times. The suspension was centrifuged twice at $20\,000 \times g$ at 4 °C for 15 min. Subsequently, glycerol was added to the clear supernatant to 10% and CHAPS to 0.05%, the extract was mixed with 0.5 ml of Talon Metal Affinity Resin (Clontech) washed previously with 3 ml of buffer A, and left overnight on a rocking platform at 4 °C, or 2–3 h on ice. The mixture was washed four times with 3–4 ml of buffer B (buffer A with 10% glycerol and 0.05% CHAPS), then three times with 3–4 ml of buffer A. Three to five elutions with 0.5 ml of buffer E (20 mM Tris–HCl, pH 7.0, 200 mM NaCl, and 200 mM imidazole) were performed.

If needed, helicase and domain 2 eluates were concentrated on Amicon Ultra filters 30 000 and 10 000 MWCO (Millipore), respectively. They were diluted 2-fold with 20 mM Tris–HCl, pH 7.0, and applied onto a 1 ml Heparin HP column (Amersham Biosciences). The column was washed with 10 ml of buffer C (20 mM Tris–HCl, pH 7.0, and 100 mM NaCl). Since the helicase does not bind to heparin and domain 2 binds to heparin only very weakly, wash fractions were collected, concentrated on Amicon Ultra filters, and stored in 20% glycerol at −20 °C. Before reutilization, the column was washed with buffer D (20 mM Tris–HCl, pH 7.0, and 1 M NaCl). Concentrations of HCV helicase and domain 2 were determined at 280 nm using molar extinction coefficients of 51 760 and 11 760 $M^{-1}\,cm^{-1}$, respectively. The extinction coefficients were calculated on the basis of protein composition by the ProtParam program from the Expasy website (http://us.expasy.org).

2.3. Protein analysis

Extracts and eluates were analyzed by 12% SDS–PAGE and proteins visualized by Coomassie staining or electrotransferred onto an Optitran BA-S 85 membrane (Schleicher and Schuell). Helicase and domain 2 were detected with HCV-positive human antiserum, followed by addition of a goat anti-human IgG(H + L)-alcaline phosphatase conjugate (Bio-Rad) as the secondary antibody. The proteins were revealed colorimetrically using chromogenic substrates (NBT/BCIP Solution, Roche).

Rabbit polyclonal anti-HCV helicase and anti-Potato virus Y (PVY) coat protein antibodies were obtained at the Veterinary Institute, Puławy.

2.4. ATPase assay

The ATPase assay was performed essentially as described [24]. Reactions were carried out in 12.5 μl of reaction buffer for 60 min at 30 °C, stopped by addition of 250 μl of activated charcoal, centrifuged, and duplicate 25 μl samples of the supernatant were Cerenkov counted.

2.5. Fluorescent helicase assay

The substrate for the fluorescent helicase test was prepared by annealing at a 1:1.2 molar ratio, a 5′ Cy3-labeled 36-mer (TAGTACCGCCACCCTCAGAACCTTTTTTTTTTTTTT) to a 3′ BHQ-2-labeled 22-mer (GGTTCTGAGGGTGGCGGTACTA), both HPLC-purified (metaBIOn, Germany) in 20 mM Tris–HCl, pH 7.5, by brief heating to 90 °C, then slow cooling to room temperature. Standard helicase assays were performed in 30 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 0.075% Triton X-100, 0.05% sodium azide, 20 nM substrate, 1 mM ATP and 250 nM capture strand (TAGTACCGC CACCCTCAGAACC). The unwinding reaction was started by addition of HCV helicase (2–20 nM) or ATP and was carried out at 37 °C for up to 90 min. The fluorescent signal increase was measured using either a Fluorescence Reader FLx (Biotek) or a fluorescent spectrofluorometer FluoroMax (Spex). The reaction volume was 200 μl for the FLx (in a 96-well microtiter plate) and 500 μl for the Spex fluorometer (in a single cuvette). The fluorescent signal was registered every 300 s (FLx) or 30 s (Spex). Since the excitation maximum for the Cy3 fluorophore is 550 nm and the emission maximum is 570 nm, the fluorophore was excited at 550 nm and helicase activity was measured at 570 nm (Spex), or at 620 nm (FLx), i.e., not at the main maximum but still within the range of the second emission maximum.

