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Characterization of ATPase Activity of a Hepatitis C Virus NS3 Helicase Domain, and Analysis Involving Mercuric Reagents

Kiyoshi Kyono*[,1], Masahiko Miyashiro[2] and Ikuhiko Taguchi[2]

[1]Medicinal Chemistry Research Laboratories and [2]Discovery & Pharmacology Research Laboratories, Tanabe Seiyaku Co., Ltd., 16-89 Kashima 3-chome, Yodogawa-ku, Osaka 532-8505

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The C-terminal two-thirds of nonstructural protein 3 (NS3) of hepatitis C virus (HCV) exhibits RNA-dependent NTPase/helicase activity. This enzyme is considered to be involved in viral replication and is expected to be one of the target molecules of anti-HCV drugs. In a search for NTPase inhibitors specific to HCV, we expressed and purified the truncated NS3 NTPase/helicase domain. Here, we report the characterization of its RNA-dependent ATPase activity. This enzyme preferred Mg^{2+} and the optimal pH was 7.0. We further investigated the effects of heavy metal ions on the ATPase activity. The mercuric ion inhibited it significantly, the 50% inhibitory concentration being 49 nM. The fact that the inhibitory profile was competitive and that this inhibition was blocked in the presence of a large excess of cysteine or dithiothreitol, suggested that a cysteine residue in the DECH box was the main target site of mercury.

Key words: ATPase activity, DEAD box protein, expression, HCV, NS3 helicase domain, mercuric reagent, purification.

Abbreviations: BVDV, bovine viral diarrhea virus; YFV, yellow fever virus; CBB, Coomassie Brilliant Blue; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, morpholinepropanesulfonic acid; DTT, dithiothreitol; PCMB, *p*-chloromercuribenzoic acid.

Hepatitis C virus (HCV) is the major etiologic agent of non-A, non-B viral hepatitis (*1*, *2*). Chronic and persistent infection by HCV often leads to liver cirrhosis and hepatocellular carcinomas (*3*, *4*). HCV is a positive-strand RNA virus and a member of family *Flaviviridae* (*5–7*). The viral genome comprises about 9,500 nucleotides and contains a single open reading frame, which encodes a polyprotein of 3,010 to 3,033 amino acids (*5*, *7*, *8*). This polyprotein is processed into structural (C, E1, and E2) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins by both host signal peptidases and viral proteases (*9–12*). While the structural proteins arise through the action of host proteinases, the nonstructural ones are produced by two viral proteinases. The NS2-NS3 junction is cleaved by a zinc-dependent proteinase composed of NS2 and the N-terminal one-third of the NS3 protein (*11–13*).

One-third of the N-terminal of the NS3 protein (20 kDa) is a serine endopeptidase (hepacivirin, EC 3.4.21.98), which cleaves the NS3-NS4A, NS4A-NS4B, NS4B-NS5A, and NS5A-NS5B junctions (*11*, *14–18*), and has a chymotrypsin-like fold (*19*, *20*). This proteinase activity is thought to be necessary for viral replication, and many researchers are focusing on it as a therapeutic target.

The C-terminal two-thirds of NS3 has conserved amino acid motifs predictive of nucleoside triphosphatase (NTPase, EC 3.6.1.15) and RNA helicase. In fact, some groups have demonstrated experimentally that this domain has RNA helicase activity and RNA-dependent NTPase activity (*21–27*).

Helicases intrinsically possess NTPase activity, which hydrolyzes nucleoside triphosphates in the presence of RNA and provides the energy for unwinding. Recently, NS3 NTPase/helicase activity has been demonstrated to be indispensable for the replication of HCV (*28*). Therefore, NS3 NTPase/helicase, as well as NS3 protease, is an attractive target for the development of anti-HCV drugs.

HCV NS3 NTPase/helicase belongs to the DEAD box protein family consisting of many RNA helicases and RNA-dependent NTPases from various organisms, ranging from *Escherichia coli* to humans and viruses, and has conserved motifs, which are necessary for enzymatic activity. However, in the spacer region, except for conserved motifs, amino acid sequences specific to virus species have been observed (*29*). The fact that even NTPases from bovine viral diarrhea virus (BVDV) and yellow fever virus (YFV) close to HCV have different requirements for polynucleotides from each other (*30*, *31*) raises the possibility of designing an inhibitor specific to HCV NTPase.

