The NS3FL showed an enhanced RNA helicase activity and NTPase, RTPase, and RNA helicase are presented. The diseases caused by DEN infections include from dengue fever, usually a self-limiting disease, to the most common infection encountered in humans (4). The diseases pose a significant threat to humans living in DEN-infected Aedes aegypti mosquitoes endemic in areas that inhabit two-thirds of world population (5).

DEN2 genome is a single-stranded RNA (10,723 nt in length for DEN2 New Guinea C strain used in this study (6)) of positive polarity. The viral RNA contains a long open reading frame coding for a polyprotein precursor. The polyprotein is processed into mature structural proteins, capsid (C), precursor membrane (prM), and envelope (E) and at least seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5, by cellular signal peptidase and viral serine protease in the endoplasmic reticulum (for review, see Ref. 7). The 5'-end of the viral RNA is modified by a type I cap structure (m7GpppN; 2'-OH moiety of N is methylated).

DEN2 NS3 is a multifunctional protein of about 69 kDa. It includes a serine catalytic triad within the N-terminal 185 amino acid residues. The protease domain is activated by the hydrophobic protein NS2B which serves as a cofactor for the protease and forms a complex in the infected cells (8–12). NS2B has three hydrophobic regions flanking a conserved hydrophilic domain. The hydrophilic domain of NS2B (NS2BH) alone is sufficient for protease activity in processing of the 2B3 site in the precursor expressed in transfected cells or in an in vitro protease assay (13, 14). NS2B is an endoplasmic reticulum resident integral membrane protein (14).

The mosquito-borne Flavivirus genus, in the Flaviviridae family, includes human pathogens of global significance. DEN2 nonstructural protein 3 (NS3) has a serine protease domain (NS3-pro) and requires the hydrophilic domain of NS2B (NS2BH) for activation. NS3 is also an RNA-stimulated nucleoside triphosphatase (NTPase)/RNA helicase and a 5'-RNA triphosphatase (RTase). In this study the first biochemical and kinetic properties of full-length NS3 (NS3FL)-associated NTPase, RTase, and RNA helicase are presented. The NS3FL showed an enhanced RNA helicase activity compared with the NS3-pro-minus NS3, which was further enhanced by the presence of the NS2BH (NS2BH-NS3FL). An active protease catalytic triad is not required for the stimulatory effect, suggesting that the overall folding of the N-terminal protease domain contributes to this enhancement. In DEN2-infected mammalian cells, NS3 and NS5, the viral 5'-RNA methyltransferase/polymerase, exist as a complex. Therefore, the effect of NS3 on the NS3 NTPase activity was examined. The results show that NS5 stimulated the NS3 NTPase and RTase activities. The NS5 stimulation of NS3 NTPase was dose-dependent until an equimolar ratio was reached. Moreover, the conserved motif, 184RKKR, of NS3 played a crucial role in binding to RNA substrate and modulating the NTPase/RNA helicase and RTase activities of NS3.
activities of NS3 of hepatitis C virus, a member of Hepacivirus genus (26–35), and the p80 of bovine virus diarrhea virus, a member of the Pestivirus genus (36, 37), have been well characterized. However, the RNA helicases of mosquito-borne flaviviruses have not been well studied. The N-terminal-truncated DEN2 (16, 20) and Japanese encephalitis virus NS3 (38) were expressed in E. coli and purified to demonstrate the RNA helicase activity. The RNA helicase activity of the full-length NS3, its RNA binding properties, the kinetic parameters or the influence of the protease cofactor, NS2B domain, on the RNA helicase activity has not been previously studied to date. In this study we report that the full-length NS3 with or without NS2B cofactor domain, expressed in E. coli and purified, exhibits a catalytically more efficient RNA helicase activity than the N-terminal-truncated NS3 helicase domain, suggesting that the protease domain enhances RNA helicase activity. A functionally active serine catalytic triad is not required for this enhancement of RNA helicase activity as shown by mutagenesis of the protease catalytic triad residue, H51A.