The enzyme activity was calculated as the initial reaction velocity from the linear part of the progress curve using the linear regression method. A linear equation $Y = A + BX$ was fitted to experimental data, where Y is the enzyme activity expressed in fluorescence units (fu), X is the reaction time and B is the slope or the initial velocity. The Origin 6.1 program (OriginLab Corporation) was used to calculate the data.

3. Results

3.1. Overexpression and purification of the HCV helicase and its domain 2

The helicase domain of the HCV NS3 protein is composed of three separate structural domains, domain 1 containing nucleoside triphosphatase (NTPase) motifs, domain 2 with several conserved motifs, among them an arginine-rich sequence possibly involved in RNA or ATP binding, and domain 3 without conserved motifs [25,26]. Using NMR spectroscopy, binding of inhibitors to the entire helicase can be detected [27], after which individual domains can be studied separately to precisely locate sites of interaction.

We decided to validate the new helicase assay by comparative studies of the full-length helicase and its domain 2 that may hypothetically exert some of the HCV helicase functions. Both constructs were expressed as N-terminal His_6-tagged fusion proteins and purified from HF cells collected ~48 h.p.i. when the level of protein expression was the highest without significant degradation. In the cell lysates, proteins of the ex-

pected size could be detected by gel electrophoresis and subsequent Western blots (Fig. 1A). Non-denaturing cell lysis was followed by native purification on Talon Resin and the proteins further purified on a Heparin HP column; 95% purity was reached as estimated by SDS–PAGE and Coomassie staining (Fig. 1B). The identity and size of the proteins were confirmed by mass spectrometry; their molecular masses were 53.3 and 21.5 kDa, respectively.

The proteins were purified in high yield, 15 $mg\,l^{-1}$ for the helicase and 20 $mg\,l^{-1}$ for domain 2, their purity and yield amenable for NMR and crystallization studies (in progress).

3.2. ATPase activity of the HCV helicase

The ATPase activity of the purified proteins was measured: it was 49-fold higher for the full-length protein than for domain 2 (10 182 ± 436 and 208 ± 293 cpm/pmol, respectively). The activity of domain 2 was insignificant, being only slightly higher than that of prolactin, a purified control protein without NTPase motifs, expressed in the insect cells [28].

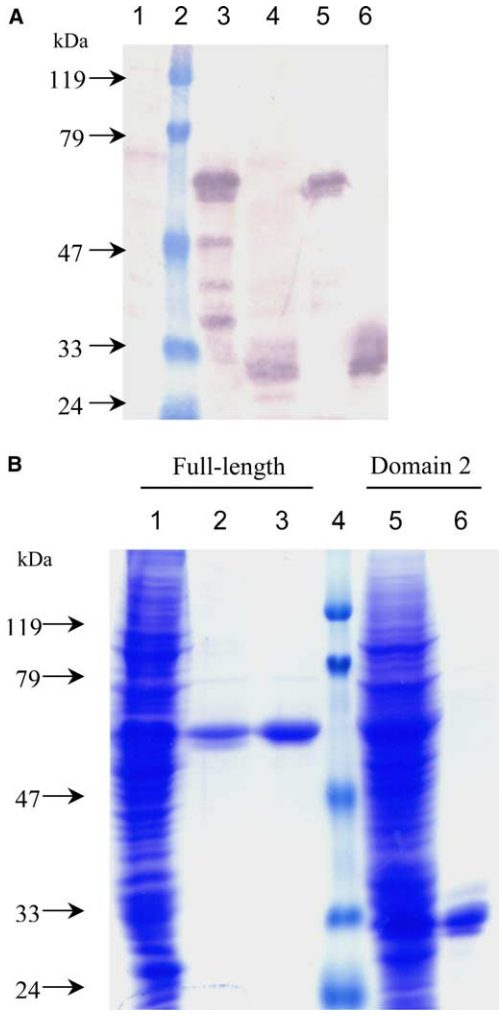

Fig. 1. Expression and purification of HCV helicase proteins. (A) Western blot analysis of the helicase and domain 2 expression in HF insect cells. Lane 1, uninfected cells; lane 2, prestained molecular marker (SM0441, Fermentas); lane 3, cells infected with recombinant virus carrying the full-length helicase gene; lane 4, cells infected with recombinant virus carrying domain 2 gene; lane 5, purified full-length helicase; lane 6, purified domain 2. (B) Enzyme purification analyzed by SDS–PAGE and detected by Coomassie staining. Lane 1, cells expressing the full-length helicase; lane 2, eluate from Talon column; lane 3, eluate from Heparin column; lane 4, prestained molecular marker (SM0441, Fermentas); lane 5, cells expressing domain 2; lane 6, domain 2 eluted from Heparin column.