In a search for HCV NS3 helicase inhibitors, we established a high throughput screening system and preparation method for a full-length NS3/4A complex (*32*). To characterize and elucidate the sites of action of inhibitors selected with this screening system, we started the development of an assay system for HCV NS3 NTPase. We intended to use the truncated NS3 NTPase/helicase domain as the enzyme source in order to examine the direct effects of inhibitors on the NTPase domain.

Here, we describe the expression and purification of the truncated NS3 NTPase/helicase domain and the characterization of its ATPase activity. We also describe the effects of divalent metals on NS3 ATPase activity and the predicted target site of mercuric ions.

*To whom correspondence should be addressed. Tel: +81-6-300-2574, Fax: +81-6-300-2593, E-mail: k-kyono@tanabe.co.jp

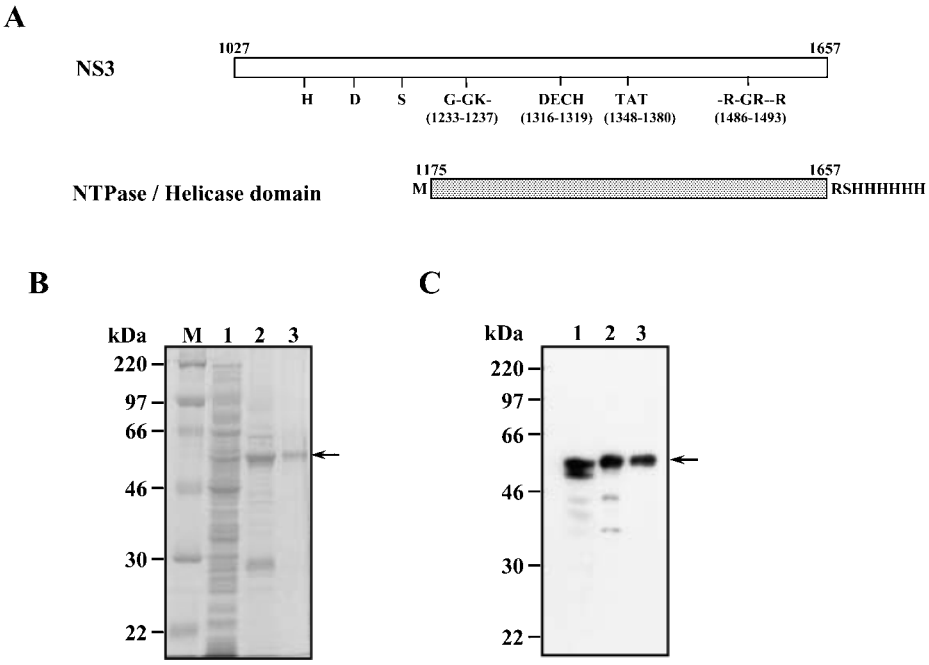

Fig. 1. **Expression and purification of the HCV NS3 NTPase/helicase domain.** (A) Schematic diagram of the NS3 protein and the constructs, which express the HCV NS3 NTPase/helicase domain. H, D, and S represent the catalytic triad amino acid residues His, Asp, and Ser, respectively, for serine protease activity. G-GK-, DECH, TAT, and -R-GR–R are the four consensus motifs required for the helicase and NTPase activities. (B) CBB-stained SDS-polyacrylamide gel. (C) Western blot analysis with the HCV NS3-specific monoclonal antibody. A total *E. coli* lysate (lane 1) was prepared from an induced culture and purified by nickel and poly(U)-Sepharose column chromatography as described under "MATERIALS AND METHODS." Lanes: 2, Ni-column elution; 3, poly(U)-Sepharose column elution.

