Characterization of the Metal Ion Binding Properties of the Hepatitis C Virus RNA Polymerase*

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The hepatitis C virus nonstructural 5B protein (NS5B) protein has been shown to require either magnesium or manganese for its RNA-dependent RNA polymerase activity. As a first step toward elucidating the nature and the role(s) of the metal ions in the reaction chemistry, we have utilized endogenous tryptophan fluorescence to quantify the interactions of magnesium and manganese ions with this protein. The association of either Mg2+ or Mn2+ ions with the enzyme resulted in a decrease in the intensity of the tryptophan emission spectrum. This decrease was used to determine the apparent dissociation constants for both ions. The apparent Kd values for the binding of Mg2+ and Mn2+ ions to the free enzyme were 3.1 and 0.3 mM, respectively. Dual ligand titration experiments demonstrated that both ions bind to a single common site, for which they compete. The kinetics of real time metal ion binding to the NS5B protein were also investigated. Based on the results of our fluorescence and near-UV circular dichroism experiments, we show that NS5B undergoes conformational changes upon the binding of metal ions. However, this process does not significantly stimulate the binding to the RNA or NTP substrates. We envisage that the ion-induced conformational change is a prerequisite for catalytic activity by both correctly positioning the side chains of the residues located in the active site of the enzyme and also contributing to the stabilization of the intermediate transition state.

Currently, hepatitis C virus (HCV) is the leading etiological agent of non-A non-B hepatitis, with more than 170 million people worldwide being infected with HCV (1). About 80% of patients with acute HCV infection will progress to chronic hepatitis. Of these, 20% will develop cirrhosis, and 1–5% will develop hepatocellular carcinoma (2–5). HCV is a positive, single-stranded RNA virus of the Flaviviridae family. The genome is ~10,000 nucleotides long and encodes a single polyprotein of about 3,010 amino acids (6). The polyprotein is processed by both host cell and viral proteases into three major structural proteins and several nonstructural proteins necessary for viral replication (6).

One key enzyme encoded by HCV is the nonstructural 5B protein (NS5B), which has been shown to be an RNA-dependent RNA polymerase (7–12). The HCV NS5B protein contains characteristic motifs, such as the GDD motif, shared by RNA-dependent RNA polymerases (13). The NS5B protein is thus believed to be responsible for the genome replication of HCV. Indeed, polymerase activity has been demonstrated with recombinant NS5B expressed in both insect cells and Escherichia coli (8, 14–26). The activity has been extensively studied because it is one of the major targets for the development of antiviral drugs. The NS5B protein can utilize a wide range of RNA molecules as template, although it appears to prefer certain homopolyribonucleotides (27). By itself, NS5B appears to lack specificity for HCV RNA and displays activity on heterologous nonviral RNA (8). This lack of specificity for HCV RNA supports the notion that additional viral or cellular factors are required for specific recognition of the viral replication signal.

Previous reports showed that both magnesium and manganese ions can support the polymerase activity (8, 14, 22–26), although manganese ions appear to be more effective in promoting optimal catalytic action (14). No information is currently available on the precise affinity of the enzyme for metal ions nor on their possible roles in catalysis, but manganese ions have recently been found in the crystal structure of NS5B (28). Metal ions have the potential to fulfill multiple functional roles in catalysis such as: (i) increasing the affinity of the enzyme for RNA; (ii) increasing the affinity of the enzyme for NTPs; (iii) inducing the proper folding of the protein; and (iv) being directly involved in catalysis by promoting the activation of nucleophiles.

As a first step toward elucidating the nature and the role(s) of metal ions in the reaction chemistry, we have utilized endogenous tryptophan fluorescence to evaluate the interactions of metal ions with the NS5B protein. Quenching of the fluorescence signals by titration of the protein with metal ions provides a straightforward and powerful technique for evaluating the binding of metal ions to proteins. Our data provide insights on the precise role of metal ions in the NS5B-mediated RNA polymerase reaction.