3.3. Fluorescent HCV helicase assay

In the new assay established here, a quenched duplex DNA substrate with a 3′ single-strand overhang was obtained by annealing two oligonucleotides, a 5′ Cy3-labeled 36-nucleotide donor and a 3′ BHQ-2-labeled 22-nucleotide quencher. Fluorescence emitted by the cyanine dye (550–650 nm) is quenched due to FRET or contact-mediated quenching between the cyanine donor and the quencher when the molecules are separated by 10–100 Å [21,22]. The quenching maximum of BHQ-2 is 579 nm, it belongs to "true dark quenchers" with much higher signal-to-noise ratios than other quencher dyes (DABCYL and TAMRA) [22]. Fluorescence emission results from the helicase activity that separates the strands leading to the arrest of FRET. The addition of excess capture strand (complementary to the quencher oligonucleotide) is crucial because it prevents reannealing of the unwound duplex and dramatically increases the reaction efficiency.

The main objective of this work was to define the optimal parameters of the HCV helicase assay; first the optimum enzyme, substrate and capture strand concentrations were determined, then the optimum temperature, pH, ATP concentration, and cation type and concentration, always using the standard helicase reaction conditions. Initial velocities of helicase reactions were calculated as described in Section 2. Reactions were carried out for 15–90 min but for calculations only the linear parts of the slopes were used. Any discrepancies from standard reaction conditions are mentioned in the figure legends.

A linear dependence of the enzyme activity as a function of incubation time was observed up to 10 min, then much before the activity reached its maximum, increase of activity was no longer linear (Fig. 2) and even some decrease in enzyme activity was observed after 60–90 min (data not shown), due probably to establishment of a steady-state equilibrium, substrate exhaustion and slow degradation of both enzyme and substrate. However, we observed no significant decrease of the

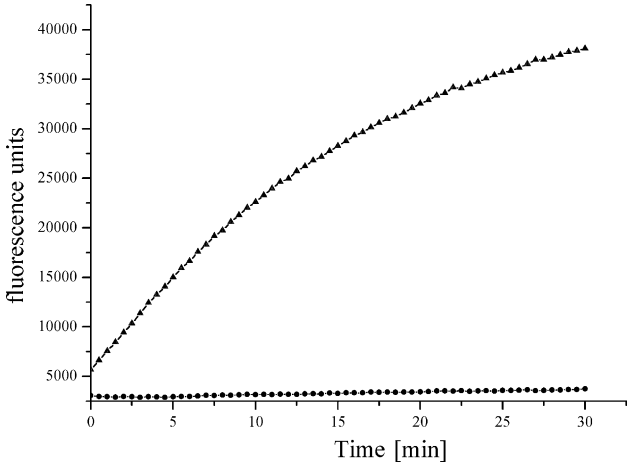

Fig. 2. Typical time-course of the helicase reaction studied using the Spex fluorometer. The fluorescent signal was measured every 30 s. Triangles: with 20 nM enzyme; circles: without enzyme.

fluorescence signal when free Cy3-labeled strand was included as control in the helicase assay. In the absence of helicase, no significant fluorescence signal increase was observed (Figs. 2 and 3). Almost linear dependence of reaction velocity on enzyme concentration was observed up to 20 nM (Fig. 3). Even at helicase concentrations as low as 2 nM, the reaction was sufficiently efficient to study the influence of inhibitors.