MATERIALS AND METHODS

Plasmid Construction—Plasmid pQEtHELI-His, used to express the truncated HCV NS3 NTPase/helicase domain (amino acids 1175–1657 of an HCV polyprotein) containing a 6xHis tag derived from the vector at the C terminus, was constructed by PCR amplification using a sense primer, 5′-GGCGCATGCACGTTGTGGGCATCTTCCGG-3′, containing an *Sph*I site, an anti-sense primer, 5′-CGGAGATCTAGTGACGACCTCCAGGTCAGC-3′, containing a *Bgl*II site, and pUNS2D34D (*32*) as a template, followed by subcloning of the PCR-generated fragment into the *Sph*I and *Bgl*II sites of pQE70 (Qiagen).

Preparation of the Truncated HCV NTPase/Helicase Domain—E. coli strain JM109 was transformed with pQEtHELI-His and then grown in LB medium containing ampicillin. The expression of recombinant proteins was induced by adding 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 30°C.

The bacterial pellet was sonicated in binding buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 20 mM imidazole), followed by centrifugation. The supernatant was applied to a nickel column equilibrated with binding buffer. After washing with binding buffer, the bound protein was eluted with Ni-elution buffer (20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 250 mM imidazole). The eluate was dialyzed against TNE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA) overnight at 4°C, and then applied to a poly (U)-Sepharose 4B column (Amersham Pharmacia Biotech) equilibrated with TNE buffer. After washing with the same buffer, the truncated NTPase/helicase domain was eluted with elution buffer (10 mM Tris-HCl, pH 8.0, 1 M NaCl, 1 mM EDTA). After dialysis against TNE buffer overnight at 4°C, the purified proteins were kept in aliquots at –20°C until use. Proteins were separated on PAGEL (ATTO, 10% gels) following Laemmli's method, and detected using a Quick CBB kit (Wako). Western blot analysis was carried out using mouse anti-NS3 monoclonal antibodies (Biodesign), a Vectastain ABC-PO Mouse IgG Kit (Vector), and an Enhanced Chemiluminescence System (Amersham Pharmacia Biotech).

Measurement of ATPase Activity—Standard ATPase reactions were carried out according to the methods described by Chan *et al.* and Henkel *et al.* (*33*, *34*) with partial modification unless otherwise indicated. The reaction mixture (40 μl) comprising 8 μl of purified enzyme, 50 mM MOPS-KOH, pH 7.0, 2 mM ATP, 2.5 mM $MgCl_2$, and 1.6 μg of poly(U) was incubated at room temperature for 30 min in a 96-well plate. The reaction was terminated by adding 160 μl of a malachite green/molybdate/polyvinyl alcohol acidic solution, followed by incubation at room temperature for 5 min. Immediately after mixing and the addition of 20 μl of 30% sodium citrate, the absorbance at 630 nm was measured (SPECTRA MAX 250, Molecular Device).

ATPase activity was calculated as the amount of Pi produced through hydrolysis of ATP using a standard curve constructed using known concentrations of potassium dihydrogen phosphate. Linearity of the standard curve was observed in the range of 0 to 7 nmol/well.

RESULTS

Expression and Purification of the Truncated HCV NS3 NTPase/Helicase Domain—To prepare the enzyme for the characterization of the HCV NS3 NTPase/helicase inhibitor, we expressed the NTPase/helicase domain (amino acid No. 1175 to 1657) in *E. coli*. To facilitate the purification, a hexa histidine tag was added to the C-terminus (Fig. 1A).

As the first purification step, we selected a nickel column. Since most of the 6xHis fusion protein was confirmed to exist in the soluble fraction (data not shown), the supernatant obtained on centrifugation of *E. coli* lysates was applied to the nickel column. Preliminary examination showed that the NTPase/helicase domain