EXPERIMENTAL PROCEDURES

HCV NS5B Expression and Purification—A plasmid for expression of a truncated form of HCV NS5B protein (NS5BΔ21) lacking the last 21 amino acids of the protein was generated by inserting the truncated NS5B gene between the BamHI and XhoI cloning sites of the pET21b expression plasmid (Novagen). In this context, the NS5B protein is fused in frame with a C-terminal peptide containing six tandem histidine residues, and expression of the His-tagged protein is driven by a T7 RNA polymerase promoter. The resulting recombinant plasmid, pET-NS5B, was transformed into E. coli BL21(DE3). A 100-ml culture of E. coli BL21(DE3)pET-NS5B was grown at 37 °C in Luria-Bertani medium containing 0.1-mg/ml ampicillin until the A600 reached 0.5. The culture was adjusted to 0.4 mM isopropyl-β-D-thiogalactopyranoside,

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1 The abbreviations used are: HCV, hepatitis C virus; NS5B, nonstructural 5B protein.

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and the incubation was continued at 18 °C for 20 h. The cells were then harvested by centrifugation, and the pellet was stored at −80 °C. All subsequent procedures were performed at 4 °C. Thawed bacteria pellets were resuspended in 5 ml of lysis buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10% sucrose), and cell lysis was achieved by the addition of lysozyme and Triton X-100 to final concentrations of 50 μg/ml and 0.1%, respectively. The lysates were sonicated to reduce viscosity, and any insoluble material was removed by centrifugation at 13,000 rpm for 45 min. The soluble extract was applied to a 2-ml column of nickel-nitrilotriacetic acid-agarose (Qiagen) that had been equilibrated with buffer A containing 0.1% Triton X-100. The column was washed with the same buffer and then eluted stepwise with buffer B (50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 10% glycerol) containing 50, 100, 200, 500, and 1000 mM imidazole. The polypeptide composition of the column fractions was monitored by SDS-PAGE. The recombinant NS5B protein was retained on the column and recovered in the 200 mM imidazole eluate. This fraction was dialyzed against buffer C (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 M NaCl, 50 mM NaCl, 10% glycerol) containing 50, 100, 200, 500, and 1000 mM imidazole. The recombinant protein was retained on the column and was recovered predominantly in the 1.0 M NaCl fraction.

Fluorescence Measurements—Fluorescence was measured using an Hitachi F-2500 fluorescence spectrophotometer. Background emission was eliminated by subtracting the signal from either buffer alone or buffer containing the appropriate quantity of substrate. The extent to which ligands bind to the HCV NS5B protein was determined by monitoring the fluorescence emission of a fixed concentration of proteins and titrating with a given ligand. The binding can be described by Equation 1.

\[
K_d = \frac{[\text{NS5B}][\text{ligand}]}{[\text{NS5B}][\text{ligand}]} \quad (\text{Eq. 1})
\]

where \(K_d\) is the apparent dissociation constant, [NS5B] is the concentration of the protein, [NS5B]-ligand is the concentration of complexed protein, and [ligand] is the concentration of unbound ligand.

The proportion of ligand-bound protein as described by Equation 1 is related to measured fluorescence emission intensity by Equation 2.

\[
\Delta F/\Delta F_{\text{max}} = \frac{[\text{NS5B}][\text{ligand}]}{[\text{NS5B}][\text{ligand}]} \quad (\text{Eq. 2})
\]

where \(\Delta F\) is the magnitude of the difference between the observed fluorescence intensity at a given concentration of ligand and the fluorescence intensity in the absence of ligand, \(\Delta F_{\text{max}}\) is the difference at infinite [ligand], and \([\text{NS5B}][\text{ligand}]\) is the total protein concentration.

If the total ligand concentration, [ligand]tot, is in large molar excess relative to [NS5B]tot, then it can be assumed that [ligand] is approximately equal to [ligand]tot. Equations 1 and 2 can then be combined to give Equation 3.

\[
\Delta F/\Delta F_{\text{max}} = \frac{[\text{ligand}]_{\text{tot}}}{[\text{NS5B}]_{\text{tot}}} \quad (\text{Eq. 3})
\]

The \(K_d\) values were determined from a nonlinear least square regression analysis of titration data by using Equation 3. The stoichiometry of binding was established from a linear version of the Hill equation.

\[
\log(\Delta F/\Delta F_{\text{max}} - \Delta F) = n \log([\text{ion}]) - \log K' \quad (\text{Eq. 4})
\]

where \(n\) is the order of the binding reaction with respect to ligand concentration, and \(K'\) is the concentration of ion that yields 50% of \(\Delta F_{\text{max}}\).