The influence of substrate concentration from 0 to 160 nM on the reaction velocity was investigated (Fig. 4) at a capture strand to substrate molar ratio of 12.5–1. No substrate inhibition was observed, as opposed to results obtained with radioactive helicase assays where non-saturating substrate concentrations were required [29,30]. Without capture strand the reaction was practically stopped (not shown), probably because of immediate reannealing of the separated strands. The reaction velocity increased linearly with capture strand concentration up to 250 nM, i.e., at 12.5:1 capture strand:substrate ratio.

The optimum temperature for the reaction was 37 °C, reaction velocities were lower at 25 and 30 °C (reaching 58% and 77% of the value at 37 °C, respectively).

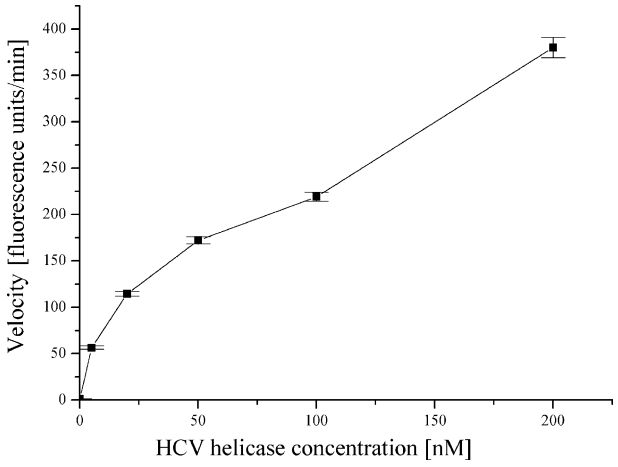

Fig. 3. Effect of HCV helicase concentration on the rate of unwinding. These experiments were repeated five times. The data are presented as means ± S.E.

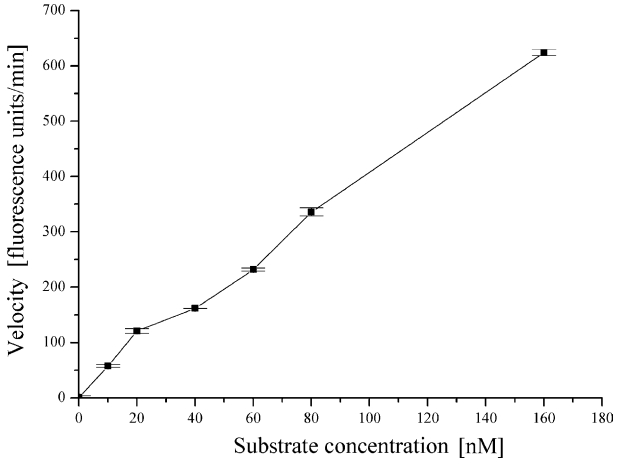

Fig. 4. Substrate dependence of the helicase reaction (20 nM enzyme). Substrate titration was carried out in triplicates. The data are presented as means ± S.E.

The effect of ATP concentration was studied using two approaches. The first was to increase the Mg^{2+} concentration with the ATP concentration (Fig. 5A), maintaining a 5:1 molar ratio. No unwinding was observed in the absence of ATP and/or divalent ions (Fig. 5A and B) confirming the catalytical mode of action of the enzyme [31]. The highest enzyme activity was reached at 2.5 mM ATP and 12.5 mM $MgCl_2$ with an abrupt decrease at higher ATP concentrations. The K_m value for ATP was 0.78 ± 0.09 mM. The second type of experiment consisted in changing only the ATP concentration, maintaining 5 mM $MgCl_2$ (not shown): the optimum helicase activity was observed at 1 mM ATP with an inhibitory effect at higher ATP concentrations ($K_m = 0.33 \pm 0.002$ mM).

Relative ATP/$MgCl_2$ concentrations seem very important, probably because Mg^{2+} and ATP bind as a complex to ATP-binding enzymes [32]. At limiting Mg^{2+} concentrations, ATP molecules were probably not sufficiently coordinated by divalent ions.