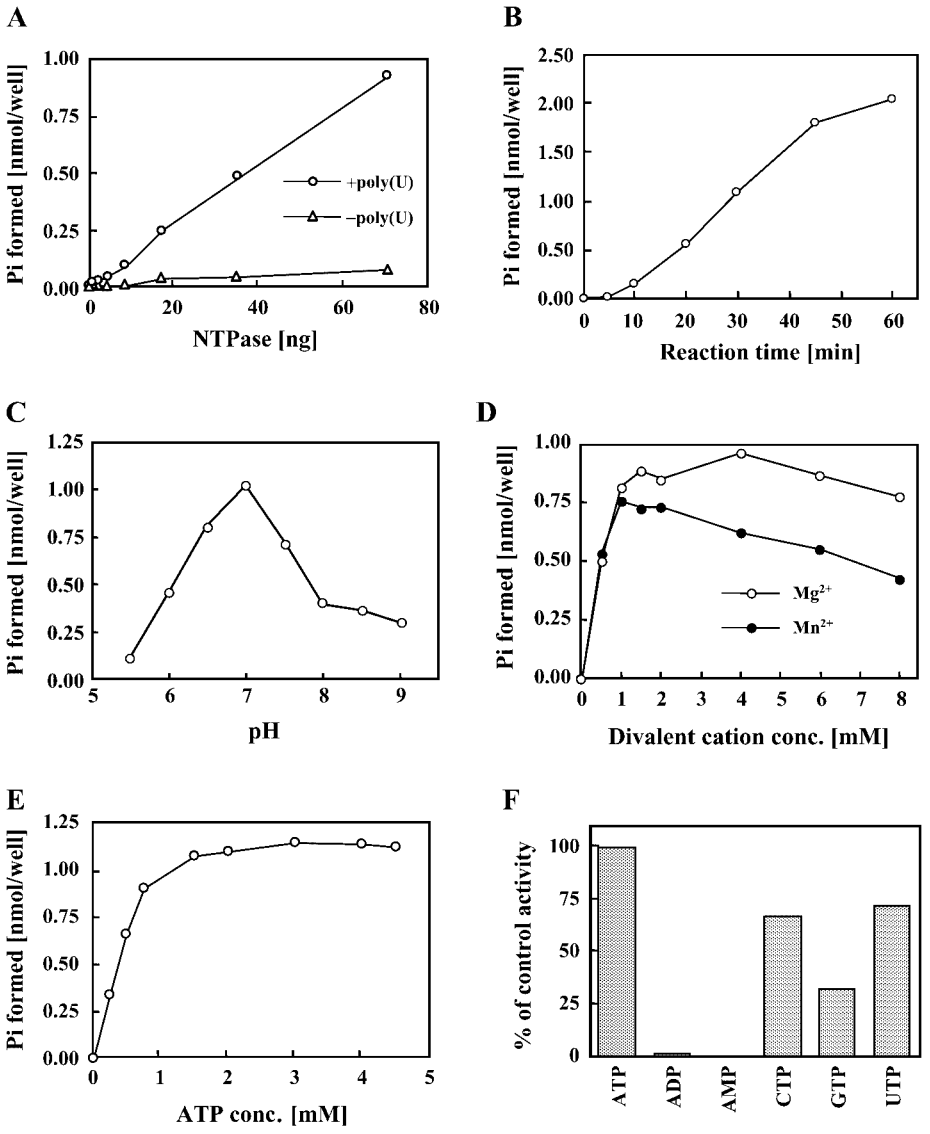

Fig. 2. **Optimization of the reaction conditions for the HCV NS3 ATPase.** (A) Effect of the enzyme concentration on ATPase activity. The ATPase reactions were carried out with the indicated amounts of the purified NS3 NTPase/helicase domain in 50 mM MOPS-KOH (pH 6.5), 2 mM ATP, and 2.5 mM $MgCl_2$, with or without poly(U). (B) Time course of ATPase activity. The ATPase reactions were carried out with 70 ng/well of purified enzyme under the conditions given above. The reactions were terminated after the indicated incubation times. (C) The optimal pH was determined in 50 mM buffer at the indicated pH with 2 mM ATP and 2.5 mM $MgCl_2$. The buffers were as follows: pH 5.5 and 6.0, MES-KOH; pH 6.5 and 7.0, MOPS-KOH; pH 7.5–9.0, Tris-HCl. (D) The optimal $MgCl_2$ and $MnCl_2$ concentrations were determined in 50 mM MOPS-KOH (pH 7.0) with 2 mM ATP. (E) The optimal ATP concentration was determined in 50 mM MOPS-KOH (pH 7.0) with 2.5 mM $MgCl_2$. (F) The hydrolytic activity of various NTPs was measured in 50 mM MOPS-KOH (pH 7.0) with 2.5 mM $MgCl_2$ and 5 mM each of NTPs, ADP or AMP. The activity is represented as a percentage of the ATP-hydrolyzing activity (control activity).