The multifunctional NS3 protein exists in a complex with NS5, which itself has two enzyme activities, the 5'-RNA O-methyl transferase involved in 5'-capping and the RNA-dependent RNA polymerase required for viral RNA replication in DEN2-infected cells (39) (for review, see Ref. 40). Therefore, we examined the role of NS5 interaction on the NTPase/RNA helicase and the RTase activity of NS3. Our results show that the NS5 stimulates the NTPase activity of NS3 in a dose-dependent manner until an equimolar stoichiometry is attained, which supports the notion that the heterodimeric NS3:NS5 complex is the functional unit involved in unwinding double-stranded RNA during replication. Furthermore, RNA binding analyses by gel shift assays show that NS3 binds to RNA, and a positively charged motif, RKKR, which is conserved in Flavivirus NS3, is required for this RNA binding activity. Mutation of this motif significantly reduced the NTPase/RNA helicase activity of NS3, suggesting that this motif is an important determinant in NTPase/RNA helicase function of NS3.

MATERIALS AND METHODS

Plasmid Constructs—The construction of the NS3pro expression plasmid with a His6 tag at the N terminus is described previously (22). NS2BH(QR)-NS3pro expression plasmid, which contains the hydrophilic domain of NS2B shown to be sufficient for activation of the NS3 protease domain (13, 14), was constructed as follows. The PCR primers used were 5'-GCCGGATCCGCCGATTTGGAACTGGAGAGAGCCGCC-3' (the BamHI site is underlined; from the 5'-TCC (Ser) codon to GCC (Ala) codon represents amino acid positions 48–57 of NS2B as described previously (14)) and 5'-TTGGCGCGCTGTTCTTCCTCTTCGTTTTTTA-CCTCCAC-3' (the BshHI site is underlined; the primer represents amino acid positions 93–95 of NS2B); the former codes for Ser-Val, and the latter codes for Ser. The primers used for generating the NS3pro fragment, the corresponding fragment of NS3FL (14), and 5'-CCAGATGTTGCGTATTTGCGAGATGCTTCCATCACCC-3' (the HindIII site is underlined). The products of these two PCR reactions were cloned into the TA vector (Invitrogen). The positive clones were identified, and the plasmid DNAs were digested with NdeI and HindIII sites, giving rise to pQE30:NS2BH(QR)-NS3pro. To generate the NS2BH(QR)-NS3FL, the NS3-pro region in the pQE30-NS2BH(QR)-NS3pro was replaced by the NS3FL as follows. pQE30-NS3FL (22) and pQE30-NS2BH(QR)-NS3pro were both digested with NdeI. After removing the NS3pro fragment, the corresponding fragment of NS3FL (reverse primer, nt positions 5071–5082, forward primer, nt positions 5103); Primer 4, 5'-AAAGTTGGAACTGGAGAGAGCCGCC-3' (the 4-amino acid region mutated spans 5071–5082, underlined in 2 and 3 is the 4M mutant region); Primer 5, 5'-CAAGATTGTTGCGTATTTGCGAGATGCTTCCATCACCC-3' (nt 5161–5184). Individual PCR reactions were carried out using primers 1 + 3 and 2 + 4, and PCR products were mixed and a third PCR was done using primers 1 and 4 as described (42). The PCR products were cloned into pGEM-T-Easy Cloning vector (Promega, Madison WI). All constructs were verified by sequencing. The PCR product (491 bp) containing the wild type NS3FL cDNA (22) between the NS1 (nt 4696) and Ba356 (nt 15168) sites within the NS3 coding sequence is shown (Fig. 1).

Purification of Recombinant Proteins—E. coli strain, Top 10F' (Invitrogen), or BL21 was transformed with appropriate expression plasmid, and the cells were grown at 37 °C in LB medium containing ampicillin (100 μg/ml) and 0.5% w/v glucose to an optical density of 0.6 at 600 nm. Cells were centrifuged at 6000 × g and resuspended in glucose-free LB + ampicillin (100 μg/ml), and isopropyl-1-thio-β-galactoside (0.4 mM) was added to a final concentration of 0.125 mM. Cells were grown for 5 h at 30 °C, harvested by centrifugation, and stored at −80 °C until use. For purification recombinant proteins, the bacterial pellets were resuspended in native lysis buffer containing 100 mM sodium phosphate buffer, pH 7.5, 300 mM NaCl, and 40% glycerol. Cell lysates were loaded onto a cobalt-based immobilized metal affinity column (Talon™, Clontech). After infection and then washing, the recombinant proteins were eluted with 500 mM imidazole. The eluted fractions containing the highest protein concentration were pooled, dialyzed against a buffer containing 50 mM sodium phosphate, pH 7.5, 300 mM NaCl, and 40% glycerol. In some cases the recombinant proteins were purified by using the fast protein liquid chromatography HiTrap Chelexing HP 1-ml column (AKTAprime system from Amersham Biosciences). The proteins were eluted with 20 mM Tris, pH 7.4, 500 mM NaCl, and 0.5 M imidazole. Peak fractions were dialyzed against the HiTrap SP FF (1 ml) column. The fractions with the highest protein concentrations were pooled and dialyzed against 20 mM Tris-HCl, pH 7.5, 75 mM NaCl, and 40% glycerol.