We determined the optimal concentration and type of ions required at 1 mM ATP (Fig. 5B). The helicase activity reached a maximum at 5 mM Mg^{2+} and decreased at higher Mg^{2+}

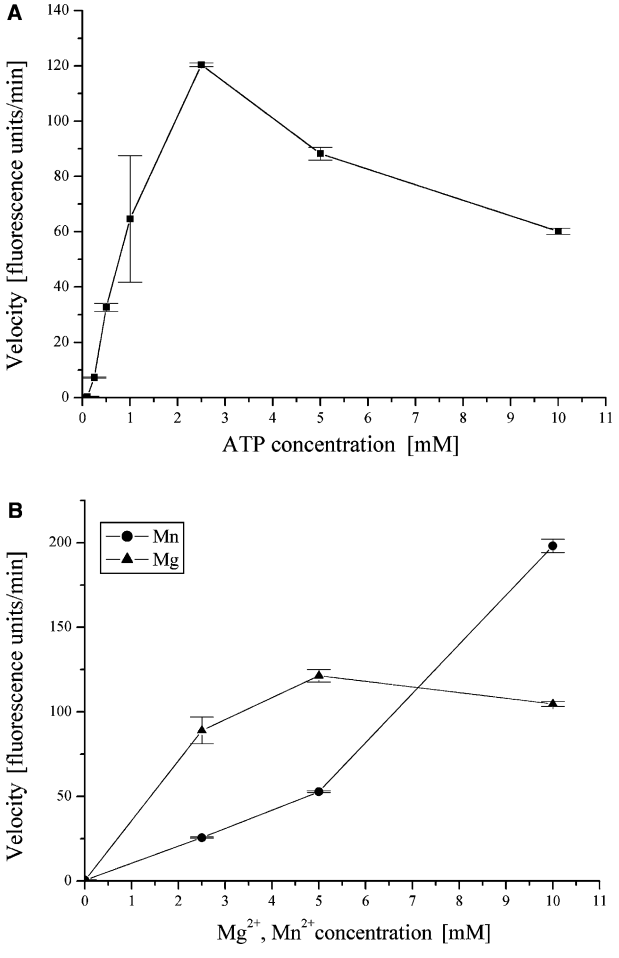

Fig. 5. Effect of ATP and divalent ion concentration on the helicase reaction velocity (20 nM enzyme). (A) ATP-dependence. The Mg^{2+} concentration was 5-fold higher than the ATP concentration. Determination of optimal ATP concentration was repeated five times. The data are presented as means ± S.E. (B) Influence of Mg^{2+} (triangles) and Mn^{2+} (circles) concentrations on unwinding. All data are means of five experiments ± S.E.

concentrations. Thus, the optimal Mg^{2+}/ATP ratio was 5:1. Surprisingly, at $\leqslant 7.5$ mM Mn^{2+}, the helicase exhibited lower activity than at identical Mg^{2+} concentrations, but at 10 mM Mn^{2+} the reaction velocity still increased, suggesting that to reach optimal conditions, 10 mM Mn^{2+} should be used. Discrepancies exist between the results of different authors concerning optimum divalent ion type and concentration, and in some studies a certain bias of the enzyme for Mg^{2+} was demonstrated [10]. This may depend not only on the concentration of divalent ions used [13] but possibly also on the type of assay and substrate (RNA or DNA) as in the FlashPlate assay involving a DNA substrate [18] in which Mn^{2+} was more efficient than Mg^{2+} at concentrations >0.25 mM.

The effect of pH between 6.5 and 9.5 on the reaction rate was examined. Lower pH values were not examined to avoid unspecific increase of fluorescence signal that occurred in these conditions; pH 6.5 seems optimal (not shown).

Based on these experiments, the optimal reaction conditions are: 2.5 mM ATP, 10 mM $MnCl_2$, and 30 mM Tris–HCl, pH 6.5 (with 0.075% Triton X-100, 0.05% sodium azide, 20 nM substrate, and 250 nM capture strand, at 37 °C). These conditions resulted in the highest reaction velocity (up to 6-fold higher than in the standard conditions, described in Section 2).

This test was verified with various helicase preparations, also overexpressed in organisms other than insect cells. The NS3 helicase purified from *Escherichia coli* using the native protocol established for the baculovirus protein was equally active in the helicase assay in contrast to preparations obtained using denaturing methods (not shown).

The activity of highly purified domain 2 of the helicase compared to the activity of the entire protein was insignificant, being 350-fold lower than that of the full-length protein, while the control protein (prolactin) demonstrated no activity (not shown).