was eluted broadly in the concentration range of 50 to 200 mM imidazole (data not shown). Therefore, sample injection and washing were carried out in the presence of 20 mM imidazole, and bound proteins were one-step eluted with 250 mM imidazole to prevent loss and dilution. The eluate contained about 30-kDa protein derived from the host, and traces of contaminants and proteolytic products of the NTPase/helicase domain besides the major 50-kDa NTPase/helicase domain (Fig. 1, B and C, lane 2).

Since the HCV NS3 NTPase/helicase domain has been reported to bind to poly(U) specifically (*35*), further purification was performed using a poly(U)-Sepharose 4B column as the second step. Our preliminary results showed that the bound NTPase/helicase domain was eluted with 250 to 700 mM NaCl (data not shown). Therefore, binding to the column and column washing were performed with the buffer containing 100 mM NaCl, and bound proteins were one-step eluted with 1 M NaCl. Judging from the CBB stained gel and Western blot analysis, the purity was expected to be more than 95% (Fig. 1, B and C, lane 3). The elution fraction was pooled, dialyzed against TNE buffer, and used for experiments as follows.

Characterization of RNA-Dependent ATPase Activity of the Truncated NS3 NTPase/Helicase Domain—We determined the optimal reaction conditions for the ATPase activity of the NS3 NTPase/helicase domain. The initial conditions [reaction buffer: 50 mM MOPS-KOH, pH 6.5, 2 mM ATP, 2.5 mM $MgCl_2$, 1.6 μg/well of poly(U); reaction conditions: room temperature, 30 min] were chosen according to Suzich *et al*. (*21*) with slight modification. At first, we investigated the optimal enzyme concentration and reaction period. Pi release was enhanced 2- to 10-fold on the addition of poly(U), and proportional to increasing concentration of the enzyme in the range of 0 to 70 ng/well (Fig. 2A). As shown in Fig. 2B, Pi release increased linearly in the range of 0 to 45-min reaction with 70 ng/well of the enzyme. Therefore, we fixed the reaction time at 30 min.

Under the conditions of 2.5 mM $MgCl_2$ and 2 mM ATP, the optimal pH was 7.0 (Fig. 2C). We carried out subsequent experiments with the pH fixed at 7.0.

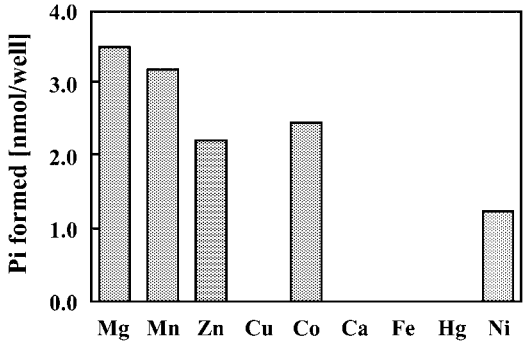

Fig. 3. **The activating effects of divalent metals on ATP hydrolysis by the HCV NS3 NTPase/helicase domain.** The ATPase reactions were performed in 50 mM MOPS-KOH (pH 7.0) containing 50 ng/well of enzyme, 2 mM ATP, 400 ng/well of poly(U), and 2.5 mM each of the indicated divalent metals.

Next, the optimal concentrations of divalent cations were investigated. The ATPase reactions were performed in the presence of various concentrations (0 to 8 mM) of either $MgCl_2$ or $MnCl_2$. As shown in Fig. 2D, the highest activity was observed at 1.0 mM for $MnCl_2$ and more than 1.0 mM for $MgCl_2$. $MgCl_2$ was more efficient than $MnCl_2$ under all conditions tested. For BVDV, it has been reported that NS3 helicase prefers $MnCl_2$ to $MgCl_2$ (*36*). Therefore, this phenomenon appears to be an inherent property of the HCV NS3 ATPase.