Circular Dichroism Spectroscopy Measurements—Circular dichroism measurements were performed with a Jasco J-810 spectropolarimeter. The samples were analyzed in quartz cells with pathlengths of 1 mm. Far-UV and near-UV wavelength scans were recorded from 200 to 250 nm and from 250 to 340 nm, respectively. All of the dichroic spectra were corrected by subtraction of the background for the spectrum obtained with either buffer alone or buffer containing metal ions. An average of six wavelength scans was presented. The ellipticity results were expressed as mean residue ellipticity, \(\theta\), in degrees-cm²-dmol⁻¹.

Analysis of Competitive Binding—Analysis of the effect of a fixed concentration of one metal ion ligand (iona) on the binding of a second ion (ionb) was performed in a manner analogous to that previously reported for analyzing the kinetics of a system in which two alternative substrates compete for the same enzyme binding site (29). The change in fluorescence (\(\Delta F\)) observed upon titration of NS5B with iona in the presence of a fixed concentration of competing substrate (ionb) can be described by Equation 5.

\[
\Delta F = \Delta F_{\text{max}}(\text{iona})/K_a + \Delta F_{\text{max}}(\text{ionb})/K_b + (\text{ionb})/K_a + (\text{ionb})/K_b \quad (\text{Eq. 5})
\]

where \(\Delta F_{\text{max}}(\text{iona})\) and \(\Delta F_{\text{max}}(\text{ionb})\) are the changes in fluorescence produced at infinite concentrations of iona and ionb, respectively. \(K_a\) and \(K_b\) are the apparent dissociation constants for iona and ionb, respectively. Equation 5 was fit to the simple ligand saturation isotherms for both iona and ionb.
5’-untranslated region) fused to the C-terminal portion of the genome (starting at nucleotide 9068 and including the 3’-untranslated region). The plasmid was linearized with XbaI (located at the 3’ end of the construct) and gel-purified. The RNA transcript (1442 nucleotides) was synthesized with the MAXIscript kit (Ambion) using T7 RNA polymerase. Radiolabeled [32P]-UTP was added to the reaction, and the RNA fragment was purified with the QIAquick nucleotide removal kit (Qiagen). The binding reaction mixtures were directly subjected to electrophoresis on 1.5% agarose gels, and the bands were visualized by autoradiography. The bands were then excised from the dried agarose gels, and the amount of radioactivity was counted with a liquid scintillation counter. The apparent dissociation constant ($K_d$) for each probe was determined according to Equation 6.

$$f_0 = \frac{[\text{NS5B}]}{K_d + [\text{NS5B}]}$$  \hspace{1cm} (Eq. 6)

Where $f_0$ represents the fraction of the shifted nucleic acids, [NS5B] is the total protein concentration, and $K_d$ is the dissociation constant for the binding reaction.

RESULTS

Expression, Purification, and Intrinsic Fluorescence Properties of HCV NS5B Protein—The HCV NS5B protein contains motifs shared by RNA polymerases, and its activity has been shown to be dependent on the presence of either magnesium or manganese ions. To further characterize the metal binding activity of the enzyme, the NS5B protein was expressed in E. coli as described under “Experimental Procedures.” A truncated form of NS5B (NS5B21) lacking the previously identified 21-amino acid hydrophobic domain was expressed and purified. SDS-PAGE analysis showed that the 65-kDa NS5B21 protein was the predominant polypeptide in the purified fraction (Fig. 1A). The amount of NS5B21 protein in this fraction was estimated to be 60 µg/ml. Immunoblotting analysis, using a monospecific antibody, also confirmed the identity of this protein (data not shown).