RNA Substrates—For the electrophoretic mobility shift assays and the 5'-RTase assays, RNA of 100 nucleotides in length was obtained from the 5'-end of DEN2 RNA (which includes the 5'-untranslated region of 98 nucleotides) was obtained by in vitro transcription of the PCR product. The template for PCR was the pSY2 plasmid (encoding the subgenomic RNA (43)). The concentration of the RNA was measured by using a spectrophotometer, and the integrity of RNA was verified by partial denaturing PAGE (3.5% containing 7 μm urea) followed by ethidium bromide staining. For RNA helicase assays two reactions were run with 500 nM RNA and 50% imidazole. The reactions containing the highest protein concentration were pooled, dialyzed against 20 mM Tris-HCl, pH 7.5, 75 mM NaCl, and 40% glycerol.

RNA Helicase Assay—For the electrophoretic mobility shift assays and the 5'-RTase assays, RNA of 100 nucleotides in length containing sequences from the 5'-end of DEN2 RNA (which includes the 5'-untranslated region of 98 nucleotides) was obtained by in vitro transcription of the PCR product. The template for PCR was the pSY2 plasmid (encoding the subgenomic RNA (43)). The concentration of the RNA was measured by using a spectrophotometer, and the integrity of RNA was verified by partial denaturing PAGE (3.5% containing 7 μm urea) followed by ethidium bromide staining. For RNA helicase assays two reactions were run with 500 nM RNA and 50% imidazole. The reactions containing the highest protein concentration were pooled, dialyzed against 20 mM Tris-HCl, pH 7.5, 75 mM NaCl, and 40% glycerol.

5'-NTPase and RTase Assays—The NTPase and RTase assays were based on the original colorimetric method described several decades ago to quantify the inorganic phosphate present in serum (45, 46) and more recently in a study of HCV NS3-associated NTPase (47). The method used in our study has slight modifications. Briefly, the reaction mixture (50 μl) consisted of 25 mM HEPEs-K+, pH 7.5, 1.5 mM MgCl₂, 1 mM NTP (unless the NTP concentration was varied), and 140 nM purified NS3 protein. For RTase assays, the NTP was substituted with triphosphorylated RNA (100 nucleotides in length). The reaction mixtures were incubated for 30–60 min (unless otherwise indicated) at 37 °C. Reactions were stopped by adding 10 μl of 2× SDS-PAGE loading buffer, heated to 95 °C for 10 min, and cooled slowly to room temperature (−3 h).

NFATPase and RTase Assays—The NFATPase and RTase assays were based on the original colorimetric method described several decades ago to quantify the inorganic phosphate present in serum (45, 46) and more recently in a study of HCV NS3-associated NTPase (47). The method used in our study has slight modifications. Briefly, the reaction mixture (50 μl) consisted of 25 mM HEPEs-K+, pH 7.5, 1.5 mM MgCl₂, 1 mM NTP (unless the NTP concentration was varied), and 140 nM purified NS3 protein. For RTase assays, the NTP was substituted with triphosphorylated RNA (100 nucleotides in length). The reaction mixtures were incubated for 30–60 min (unless otherwise indicated) at 37 °C. Reactions were stopped by adding 10 μl of 2× SDS-PAGE loading buffer, heated to 95 °C for 10 min, and cooled slowly to room temperature (−3 h).

Functional Interactions of Flavivirus Replicase Components
amounts were varied), and 5 mM substrate. The reaction was carried out at 37 °C for various times and then terminated by adding 5 μl of 5× RNA loading dye (100 mM EDTA and 0.7% SDS). Samples were loaded onto a 12% native polyacrylamide gel. The gel was electrophoresed at 100 V for 1.5 h, dried, and subjected to autoradiography. The ratio of released single strands versus the total of single and double strands was quantified by PhosphorImager (Molecular Dynamics).