Finally, the optimal concentration of ATP was determined in the presence of 2.5 mM $MnCl_2$ at pH 7.0. ATPase activity increased in a concentration-dependent manner up to 0.75 mM ATP, forming a plateau at concentrations higher than 1.5 mM (Fig. 2E).

Next, we investigated the requirement of HCV NS3 truncate NTPase/helicase for nucleoside triphosphates (NTPs) other than ATP. As shown in Fig. 2F, HCV NTPase did not utilize ADP or AMP as a substrate. Among NTPs, CTP and UTP supported 70% of the control activity, but GTP only 30% of the control activity. This is consistent with the report by Suzich *et al*. (*21*). While the nucleotide requirement of BVDV NTPase has been shown to be ATP>UTP>CTP, GTP (*30*), that of YFV NTPase has been reported to be GTP>ATP>CTP>UTP (*31*). The fact that there are differences in the nucleotide requirements among NTPases from closely related viruses suggests that it is possible to develop inhibitors that are specific to individual NTPases.

Table 1. **The inhibitory effects of divalent metals on the ATPase activity of the HCV NS3 NTPase/helicase domain.**

Divalent metal	IC_{50} (μM)
$MnCl_2$	–[a]
$ZnCl_2$	–
$CuCl_2$	130
$CoCl_2$	–
$CaCl_2$	–
$FeCl_2$	750
$HgCl_2$	0.049
$NiCl_2$	–

[a]IC_{50} > 1 mM.

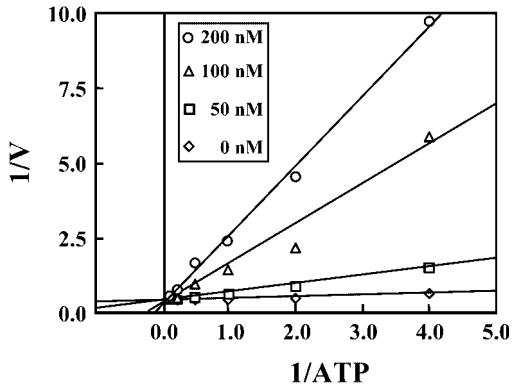

Fig. 4. **Inhibition profile of $HgCl_2$ as to ATPase activity.** The activity was measured in 50 mM MOPS-KOH (pH 7.0) containing 40 ng/well of enzyme, 2.5 mM $MgCl_2$, 400 ng/well of poly(U), and various concentrations of ATP (0.25, 0.5, 1.0, 2.0, 4.0, 8.0 mM) in the presence of the indicated concentrations of $HgCl_2$.

The Activating Effects of Various Divalent Metals on ATPase Activity of the Truncated HCV NS3 NTPase/Helicase Domain—We further examined the effects of divalent metals other than $MgCl_2$ and $MnCl_2$ on ATPase activity. ATPase activity was measured in the presence of 2.5 mM of various divalent metals. When ATPase activity in the presence of $MgCl_2$ was defined as 100%, the relative activity values in the presence of each of $ZnCl_2$, $CoCl_2$, and $NiCl_2$ were 64%, 72%, and 36%, respectively (Fig. 3). The fact that the addition of $CaCl_2$, $CuCl_2$, $FeCl_2$, or $HgCl_2$ had no effect suggested that the heavy metals required for ATPase activity and formation of a complex with NTP were limited.

The Inhibitory Effects of Various Divalent Metals on ATPase Activity of the Truncated HCV NS3 NTPase/Helicase Domain—Next, we investigated the effects of these heavy metals on ATPase activity in the presence of 2.5 mM $MgCl_2$ (Table 1). While $CuCl_2$, $FeCl_2$, and $HgCl_2$ showed an inhibitory effect, other metals had a negligible effect. The inhibitory effect of $HgCl_2$ was especially sig-