The fluorescence emission spectrum of purified NS5B in standard buffer at 22 °C is shown in Fig. 1B. To obtain the maximal emission peak at the low concentrations of protein required to accurately determine $K_d$ values, excitation was carried out at 290 nm. Both tyrosine and tryptophan absorb at this wavelength (29). However, varying the excitation wavelength from 254 nm, where the contribution of tyrosine fluorescence to the emission spectrum would be the greatest, to 295 nm, where the emission spectrum would arise almost exclusively from tryptophan, produced no change in the position of $\lambda_{\text{max}}$ (335 nm) or in the spectral bandwidth (55 nm at half-height) (data not shown). Thus, despite the fact that NS5B contains 22 tyrosines in addition to the 9 tryptophans, the emission spectrum is dominated by the indole fluorophores. This dominance is due, in part, to the higher extinction coefficient of tryptophan and to resonance energy transfer from tyrosine to tryptophan.

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The emission maximum of the enzyme (335 nm) is blue-shifted relative to that of free L-tryptophan, which under the same conditions is observed to be at 350 nm. The $\lambda_{\text{max}}$ of

![Fig. 2. Titration of NS5B with Mg$^{2+}$ ions. A, increasing amounts of MgCl$_2$ were added to a 250 nM solution of the enzyme in binding buffer (50 mM Tris-HCl, 50 mM KOAc, pH 7.5), and the emission spectrum was scanned from 310 to 440 nm. B, a saturation isotherm can be generated from these data by plotting the change in fluorescence intensity at 335 nm as a function of added MgCl$_2$. C, the effect of increasing ionic strength on the apparent dissociation constant of NS5B for MgCl$_2$ was investigated. Increasing concentrations of KCl were added to the reactions to generate the desired ionic strength. D, kinetic analysis of real time binding of MgCl$_2$ to the NS5B protein. A 300 µl solution of the enzyme was incubated with 150 µl MgCl$_2$. Emission was monitored for 30 s at 335 nm, and excitation was performed at 290 nm.](image)
tryptophan is highly sensitive to the polarity of the microenvironment in which its indole side chain is localized. Blue shifts of protein emission spectra have been ascribed to shielding of the tryptophan residues from the aqueous phase (30). This shielding is the result of the three-dimensional structure of the protein. Accordingly, denaturation of NS5B with 8 M urea results in a red shift of λmax toward 350 nm (Fig. 1B).

The molar intensity of the fluorescence emission spectrum of NS5B was also determined. This spectrum was determined to see whether significant protein aggregation or whether the loss of protein from solution through adhesion could influence the data. As can be seen in Fig. 1C, a decrease in fluorescence is observed with decreasing concentrations of NS5B. A linear change of 0.16 fluorescence intensity units/nM of protein was observed over the range examined. This relatively small change can probably be attributed to small losses of proteins from solution through adhesion. Alternatively, some photo-bleaching may occur over the period of time in which the experiment is carried out. All of the binding experiments were thus performed at a protein concentration of 250 nM, with the assumption that the binding equilibrium was not complicated by the presence of an aggregation equilibrium.

**Binding of Mg2⁺ and Mn2⁺ Metal Ions to the HCV NS5B Protein**—The binding of metal ions to free enzymes has been shown to result in a significant decrease in emission fluorescence intensities (31–33). We observed that the binding of both Mg2⁺ and Mn2⁺ ions to the NS5B protein resulted in a modification of the intensity of the intrinsic fluorescence of this protein. As a consequence we were able to evaluate $K_d$ values for both Mg2⁺ and Mn2⁺ ions, the cofactors necessary for the NS5B-mediated polymerase activity, by titrating the binding of increasing amounts of metal ion to a fixed concentration of the NS5B protein. Typical emission spectra obtained from the titration of MgCl₂ are shown in Fig. 2A. The addition of increasing amounts of Mg2⁺ produced a decrease in the fluorescence intensity, but the emission maximum (335 nm) and spectral bandwidth were unaffected. The corresponding saturation isotherm generated by plotting the change in fluorescence intensity at 335 nm as a function of added MgCl₂ is shown in Fig. 2B. Quenching saturated at millimolar Mg2⁺ concentrations, and a 3.1 mM $K_d$ value could be estimated for Mg2⁺ from a fit of Equation 3 to the generated saturation isotherm. About 50% of the intrinsic protein fluorescence was accessible to the quencher Mg2⁺ ion (Fig. 2B). Analysis of a Hill plot generated from the Mg2⁺ ion binding data yielded a Hill coefficient of 1.04, indicating a lack of cooperativity (Table I). Furthermore, a Scatchard plot of Mg2⁺ binding data is linear, providing no evidence for multiple classes of independent magnesium ion binding sites or of cooperative binding sites (data not shown). Note that binding of the Mg2⁺ ions could not be detected after heating the enzyme at 60 °C in the presence of 0.1% SDS prior to the titration (data not shown). The addition of EDTA to the reaction reversed the effects on fluorescence and showed that the change in fluorescence observed by the addition of the metal ion is not solely due to a change in the ionic strength of the solution (data not shown). Accordingly, electrostatic interactions appear to make only minor contributions to the overall binding energy, as illustrated by the minimal effect of ionic strength on the apparent $K_d$ value of NS5B for Mg2⁺ ions (Fig. 2C).