**RESULTS**

Expression and purification of DEN2 NS3FL proteins. A previous study from our laboratory indicated that the N-terminal-truncated DEN2 NS3 (NS3del2, referred to as NS3a160) protein had basal NTPase that was stimulated by the addition of poly(A). NS3Δ160 had the deletion of the serine protease domain but contained all the conserved domains attributed to NTPase/RNA helicases of DENV family members. The kinetic constants of the NTPase activity of NS3Δ160 were close to those of other flaviviral NTPases. However, the RNA helicase activity of NS3del2 required 2.7 μM concentrations of purified NS3del2 protein to unwind less than 5% of a 29-bp RNA duplex. On the other hand, the hepatitis C virus NS3 and the bovine diarrhea virus ψ80 RNA helicases required 0.1–1 pmol (−10–100 nM) of enzyme for unwinding similar substrates (27, 36). We also reported that the RNA-stimulated NTPase activity of NS3del2 was abolished by mutation of the positively charged motif, RSRRK → QNGN, of NS3 or by deletion of an additional 20-amino acid residues from NS3Δ160 (NS3Δ180) even though the latter mutant still contained the RSRRK motif very close to the N terminus. In either of these two mutants the basal NTPase activity was still retained (16). From these results we concluded that the RSRRK motif, although not required for basal NTPase activity, played an important role in the RNA-mediated stimulation of NTPase. The mechanism for requirement of the positively charged motif in RNA-stimulated NTPase was unknown. In this study we hypothesized that the weak RNA helicase activity associated with the NS3Δ160 protein might be due to suboptimal folding of the NS3Δ160 protein and/or binding to double-stranded RNA substrate in contrast to the RNA helicases associated with the full-length NS3 of hepatitis C virus or the ψ80 of bovine viral diarrhea virus. We also considered the alternate possibility that the DEN2 RNA helicase may be stimulated by interaction with other viral components such as NS5, the RNA-dependent RNA polymerase, with which NS3 forms a stable complex in Flavivirus-infected cells (39, 48). Moreover, none of the mosquito-borne flaviviral NS3 RNA helicases have been studied in detail to date.

In this study we launched detailed biochemical and kinetic analyses of NTPase/RNA helicase activities of full-length NS3 (NS3FL) containing both the serine protease domain at the N terminus and the conserved NTPase/RNA helicase domains at the C terminus. Because the protease domain of NS3 interacts with the hydrophilic domain of NS2B (NS2BH), we sought to examine the effect of this interaction on NTPase and RNA helicase activities. To this end we also constructed the expression plasmid, NS2BH(QR)-NS3FL, encoding the precursor protein in which the NS2BH is linked to the protease domain of NS3 flanking the two amino acid residues, QR, occupying the P2 and P1 positions of the protease cleavage site. The recombinant protein expressed from this plasmid underwent cis cleavage at the protease-sensitive site QR ↓ due to interaction of NS2BH with the NS3 protease domain to produce the non-covalent binary complex, NS2BH:NS3FL. We also constructed the NS2BH(QR)-NS3FL(H51A) expression plasmid containing the mutant catalytic triad residue (H51A). The protein expressed from this plasmid did not undergo cis cleavage and was in the precursor form (see Fig. 2A) as expected. To analyze the role of 184RSRKK motif in NTPase and RNA helicase activities of NS3FL, the NS3FL containing the mutant motif RSRRK→QNGN was constructed (Fig. 1). The transformed E. coli TOP10F cells were grown, and the proteins were expressed and purified as described under “Materials and Methods.”

