The helicase of hepatitis C virus (HCV) unwinds nucleic acid using the energy of ATP hydrolysis. The ATPase cycle is believed to induce protein conformational changes to drive helicase translocation along the length of the nucleic acid. We have investigated the energetics of nucleic acid binding by HCV helicase to understand how the nucleotide ligation state of the helicase dictates the conformation of its nucleic acid binding site. Because most of the nucleotide ligation states of the helicase are transient due to rapid ATP hydrolysis, several compounds were analyzed to find an efficient unhydrolyzable ATP analog. We found that the β-γ methylene/amine analogs of ATP, ATPγS, or [αF3]ADP were not effective in inhibiting the ATPase activity of HCV helicase. On the other hand, [BeF3]ADP was found to be a potent inhibitor of the ATPase activity, and it binds tightly to HCV helicase with a 1:1 stoichiometry. Equilibrium binding studies showed that HCV helicase binds single-stranded nucleic acid with a high affinity in the absence of ATP or in the presence of ADP. Upon binding to the ATP analog, a 100-fold reduction in affinity for ssDNA was observed. The reduction in affinity was also observed in duplex DNA with 3' single-stranded tail and in RNA but not in duplex DNA. The results of this study indicate that the nucleic acid binding site of HCV helicase is allosterically modulated by the ATPase reaction. The binding energy of ATP is used to bring HCV helicase out of a tightly bound state to facilitate translocation, whereas ATP hydrolysis and product release steps promote tight rebinding of the helicase to the nucleic acid. On the basis of these results we propose a Brownian motor model for unidirectional translocation of HCV helicase along the nucleic acid length.

Helicases are molecular motors that use the energy of ATP hydrolysis to unwind nucleic acid. HCV codes for a helicase that constitutes the C-terminal domain of NS3 protein. Here we have studied the properties of the helicase domain of NS3 protein (NS3h) expressed as a separate polypeptide. NS3h is a 3' to 5' helicase that can unwind both RNA and DNA (1, 2). Nucleic acid unwinding is a stepwise process involving translocation and local separation of the duplex nucleic acid strands (3). In the wedge mechanism, a simple mechanism of unwinding, strand separation is accomplished by unidirectional translocation of the helicase and capture of the ssDNA (3, 4). In such a mechanism, the basic activity of the helicase is unidirectional translocation driven by the ATPase cycles. The mechanism by which ATPase drives directional translocation of the helicase is however not known.

Each cycle of the ATPase reaction consists of a number of steps including ATP binding, hydrolysis, phosphate release, ADP release, and rebinding of ATP.

\[
E \rightarrow E\cdot ATP \rightarrow E\cdot ADP \cdot Pi \rightarrow E\cdot ADP \rightarrow E \rightarrow \ldots
\]

Scheme I

The ATPase cycle allows the helicase to adopt various nucleotide ligation states that allosterically control the conformation of the nucleic acid binding site. Thus, it is believed that the ATPase cycle causes conformational changes in the nucleic acid binding site to drive the movement of the helicase along the length of the nucleic acid. Several structural features including the oligomeric state of the helicase and the number of ATP and nucleic acid binding sites influence the helicase mechanism. HCV helicase does not form stable oligomers, and each helicase molecule has one nucleic acid binding site (5–7). Although sequence and crystallographic data support a single NTP binding site (8, 9), it was proposed that the HCV helicase molecule has two ATP binding sites (10). In this paper we explore the ATP binding stoichiometry with direct nucleotide binding assays.

To understand the mechanism of helicase translocation, it is important to identify as well as to characterize biochemically and structurally the conformational states of the nucleic acid binding site. To this end, it is necessary to determine the nucleic acid affinity of the helicase in its various nucleotide ligation states. In a recent article, we studied the nucleic acid binding properties of HCV helicase in the nucleotide-free state and found that NS3h binds DNA and RNA with a high affinity in the absence of nucleotide (7). The minimal DNA binding site of NS3h is 8 bases, and it binds DNA with a microscopic \(K_r\) of 4 nm (7). The studies of nucleic acid interactions in the presence of ATP or in the E-ATP state are more difficult because the ATPase rate of HCV helicase is high and therefore E-ATP intermediate is short-lived. One way to overcome this problem is to use an unhydrolyzable ATP analog. In this article we report the interactions between NS3h protein and unhydrolyzable ATP analogs and their effect on nucleic acid affinity. The results provide insights into the mechanism by which ATP hydrolysis...
binding and hydrolysis modulate the binding of NS3h to nucleic acid.