The kinetics of real time Mg2⁺ ion binding to the NS5B protein were investigated by monitoring the intrinsic protein fluorescence of NS5B following the addition of Mg2⁺ ions (Fig. 2D). The progress of the binding reaction was followed and showed that there was a rapid exponential decrease in fluorescence following the addition of the Mg2⁺ ions. An apparent association rate of 0.2 $\mu M^{-1} s^{-1}$ was estimated from the data. Half-maximal quenching was observed at $-2 s$, whereas maximal quenching was achieved after 5 s of incubation with Mg2⁺ ions and remained constant thereafter. The exponential decrease in fluorescence observed following the addition of metal ions was not due to photobleaching, because similar results were obtained when the NS5B protein was incubated away from the light source.

The binding of Mn2⁺ ions to the NS5B protein was investigated in an analogous manner by monitoring the decrease in the intrinsic protein fluorescence following binding to the ion. Again quenching saturated at millimolar Mn2⁺ concentrations, and a 0.3 mM $K_d$ value was estimated from a fit of Equation 3 to the generated saturation isotherm (Table I). About 25% of the intrinsic protein fluorescence was accessible to the quencher Mn2⁺ ion. A Hill plot was generated from the Mg2⁺ ion binding data and yielded a Hill coefficient of 0.98 (Table I). Finally, the addition of Mn2⁺ ions resulted in a rapid exponential decrease in fluorescence yielding an apparent association rate of 0.3 $\mu M^{-1} s^{-1}$ (Table I).
The presence of multiple tryptophan residues in the NS5B protein allowed binding assays to be performed with a high degree of sensitivity. Interpretation of the quenching data in terms of spatial relationships is complicated because the tryptophan residues are distributed rather uniformly throughout the protein. To further characterize the interaction between the metal ions and NS5B, far- and near-UV CD spectra were recorded both in the presence and the absence of metal ions. Analysis of the far-UV CD spectra (Fig. 3A) revealed that the binding of Mg\(^{2+}\) ions to the NS5B protein does not induce a significant modification of the secondary structure component of the protein. The far-UV CD spectra thus suggest that the NS5B protein maintains a comparable ordered secondary structure following the binding of the metal ions. Although the far-UV CD data indicate that no significant changes in secondary structure are occurring, analysis of the near-UV CD spectra was performed to verify that the decrease of fluorescence intensity observed upon binding of metal ions is indeed reflecting conformational changes. Analysis of the near-UV CD spectra of the NS5B protein in both the absence and presence of Mg\(^{2+}\) ions was performed from 250 to 340 nm. As can be seen in Fig. 3B, a significant reduction of the amplitude of the signal is observed over the 280–300-nm region when the protein is incubated with Mg\(^{2+}\) ions. Overall, the CD spectra suggest that the protein undergoes a subtle conformational change upon the binding of metal ions rather than a radical modification of the overall protein architecture.