**NTPase Activities of NS3FL**

**Poly(A) Stimulation of the NTPase Activity of NS3FL.**—Our previous study indicated that the NTPase activity of the NS3Δ160 deletion mutant was stimulated by poly(A), which required not only the region between the 161–180 amino acid...
residues but also the 184RKKR motif (16). This conclusion was reached based on our results that the poly(A)-stimulated NTPase activity was abolished with the NS3Δ160(4M) mutant protein containing the 184RKKR → 184QNGN mutation or with the deletion of an additional 20 amino acid residues from NS3Δ160 protein (NS3Δ180 protein), although the basal NTPase activity of either protein was unaffected. We sought to examine whether the basal NTPase activity of the NS3FL protein is also stimulated by poly(A) and whether the 184RKKR → 184QNGN mutation has any effect on the poly(A)-stimulated NTPase activity. We expressed and purified the NS3FL(4M) mutant protein under conditions similar to those for NS3FL (data not shown) and assayed its NTPase activity. Fig. 4 shows that poly(A) stimulated the basal activity of NS3FL protein but not the basal activity of the NS3FL(4M) mutant. However, the degree of stimulation of the NTPase activity of NS3FL was only about 2-fold compared with 5–7-fold for the NS3Δ160. This can be explained due to a higher basal NTPase activity of NS3FL than the NS3Δ160. Moreover, the positively charged motif 184RKKR was also important for RNA-stimulated NTPase activity of NS3FL because mutation of this motif abolished this stimulation (Fig. 4). This result suggested that the 184RKKR motif is involved in RNA binding, and the resultant conformational change is required for stimulation of NTPase activity.

**Characterization of the RNA Helicase of NS3FL Protein**—Next, we examined the RNA helicase activities associated with NS3FL and NS3FL(4M) (Fig. 5). The helicase assay utilized synthetic double-stranded RNA substrate having a 3′-single-stranded terminus, formed by annealing of a synthetic 35P-labeled 30-mer RNA to a 15-mer complementary RNA according to the method described previously (4). We used a 100-fold excess of the enzyme to substrate in all the experiments shown in Fig. 5, A–D. The results shown in Fig. 5 indicated that the presence of the protease domain in the NS3FL had an enhancing effect on the RNA helicase activity compared with that of the protease domain-deleted NS3 (NS3Δ160) protein described previously (16). The NS3Δ160 protein was required at 2.7 μM for RNA unwinding of less than 5% of the substrate RNA (16). Interestingly, the mutation of the 184RKKR motif significantly reduced the RNA helicase activity of NS3FL (Fig. 5D). This is the first report for the function of this positively charged motif in RNA helicase activity of any flaviviral NS3 protein.

We also examined whether the presence of the protease cofactor, NS2BH, in a binary complex with NS3FL (NS2BH/QR/NS3FL), which was formed after cleavage of the QR↓ site, had any effect on the RNA unwinding activity catalyzed by NS3FL. Our results showed that the presence of NS2BH cofactor domain at the N terminus of NS3FL protease domain in the binary complex did not have a significant en-

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**TABLE I**

The calculations were carried out using Sigma Plot (v8) with a kinetic module.

| Kinetic parameters of NS3
| NTPase | 5′-RTPase |
|----------------|----------|
| V_{max} (nmol/s/μg of protein) | 6.05 ± 0.4 | 0.65 |
| K_{cat} (pmol of P_i released/s/pmol of enzyme) | 0.92 ± 0.35 | 0.09 |
| K_{m} (μM) | 191.3 ± 24.9 | 29.3 |
| K_{cat}/K_{m} (μM/s) | 4788.8 ± 1237.3 | 3185.2 |

**FIG. 2.** Purification of NS3 proteins. The purification of NS3 proteins with an N-terminal His tag was as described previously for NS3FL (22) and as described under “Materials and Methods.” A and B, Coomassie Blue-stained SDS-PAGE gels of purified proteins. A, lane M, protein molecular weight markers; lane 1, NS2BH(QR)-NS3FL; lane 2, NS2BH(QR)-NS3FL(H51A). B, lane 1, molecular size markers; lane 2, purified NS3FL (wild type), lane 1, molecular size markers; lane 2, purified NS3FL(4M), lane M, molecular size markers; lane 1, NS2BH(QR)NS3FL after dialysis at pH 9.0; lane 2, the same protein as in lane 1 before dialysis. C, Western blot of the proteins on the gel in D with rabbit polyclonal anti-NS2B antibody (14). D, Western blot of the proteins on the gel in D with rabbit polyclonal anti-NS3 antibody (22).

**FIG. 3.** Analysis of NTPase activity of NS3FL. The NTPase assays of NS3FL with different substrate (ATP) concentrations in triplicate were performed under “Materials and Methods.” From the Lineweaver-Burk plots (inset), the kinetic parameters were calculated as shown in Table 1.