MATERIALS AND METHODS

The helicase domain of HCV (strain HCV-1) containing a C-terminal His-tag (11) was expressed in Escherichia coli, purified by nickel-chelating and anion exchange chromatography and quantitated as described previously (6). MOPS-free acid was purchased from Sigma, and [α-32P]ATP was from Amersham Biosciences. Ultra-pure Tween 20 was synthesized and purified by Pharmacia Research (Lafayette, CO) and was deprotected according to the supplied protocol. The concentration of nucleic acids was determined spectrophotometrically using extinction coefficients calculated from nucleotide compositions. The fluorescent nucleotide analog mantADP was a kind gift from Dr. Dong-Eun Kim. All experiments were performed at 22 °C in 50 mM MOPS-NaOH, 5 mM MgCl2, 5 mM dithiothreitol, and 0.1% Tween 20, pH 7.0, unless mentioned otherwise.

ATPase Assay—The ATPase activity was measured at room temperature (about 22 °C). NS3h protein was preincubated with 0.1 μM poly(U), and the reaction was initiated by the addition of [α-32P]ATP and stopped with 0.5 mM formic acid (final concentration). The reaction products were separated by TLC on PEI Cellulose F sheets (from EM Science) in 0.4 M potassium phosphate buffer, pH 3.4, and quantitated with a PhosphorImager (Amersham Biosciences). The ATPase time course was analyzed to determine initial rates.

Fluorimetric Assays—Fluorescence was measured using FluoroMax-2 spectrofluorometer (Jobin Yvon, Spex Instruments S.A., Inc.) in a 10 × 10 × 40-mm quartz cuvette with a magnetic stir bar in 2.8 ml of the reaction buffer. The intrinsic NS3h protein fluorescence at 340 nm was measured by exciting the sample at 280 nm. MantADP binding was measured by exciting NS3h at 280 nm and measuring the fluorescence of mantADP at 443 nm due to FRET. The fluorescence of etheno-DNA at 405 nm was measured by exciting the sample at 330 nm. The observed fluorescence intensity values, Fobs, were corrected for sample dilution and inner filter effect according to Equation 1,

\[ F = F_0 \frac{V_s + V_i}{V_s} \times 10^{-\frac{(A_{284} + A_{340})}{2}} \]  

(Eq. 1)

where F is corrected fluorescence intensity, V0 is initial sample volume, \( V_s \) is total volume of titrant added, and \( A_{284} \) and \( A_{340} \) are absorbance values at the excitation and emission wavelengths, respectively.

In titrations with constant amount of fluorescent compound, the fluorescence intensity values were fit to Equation 2,

\[ F = F_s + f_c C \]  

(Eq. 2)

where \( F_s \) is the starting fluorescence of the reaction mixture, \( f_c \) is fluorescence coefficient of complex formation, and C is concentration of the complex.

In titrations with increasing amount of fluorescent compound, the fluorescence intensity values were fit to Equation 3,

\[ F = F_s + f_c D + f_D C \]  

(Eq. 3)

where \( f_c \) is fluorescence coefficient of free dye and D is total concentration of the dye.

Complex formation by simple one-to-one binding is described by Equation 4,

\[ C = \frac{K_0 + E}{f_0} = \frac{E + D}{f_0 + D} \]  

(Eq. 4)

where E is total protein concentration.

RESULTS

[BeF3]ADP Is an Efficient Unhydrolyzable ATP Analogue of NS3h Helicase—The NS3h-ATP complex has a lifetime of less than 25 ms because of rapid ATP hydrolysis, which makes it difficult to investigate the DNA binding properties of NS3h in its ATP-bound state. An unhydrolyzable ATP analog can solve this problem. Ideally the ATP analog should bind the ATP binding site of NS3h as tightly as ATP, and by imitating the shape and surface properties of ATP, it should cause the same conformational changes in the NS3h structure. Thus far, there has been no detailed study of an efficient unhydrolyzable ATP analog of HCV helicase. Therefore, we screened several compounds that have been used as unhydrolyzable ATP analogs in other NTPases.