**Binding of Other Divalent Cations and Competitive Binding**—Previous studies showed that other divalent cations, such as zinc, cobalt, copper, and calcium, do not support the NS5B-mediated RNA polymerase activity (14, 25, 26). However, it has been noted that these ions can efficiently inhibit both the Mn\(^{2+}\)- and the Mg\(^{2+}\)-dependent RNA polymerase activity (14, 25, 26). Inhibition by these ions presents several interesting scenarios: (i) Do these ions bind to the same active site as Mn\(^{2+}\) and Mg\(^{2+}\)? or (ii) Do they bind at a different site on the protein, thereby somehow allosterically hindering the polymerase reaction? To answer these questions, we attempted to evaluate the binding of other divalent cations to the NS5B protein using fluorescence spectroscopy. However, interaction of the protein with Zn\(^{2+}\), Ca\(^{2+}\), and Cu\(^{2+}\) could not be efficiently and repeatedly evaluated. Significant quenching of the intrinsic protein fluorescence was only observed in the presence of Co\(^{2+}\) ions. Analysis of the binding data yielded a $K_d$ value of 35 mM (Table I).

Competitive alternative ligand binding experiments were carried out to determine whether the different metal ions compete for a common binding site. In the first experiment, the combined $\Delta F$ produced at 335 nm by the addition of various manganese concentrations was plotted against the concentra-
tion of Mg$^{2+}$ ions. Three concentrations of Mn$^{2+}$ were used in this competition experiment. The saturation isotherm resulting from the competitive ligand experiment conducted with Mg$^{2+}$ as ion$_a$ and Mn$^{2+}$ as ion$_b$, is shown in Fig. 4. The pattern of lines is in accordance with a model in which the binding of Mg$^{2+}$ and Mn$^{2+}$ is mutually exclusive (29). A quantitative analysis of the compliance of the experiment with the competitive binding model was made by comparing the $K_d$ and $\Delta F_{\text{max}}$ values derived from the fit of Equation 5 to the dual titration data with the $K_d$ and $\Delta F_{\text{max}}$ values determined in single ligand titrations. In each case, the values were statistically indistinguishable. The reverse experiment, using Mn$^{2+}$ as ion$_a$ and Mg$^{2+}$ as ion$_b$, yielded similar conclusions (data not shown). Similar competitive experiments also demonstrated that Co$^{2+}$ ions are competing for the same site as Mn$^{2+}$ and Mg$^{2+}$ ions (data not shown).

**Importance of the Metal Ions in the Nucleic Acid Binding Activity of NS5B**—Magnesium and manganese ions have previously been shown to be essential for the NS5B-mediated polymerase activity (8, 14–22–26). We were thus interested in determining whether the ion binding activity is necessary for the binding of NS5B to the HCV RNA template, an essential step in the initiation of polymerization. An electrophoretic mobility shift assay was thus performed to evaluate the binding to RNA in both the presence and the absence of metal ions.

The binding of the HCV NS5B protein to a single-stranded RNA transcript containing the NS5B gene flanked by the 5’- and 3’-terminal sequences found in the HCV genome was initially evaluated in the presence of 10 mM MgCl$_2$ (Fig. 5A). The amount of the shifted species reached a maximum around 15 nM. Higher concentrations of the protein did not further enhance the amount of shifted RNA, suggesting that the reaction had come to an equilibrium. An apparent $K_d$ of 10 nM could be estimated for this RNA substrate (Fig. 5B). Note that the binding to an HCV-unrelated single-stranded RNA sequence was also evaluated and yielded a similar apparent $K_d$ value (8 nM), indicating that NS5B does not interact with RNA substrates in a sequence-specific manner (data not shown).

The divalent ion requirement for the binding of NS5B protein to nucleic acids was then investigated using 5 nM of NS5B protein, an amount near the $K_d$ value and thus in the linear range of the binding reaction. The results showed that RNA binding can occur in the absence of ions and that binding is only modestly stimulated by lower concentrations of magnesium (Fig. 5C). The data indicated that the binding activity reached a maximum at ~5 mM of MgCl$_2$ and that a decrease was noted at higher concentrations. On the other hand, Mn$^{2+}$ ions were able to support the binding activity over a wide range of concentrations ranging from 0 to 25 mM (Fig. 5D). Overall, these binding studies indicated that NS5B can bind to RNA substrates in the absence of Mg$^{2+}$ or Mn$^{2+}$ ions and that the presence of these ions only modestly affects the binding reaction.