Preincubation of the helicase with the substrate (with ATP added to start the reaction at the end of preincubation) moderately increased the rate of unwinding. Whereas the reaction velocity without preincubation was 19.5 ± 1.4 fu/min, after 10 min of preincubation at room temperature it increased to 26.0 ± 7.6 fu/min, a velocity increase of 33%. A longer preincubation (30 min) had a much lower or even negative effect on enzyme activity, probably due to decreased enzyme stability at room temperature in the absence of ATP.

Inhibition of enzyme activity was observed in the presence of anti-HCV helicase antibodies, using antibodies against PVY coat protein (also carrying a His-tag) as negative control. Enzyme activity (compared to enzyme incubated without antibodies) was reduced in both samples, 9- and 5-fold with the specific and the non-specific antibodies, respectively; this low difference in the inhibition level could be due to the presence of a certain level of anti-His antibodies in both sera. Potassium ions (200 mM), shown previously to inhibit the helicase activity [13,19], decreased the unwinding efficiency to 13.5%.

4. Discussion

Searching for a suitable system to screen a variety of possible HCV helicase inhibitors, and to prepare material for NMR and crystallization studies of complexes of this enzyme with selected inhibitors, the entire helicase and its central domain 2 were overexpressed in a baculovirus system. This approach was chosen to avoid problems with low solubility and yield of proteins expressed in *E. coli* that makes their purification in active form difficult. The yield and purity of the full-length helicase and domain 2 make them suitable for structural and enzymatic studies as demonstrated here.

A helicase assay was developed to enable high throughput screening of helicase inhibitors. One of the main advantages of this test is its rapidity: even 15 min after the start of the reaction, the results can be analyzed, helicase activities of various samples compared, conditions modified, and a new test initiated. This is a simple and rapid test, the only limitation being access to a fluorometer. The test is sensitive because 2 nM helicase and 10 nM substrate are sufficient to study the influence of various chemical compounds on enzyme activity as compared to other methods that require 10–100-fold higher enzyme concentrations [14,16,19,33]. The helicase reaction belongs to relatively inefficient reactions and in most assays a large excess of helicase over substrate is required, even as high as 20 nM helicase versus 4.7 pM substrate because of substrate inhibition [16]; in these conditions, the helicase acts in a stoichiometric, non-catalytic manner as opposed to our assay where this is avoided by adding excess capture strand; enzyme concentrations may be lower than those of the substrate, better mimicking normal replication conditions. It should also be stressed that no toxic compounds and waste products are produced, and the fluorescent substrate is stable for at least one year at −80 °C.

This type of assay may have some limitations due to autofluorescence of certain compounds submitted to tests but this problem may be partially solved using various fluorophores and appropriate sets of excitation/emission filters selected on the basis of the autofluorescence spectrum. In addition, in each test, the background is measured and the final results are corrected by subtraction of the background value. We have already tested another pair of fluorophore/quencher (FAM/BHQ-1) and applied it successfully to test the inhibitory effect of autofluorescent epirubicin [an anthracycline cytotoxic agent (the 4′-epimer of doxorubicin), used widely in cancer therapy] on the HCV helicase activity (to be published elsewhere).

Both helicase and ATPase assays confirmed that significant enzymatic activity is observed only with the full-length helicase, domain 2 showing only residual ATPase activity. Domain 2 does not contain canonical NTPase motifs, although binding of ATP-Mg^{2+} is believed to occur in the cleft between domains 1 and 2, and a conserved arginine-rich sequence in domain 2 may be involved in this reaction [16,25]. Newly identified motifs in domain 2, an Arg-clamp and a Phe-loop, are critical for DNA/RNA binding and substrate unwinding [33]. Moreover, a second nucleoside/nucleotide binding site [16,34] could be located in domain 2 and be responsible for the residual ATPase activity detected.

Using the present assay, we have demonstrated that two other putative helicases overexpressed in *E. coli*, those of PVY and *Saccharomyces cerevisiae* Ja2, possess DNA helicase activity although lower than that of the HCV NS3 protein in the conditions established for the flavivirus protein (to be presented elsewhere). Thus, this assay could serve to study helicase activities of proteins from various organisms.

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