Potential candidates of an ATP analog were screened by testing their ability to inhibit the ATPase activity of NS3h. The steady-state ATPase rate of 10 nM NS3h was measured in the presence of 0.2 μM poly(U) and 1.5 mM ATP in the absence and in the presence of an 0.5 mM potential inhibitor. As shown in Fig. 1, the addition of ADP, ADPCP, ADPNP, ATP-γ-S, or [AlF4]ADP did not have a significant effect on the ATPase rate of NS3h. However, the addition of [BeF3]ADP reduced the ATPase rate of NS3h more than 10-fold. A 2-fold decrease in rate was observed also with BeCl2. The effect of ATP analogs was also determined at higher protein (90 nM) and inhibitor (1 mM) concentrations; the results showed that [BeF3]ADP reduced the ATPase rate to the highest extent.

To determine the mechanism of inhibition by [BeF3]ADP, the ATPase \( K_m \) and \( k_{cat} \) values were measured in the absence and presence of [BeF3]ADP. These experiments showed that 20 μM [BeF3]ADP increased the NS3h ATPase \( K_m \) more than 4-fold but did not change the \( k_{cat} \) (data not shown), indicating that [BeF3]ADP inhibits NS3h ATPase by a competitive mechanism. The competitive inhibition constant, \( K_I \), for [AlF4]ADP and [BeF3]ADP was determined by measuring the ATPase rate as a function of aluminum or beryllium fluoride concentrations (Fig. 2). The data were fit to a competitive inhibition equation.
Fluorimetric titrations were carried out to measure the equilibrium binding of the ATP analog. The protein was excited at 280 nm, and emission of mantADP due to FRET was measured at 443 nm. Under these conditions the background fluorescence from free mantADP was minimal. Three components participate in the formation of the NS3h-[BeF_3]ADP complex; hence, the titration can be carried out in several different ways. Titration with BeCl_2 is shown in Fig. 4A. In this experiment a constant amount of NS3h, NaF, and NaF was titrated with increasing concentrations of BeCl_2. The resulting binding data showed that the apparent K_d of BeCl_2 is about 26 μM. In a second type of titration, constant amounts of NS3h, NaF, and 0.5 mM BeCl_2 were titrated with increasing concentrations of BeCl_2. Such titration provided an apparent K_d of mantADP close to 80 μM and the stoichiometry of [BeF_3] mantADP binding close to one (Fig. 4B). These results indicate that the affinity of NS3h for ATP is high and that there is only one ATP binding site on the NS3h molecule.

We further characterized the NS3h-[BeF_3]ADP complex by measuring its rate of formation and dissociation. The rate of [BeF_3]ADP binding was measured by monitoring the increase in FRET (Fig. 5). The apparent rate of complex formation at 1 μM NS3h, 2 μM mantADP, 100 μM BeCl_2, and 5 mM NaF is about 0.02 s^{-1}. The dissociation rate of mantADP from the
NS3h-[BeF₃]mantADP complex was measured by chasing with a high concentration of ADP. The dissociation rate was found to be slow (0.005 s⁻¹) as shown in Fig. 5. Thus, [BeF₃]ADP is a tightly binding and slowly dissociating inhibitor.