Preliminary studies showed that the binding of RNA to the NS5B protein results in a significant decrease in emission fluorescence intensities (data not shown). The kinetics of RNA binding to the NS5B protein were thus investigated in both the presence and the absence of Mg$^{2+}$ or Mn$^{2+}$ ions by monitoring the intrinsic protein fluorescence of NS5B following the addition of RNA. The progress of the binding reaction was analyzed and showed that there was an exponential decrease in fluorescence upon the addition of RNA, followed by a slower linear decrease (Fig. 5E). Half-maximal quenching was observed at ~2 min, whereas maximal quenching was achieved after 6 min of incubation with RNA and remained constant thereafter ($t_{1/2}$ = 2 min). The presence of either Mg$^{2+}$ or Mn$^{2+}$ ions did not significantly influence the rate of binding to the RNA substrate (data not shown). Again, the exponential decrease in fluorescence observed following the addition of RNA was not due to photobleaching because similar results were obtained when the NS5B protein was incubated away from the light source.

To evaluate the stoichiometry of NS5B binding to nucleic acids, the extent of binding was evaluated by monitoring the intrinsic fluorescence of NS5B upon interaction with RNA. The binding site size ($n_{\text{app}}$) was determined from fluorescence titration curves in which increasing amounts of RNA were added to a fixed amount of protein (Fig. 5F). A plot of the absolute fluorescence change, normalized by the initial fluorescence in the absence of any RNA, versus the ratio of total RNA (nucleotides) to total protein was generated. Linear approximations were fitted to the initial and final slopes of the curves, and the intersection of the two lines corresponds to the apparent binding site size (34). The apparent binding site size of NS5B on the RNA substrate was estimated to be 8 nucleotides. The addition of various concentrations of metal ions did not significantly alter this value (data not shown). Taken together, the results indicate that metal ions do not influence the RNA binding activity of the NS5B protein.

**Importance of the Metal Ions in the Nucleotide Binding Activity of NS5B**—The effect of magnesium and manganese ions on the binding of free nucleotides was also investigated by fluorescence spectroscopy. Initial experiments, performed in the absence of added metal ions, indicated that binding of NTP ligands to the NS5B protein results in a significant decrease in the fluorescence emission intensity. A typical titration experiment, using GTP as the ligand, is shown in Fig. 6. An apparent $K_d$ value of 65 $\mu$M could be estimated from the generated saturation isotherm. To determine the role of metal ions in NTP binding, the enzyme was titrated with GTP in the presence of increasing concentrations of either MgCl$_2$ or MnCl$_2$. The addition of increasing concentrations of either ion, up to 10 mM, did not significantly modify the apparent $K_d$ value for GTP (data not shown). Similar conclusions were drawn when titration experiments were performed with other NTPs (data not shown). These results clearly indicate that both Mg$^{2+}$ and Mn$^{2+}$ do not influence the RNA binding activity of the NS5B protein.

**DISCUSSION**

The enzymatic activity of the HCV NS5B protein has been extensively characterized (8, 14–26). The protein is believed to
be responsible for the genome replication of HCV and is thus a critical protein of the virus. Magnesium and manganese ions have been shown to support the polymerase activity mediated by NS5B (8, 14, 22–26), and manganese has recently been found in the crystal structure of this protein (28). However, the precise role of these ions in the reaction chemistry has not been determined. As a first step toward elucidating the nature of the metal ion binding to the NS5B protein, we have utilized the endogenous tryptophan fluorescence to precisely quantitate the interactions of metal ions with the enzyme.

Quenching of the fluorescence signals by titration of the protein with metal ions provides a straightforward technique for determining apparent $K_d$ values. The high intrinsic fluorescence signal of the NS5B protein allowed binding assays to be carried out with a high degree of sensitivity. The decrease in fluorescence intensity observed upon saturation of the enzyme with these ions can be produced by contact of the quenching agent with the indole side chain of a tryptophan and/or by inducing a conformational change in the enzyme that results in alterations in the microenvironments of tryptophan residues distal to the ion binding site. The fact that no tryptophan residues appear to be located in the active site of the NS5B crystals suggests that the decrease in fluorescence intensity is not solely the result of the selective quenching of a specific subpopulation of indole fluorophores but that it also involves a subtle conformational change. Similar findings have been noted with other ion-binding proteins (35). Although crystallographic studies have not yet identified different conformations of NS5B (28, 36, 37), analysis of the near-UV CD data clearly suggests that a conformational change is induced upon the binding of metal ions to the NS5B protein. In fact, comparisons with closely related polymerases strongly suggest that conformational changes are required for NS5B to efficiently initiate polymerization (28). However, analysis of the crystal structure of the RNA polymerase of the rabbit hemorrhagic disease virus, a virus closely related to HCV, revealed the presence of both active and inactive conformations within the same crystal form (38). The conformations adopted by the rabbit hemorrhagic disease virus polymerase can vary dramatically depending on the ions present in the crystallization solution. It was suggested that these structural changes may be important for the enzymatic activity of the protein (38). As reported previously for DNA polymerases (39, 40), these conformational changes are probably required for the catalytic activity of rabbit hemorrhagic disease virus and HCV polymerases.