**FIG. 4.** The basal NTPase activity of NS3FL but not NS3FL(4M) mutant was stimulated by poly(A). The standard ATPase assays were carried out as described under “Materials and Methods” with purified NS3FL or NS3FL(4M) protein in the absence or presence of increasing concentrations of poly(A). The assays were done in triplicate in 96-well microtiter plates, and the average values of K_{cat}/s were plotted. Φ, NS3FL; ○—○, NS3FL(4M).
hancing effect on the RNA helicase activity of NS3FL. We surmised that in NS2BH(QR)/NS3FL, the activated protease in the binary complex had an opposing effect on the helicase activity as seen from the time course of RNA unwinding by NS3FL versus NS2BH(QR)/NS3FL. Therefore, we constructed the NS2BH(QR)-NS3FL (H51A) plasmid in which the protease domain was inactivated by mutagenesis of the catalytic triad residue, H51A and purified the mutant protein as an uncleaved precursor (Fig. 2A). This protein had reproducibly, an enhanced RNA helicase activity over the NS3FL (Fig. 5, A versus B; see also E).

We sought to determine whether changing the ratio of the enzyme to substrate had any effect on the rate of unwinding. The unwinding of the substrate by the NS2BH(QR)/NS3FL protein was carried out at an [E:S] ratio of 40 and 400. As shown in Fig. 5F, increasing the enzyme to substrate ratio significantly increased the activity of the NS3 RNA helicase. These results are consistent with a recent study which revealed that a large excess of the HCV NS3 helicase was required for optimal unwinding of the substrate (35).

The NTPase Activity of NS3FL Is Stimulated by Interaction with NS5, the RNA-dependent RNA Polymerase—In DEN2-infected cells, NS3 and NS5 exist as a complex and are thought to be important components of the viral replicase involved in RNA replication (39). However, the functional consequence of this interaction and which step this complex is required for viral replication have not yet been established. In this study we asked whether the NS5 protein has any influence on the NTPase activity of NS3. The NTPase activity of NS3FL was assayed in the absence and presence of increasing amounts of NS5. The results shown in Fig. 6 indicate that NS5 stimulated the NTPase activity of NS3FL in a dose-dependent manner until the molar ratio of 1:1 was reached. At this point the addition of further amounts of NS5 had no effect on the NTPase activity of NS3. From these results we conclude that a 1:1 stoichiometric complex of NS3/NS5 is the functional NTPase. Next, we examined whether the basal NTPase activity of NS3FL(4M) mutant can also be stimulated by the addition of NS5. The results shown in Fig. 6 indicate that the NTPase activity of the NS3FL(4M) mutant lost the ability to be stimulated by NS5, suggesting that stimulation of basal NTPase activity by RNA and NS5 is intimately linked.

The 5′-RTPase Activity of NS3 Is Also Stimulated by Interaction with NS5—We previously reported that the NS3FL has 5′-RTPase, the cleavage of the γ-β phosphodiester bond of a triphosphorylated RNA (22), and the same active site was
involved in NTPase and 5'-RTPase activities of NS3 (20, 22). The 5'-RTPase is the first enzymatic step required in the 5'-cap addition of viral RNA. The N-terminal region of NS5 has been reported to possess the 2'-O-methyltransferase, an activity involved in the formation of type I cap, and the crystal structure of this domain was reported previously (51). We surmised that the 5'-cap addition is carried out by an enzyme complex of NS3 and NS5 and that this interaction, which enhanced the NTPase activity of NS3, might also influence its 5'-RTPase activity. Fig. 7 shows that the addition of submolar amount of NS5 (50 nM) to 280 nM NS3 stimulated the RTPase activity of NS3 ~5-fold on a triphosphorylated RNA substrate (100 nucleotides in length). The apparent $K_m$ of DEN2 NS3 for triphosphorylated RNA substrate was 29.3 μM, and the $V_{max}$ was 0.65 nmol of substrate hydrolyzed/s. The apparent $K_{cat}$ for the RTPase was 0.09/s, and the catalytic efficiency, $K_{cat}/K_m$, was 3185 m$^{-1}$ s$^{-1}$. The turnover number of the RTPase activity was 10-fold lower than that of the NTPase activity of NS3$_{FL}$, although the catalytic efficiency for the two reactions was approximately similar (Table I).