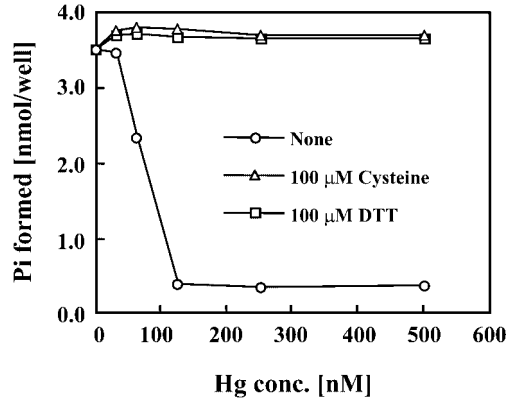

Fig. 5. **The inhibitory effects of $HgCl_2$ on the HCV NS3 ATPase activity in the presence of DTT or cysteine.** The activity was measured in 50 mM MOPS-KOH (pH 7.0) containing 40 ng/well of enzyme, 2 mM ATP, 2.5 mM $MgCl_2$, 400 ng/well of poly(U), and the indicated concentrations of $HgCl_2$, with or without 100 μM DTT or cysteine.

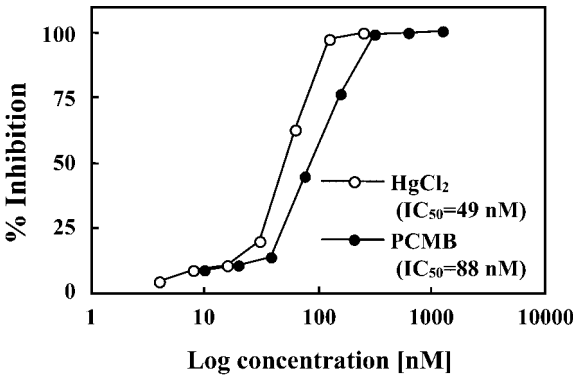

Fig. 6. **Titration curves for $HgCl_2$ and PCMB.** The ATPase reaction was carried out in 50 mM MOPS-KOH (pH 7.0) containing 40 ng/well of enzyme, 2 mM ATP, 2.5 mM $MgCl_2$, 400 ng/well of poly(U), and the indicated concentrations of $HgCl_2$ or PCMB.

nificant (IC_{50} = 49 nM). We speculated that this inhibitory effect was caused by a direct action on the enzyme rather than prevention of complex formation of $MgCl_2$ with ATP, since the reaction mix contained large molar excesses of ATP (2 mM) and $MgCl_2$ (2.5 mM). To clarify the site of action of $HgCl_2$, we performed the subsequent experiments.

Determination of the Site of Action of $HgCl_2$ on HCV NS3 NTPase—Figure 4 shows an inhibition profile for $HgCl_2$. A Lineweaver-Burk plot revealed that the inhibitory profile comprised typical competitive inhibition against ATP. This inhibitory effect was blocked with the coexistence of 100 μM cysteine or DTT (Fig. 5). PCMB, which is a modifying agent for cysteine residues, showed strong inhibition (IC_{50} = 88 nM) comparable to that of $HgCl_2$ (Fig. 6A). These results suggest that $HgCl_2$ inhibits its NS3 ATPase activity by acting through a cysteine residue that exists in the proximity of the ATP binding site.

DISCUSSION

To establish a HCV ATPase assay system, we expressed the NTPase/helicase domain in *E. coli* as the enzyme source. In this study, the NTPase/helicase domain was also expressed successfully in a soluble form, as previously reported (*22*, *26*, *27*). We performed one-step purification by nickel column chromatography from the soluble fraction. When we measured the ATPase activity of the eluted fraction, significant Pi release was observed. The fact that the same fraction derived from lysates of *E. coli* carrying only the vector showed no activity indicated that the observed Pi release was caused not by ATPase from the host, but by the expressed HCV NTPase/helicase domain (data not shown). Since this eluted fraction contained some contaminants, we further purified it by poly(U)-Sepharose 4B column chromatography to homogeneity (Fig. 1B, lane 3).