Analysis of the crystal structure of NS5B revealed that the protein is folded into characteristic fingers, palm, and thumb subdomains (36, 37). The particular fold adopted by the palm subdomain is shared by many proteins that bind nucleotides and/or nucleic acids (41). The crystal structure of DNA polymerase I from *E. coli* complexed with nucleotides showed that it contains two absolutely conserved aspartic acid residues that coordinate two Mg$^{2+}$ ions in the active site of the protein (41). These two metal ions are in contact with both the phosphate of the nucleotide and several acidic amino acids residues (41). Two metal ions are also bound in the active site of the rabbit hemorrhagic disease virus (38) and HCV polymerases (28). A catalytic mechanism has been proposed in which one metal ion is involved both in positioning the substrate and in the activation of an incoming nucleophile (42). Nucleophilic attack then generates a trigonal bipyramidal transition state that is stabilized by both metal ions. The second metal ion also stabilizes the negative charge that appears on the leaving 3’ oxygen, thus facilitating its departure from the phosphate. Analysis of the DNA polymerase I crystal structure indicated that the two metal ions are about 4.0 Å apart in the active site of the protein (41). Metal ion binding thus seems to be limited to the active site region and does not involve other subdomains of the protein. Our titration and competition experiments performed with the NS5B protein suggest a similar mechanism, as illustrated by the data indicating that the metal ions bind to single site on NS5B.

Based on the results of our fluorescence and near-UV CD experiments, we demonstrated that NS5B undergoes conformational changes upon the binding of metal ions. This process, however, does not significantly stimulate NS5B binding to its RNA or NTP substrates. Far-UV circular dichroism measurements also revealed that the binding of metal ions does not significantly modify the secondary structure of the protein. This is in agreement with various studies that showed that NS5B has a preformed active site (36, 37, 43) in contrast to many other polymerases, in which major domain rearrangements are needed to form a catalytically active site (40, 44). We thus envisage that the ion-induced conformational change is a prerequisite for catalytic activity by correctly positioning the side chains of residues located in the active site of the enzyme, while at the same time contributing to the stabilization of the intermediate transition state. A number of acidic amino acid residues located in the active site of the NS5B protein have the potential to coordinate metal ions through a network of hydrogen bonds. Following the binding of metal ions, the original hydrogen bonding interactions would be replaced by interactions with metal ions and possibly with water molecules, positioning the residues for efficient enzymatic catalysis. Further analysis by site-directed mutagenesis could precisely identify the role of these residues and provide additional information on the enzyme mechanism.

Fluorescence spectroscopy has tremendous potential for the screening of antiviral drugs aimed at inhibiting the NS5B-mediated RNA polymerase activity. The availability and simplicity of data acquisition and analysis are important practical features behind this possibility. Many aspects, including characterization of the binding of various ligands, analysis of the thermodynamics of the interactions, and competition experiments can easily be performed by fluorescence spectroscopy analysis. Although the understanding of the mechanisms underlying HCV replication and the cellular and viral factors required for these processes is still incomplete, characterization of the biochemical properties of NS5B should provide the basis for further studies in this direction. Replication of the HCV genome is a complex event that probably requires not only the polymerase but also additional viral and cellular factors to form a functional replicative complex. Structural and enzymatic studies are beginning to reveal the essential features of the polymerase reaction and should ultimately lead to the design of effective antiviral drugs.

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Metal Ion Binding Activity of NS5B

3875

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