**FIG. 6.** The NTPase activity of wild type NS3$_{FL}$ but not NS3$_{FL}(4M)$ is stimulated by the addition of NS5. The standard ATPase assays were carried out in the presence of purified NS3$_{FL}$ or NS3$_{FL}(4M)$ (140 nM) as described under “Materials and Methods” in the absence or presence of increasing concentrations of purified NS5. The assays were done in triplicate in 96-well plates, and the average values of $K_{cat}/s$ were plotted. ○, NS3$_{FL}$; □—□, NS3$_{FL}(4M)$.

**DISCUSSION**

In this study we launched a detailed biochemical and kinetic analysis of the RNA-stimulated NTPase RNA helicase and 5'-RNA triphosphatase activities of full-length DEN2 NS3 protein. We established the following key findings. 1) Full-length NS3 with the serine protease domain at the N terminus and the conserved motifs found in several RNA-stimulated NTPases/ RNA helicases at the C-terminal region of the protease domain is a more active RNA helicase than the N-terminal-truncated or the glutathione S-transferase-NS3 fusion protein that was reported previously (20, 22, 52). 2) Moreover, the interaction of NS3$_{FL}$ with NS5 stimulated the NTPase in a dose-dependent manner up to the point when a 1:1 stoichiometry was reached, and beyond this point NS5 had no effect on the NTPase activity, suggesting that the NS3-NS5 complex is the functional unit for the NTPase activity of NS3, which is coupled to RNA unwinding (3). The positively charged motif 184RKKR plays an important role in RNA binding, and this motif modulates both the RNA-stimulated NTPase, RNA helicase, and the 5'-RTPase activities, all requiring interaction with RNA. Recently, the structure of RNA helicase of yellow fever virus has been solved, which suggests that the positively charged side of the helicase structure might play a role in single-stranded nucleic acid binding. However, the RKKR motif is not proximal to the NTP binding site nor to the presumed double-stranded RNA binding region. Because our data clearly show that the poly(A)-stimulated NTPase activity of NS3Δ160 (16) as well as NS3$_{FL}$ (this study) is abolished by mutation of RKKR → QNGN, it is possible that the poly(A) binds to the interdomain clef$^2$ where single-stranded nucleic acid is bound as in other helicases including the HCV enzyme (53–55), and the resultant conformational change is involved in NTPase stimulation. The notion

*J. Smith, Purdue University, personal communication.*
that a conformational change is required for NTPase stimulation is supported by our previous observation (16) that NS3A180 protein, which had a deletion of the N-terminal 180 amino acid residues, did not exhibit RNA-stimulated NTPase activity even though this protein has the positively charged motif situated close to the N terminus. Recent biochemical and kinetic analysis of HCV NS3 RNA helicase supports that multiple molecules of NS3 bound to the single-stranded region or the junction of single-stranded and double-stranded regions of the substrate which seemed to be required for optimal unwinding (35). Blunt-end duplex is not an optimal substrate for unwinding by HCV NS3 helicase (44). Therefore, we propose that the positively charged motif in Flavivirus NS3 might play a role in the initial binding of the enzyme to the single-stranded region of the substrate RNA before unwinding of the duplex region. Our observation that mutation of this positively charged motif significantly reduced the RNA helicase activity is also consistent with this notion.

We examined the conservation of this positively charged motif in NS3 protein of other flaviviruses. As shown in Fig. 9, this motif is conserved across the flaviviral NS3 NTPase/RNA helicases, suggesting an important role in viral life cycle. Interestingly, the amino acid sequences in the C-terminal side of this positively charged motif are also conserved, suggesting that for a conformational change induced by single-stranded nucleic acid binding, additional amino acid residues might also be involved. However, in contrast to our results, in another study using glutathione S-transferase fusion proteins of DEN2 NS3 and mutants within and outside the helicase motifs, the authors concluded that mutation of this positively charged motif enhanced the RNA helicase activity (52). The reason for this discrepancy is unclear and might be attributed to differences in the nature of the recombinant proteins and RNA substrates used in the two studies. Our observation that mutation of this positively charged motif abolished the NS3-mediated stimulation of NTPase and 5'-RTase activities of NS3 when the two proteins are present in stoichiometric amounts suggested that this motif is also involved in stabilization of NS3/NS5 interaction to form a functional complex. Further work is needed to establish the role of this motif and the conserved amino acid residues juxtaposed with this motif in NS3/NS5 interaction in the presence and absence of RNA.
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