The ATPase activity of the NS3 NTPase/helicase domain we prepared was optimal at pH 7.0 (Fig. 2C). This profile differs from the pH optimum of 6.5 previously reported by Suzich *et al.* (*21*). Jin *et al.* and Preugschat *et al.* measured the ATPase activity at pH 6.5 and pH 7.0, respectively. However, there has been no mention of the optimal pH. With respect to the requirement of divalent cations, our enzyme slightly preferred Mg^{2+} to Mn^{2+} at all concentrations tested. While no comparative data for Mg^{2+} and Mn^{2+} as to the ATPase activity of the truncated NTPase/helicase domain have been reported, there have been previous reports on the effects of Mg^{2+} and Mn^{2+} on helicase activity. Kim *et al.* reported that NS3 RNA helicase showed a slight bias for Mg^{2+} compared to Mn^{2+} (*22*). Tai *et al.* reported that with lower concentrations of divalent ions (up to 1.5 mM), RNA helicase preferred Mn^{2+} to Mg^{2+}, but increased concentrations had an adverse effect (*27*). The discrepancies regarding the optimal pH and divalent cation requirement may reflect the different HCV clones used in different laboratories.

The K_m, V_{max}, k_{cat}, and k_{cat}/K_m values for ATP with poly(U) were 151 μM, 80.9 pmol/min, 107 min^{-1}, and 710 min^{-1} mM^{-1}, respectively. The K_m value agreed with the value of 160 μM reported previously (*21*). However, the k_{cat} and k_{cat}/K_m values were lower than those reported by Suzich *et al.* (*21*). The reason for this may be the difference in the HCV clone or the assay method used. For BVDV and YFV, which are close to HCV, the k_{cat}/K_m values for ATP with poly(U) or poly(A) were reported to be 3,755 min^{-1} mM^{-1} and 9,768 min^{-1} mM^{-1}, respectively (*30*, *31*). These results suggest that BVDV and YFV may replicate more effectively than our HCV clone in their host cells.

Since there has been no previous report on the effects of various divalent cations on ATPase activity, we investigated the requirement of seven divalent cations in addition to Mg^{2+} and Mn^{2+}. We found that in addition to Mg^{2+} and Mn^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} also supported the ATPase activity. However, their activating effect was lower than that of Mg^{2+} and Mn^{2+} (Fig. 3). On the other hand, we found that Cu^{2+}, Fe^{2+}, and Hg^{2+} inhibited the ATPase activity in the presence of 2.5 mM $MgCl_2$. The inhibitory effect of Hg^{2+} was especially very strong (IC_{50} = 49 nM), and its inhibition profile was competitive against ATP (Fig. 4). The fact that this inhibitory effect was blocked by the addition of reducing agents (Fig. 5) and that PCMB (a modifying agent for cysteine residues) also showed strong inhibition (IC_{50} = 88 nM) (Fig. 6) suggested that the site of action of Hg^{2+} was a cysteine residue that exists in the proximity of the ATP binding site.

RNA-dependent helicases/NTPases in RNA viruses are classified into three superfamilies (SF I, II, and III) (*37*, *38*). The NTP binding motifs that are the conserved regions in these superfamilies, are the Walker A motif and B motif (*39*). The A motif and B motif are thought to be involved in binding with β- or γ-phosphate of NTP, and the chelation of Mg^{2+} of the Mg-NTP complex.

Therefore, the site of action of Hg^{2+} on HCV NTPase was suggested to be located in the proximity of the A motif or B motif. Since only one cysteine residue (Cys-1318) exists in both motifs, we speculated that this cysteine was the target site of $HgCl_2$. In fact, mutational studies demonstrated that the replacement of Cys-1318 with Gly or Ser led to a significant decrease in ATPase activity (*40*), and the replacement with Ala resulted in a 50% decrease in the wild-type activity (*41*). Considering that the B motifs in BVDV and YFV NTPase are DEYH and DEAH, respectively, this cysteine residue may be an attractive target site for designing NTPase inhibitors specific to HCV.

Although $HgCl_2$ is a strong HCV NS3 NTPase inhibitor, it is impossible to develop it as an anti-HCV drug in terms of toxicity. Therefore, it is necessary to find inhibitors that are specific to the DECH motif of HCV NTPase and have few side effects. The knowledge obtained in the present study will be useful for designing such inhibitors in the future.

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