(BeF₃)ADP Binding Decreases the Affinity of NS3h for ssDNA—Our previous studies have shown that NS3h binds ssDNA in the absence of ATP with a microscopic $K_d$ of 4 nM and with a minimal ssDNA binding site of 8 nucleotides. We have used [BeF₃]ADP, a tightly binding ATP analog, to determine the effect of ATP binding on NS3h affinity for ssDNA, dsDNA, ss/dsDNA, and RNA (Table I). The $K_d$ values were determined by fluorimetric titrations both in the absence and the presence of 0.1 mM ADP plus 0.5 mM BeF₃⁻. As shown in Fig. 6, NS3h binds a (dT)₁₂ DNA with a $K_d$ of 0.4 nM in the absence of nucleotide. In the presence of [BeF₃]ADP, NS3h binds (dT)₁₂ with a 100-fold weaker affinity. The same effect was observed with a ss/dsDNA. In the absence of ATP, NS3h binds the ss/dsDNA substrate (HP7, Table I) with a 0.6 nM $K_d$. In the presence of [BeF₃]ADP, the affinity of NS3h for ss/dsDNA substrate was reduced about 70-fold. At the same time, NS3h affinity to a dsDNA substrate is weak and remains almost unchanged by the presence of [BeF₃]ADP. A control experiment in the absence of BeCl₂, but in the presence of ADP and NaF, showed tight affinity to ssDNA (data not shown). These results indicate that the binding to ssDNA becomes weaker only when BeCl₂ is present in addition to ADP and NaF. Similar results were observed with RNA. The affinity of (U)₁₅ decreased 24-fold in the presence of the ATP analog. These results indicate that NS3h binds ss nucleic acid or ss/dsDNA very tightly in the absence of nucleotide or in the presence of ADP, but the affinity decreases manyfold when NS3h binds ATP, showing that ATP binding and hydrolysis trigger partial DNA release and tight rebinding, respectively.

A different type of fluorimetric titration was conducted to confirm that [BeF₃]ADP binding to NS3h indeed reduces its affinity for ssDNA. The equilibrium binding of ssDNA was carried out using an etheno-labeled fluorescent DNA substrate (Fig. 7). Previously, we reported the titration of 100 nM NS3h helicase with increasing amount of etheno-labeled 20-mer (7). The same DNA was used to titrate NS3h in the presence of 0.1 mM ADP and 0.5 mM BeF₃⁻. As shown in Fig. 7, [BeF₃]ADP reduces the affinity of NS3h helicase for ssDNA. The $K_d$ of ssDNA in the absence of ATP analog is less than 1 nM, and it increases to $57 \pm 26$ nM in the presence of [BeF₃]ADP, a value similar to the $39 \pm 1.3$ nM obtained by intrinsic protein fluorimetric titration for (dT)₁₂. Etheno-labeled DNA titration data therefore confirms that [BeF₃]ADP binding to NS3h reduces its affinity for ssDNA.

**DISCUSSION**

Helicases unwind long stretches of duplex nucleic acid by a stepwise process involving unidirectional translocation and local separation of the nucleic acid strands. Both of these activities are fueled by NTP hydrolysis, but their mechanisms are not known. One of the simplest mechanisms of strand separation, the “wedge mechanism,” involves the helicase moving unidirectionally along one of the strands while pulling the other strand away (3, 16). Thus, translocation is likely to be the basic activity of helicases, and understanding it is necessary for deciphering the mechanism of nucleic acid unwinding.

It is believed that NTP binding, hydrolysis, and product release steps induce conformational changes in the helicase nucleic acid binding site. These conformational changes drive stepwise unidirectional translocation of the helicase along the nucleic acid length. To understand how the NTPase reaction is coupled to translocation and unwinding, it is necessary to determine how the NTPase reaction modulates the affinity of the helicase for nucleic acid. Our previous studies have characterized in detail the interaction of NS3h with ssDNA in the absence of ATP (7). Here we determine how the ssDNA affinity changes when NS3h is in the ATP-bound state. The ATP-bound state of NS3h is transient, because NS3h hydrolyzes ATP at a fast rate. Therefore, to measure the energetics of the NS3h-ATP-DNA complex, we tested several unhydrolyzable ATP analogs to find one that would bind tightly and mimic the ATP state of NS3h.

Our studies revealed that [BeF₃]ADP is an efficient inhibitor of NS3h ATPase. Inhibition of HCV helicase by beryllofluoride has not been reported previously. It has been reported that NaF, which is used to make beryllofluoride, inhibits the ATPase activity of HCV helicase. The inhibitory effect of NaF was not observed, however, in our studies, perhaps because relatively low concentrations of NaF (5 mM) were used. Interestingly, [BeF₃]ADP inhibits the ATPase activity of NS3h, but the commonly used unhydrolyzable ATP analogs including ADP·NP, ADP·CP, ATP·γ·S, and [AlF₄]ADP were not as effective in inhibiting the ATPase activity of NS3h. Thus, different helicases seem to have different preferences for binding unhydrolyzable ATP analogs, which cannot be predicted simply from their protein structures.

Beryllofluoride along with ADP has been used as an ATP analog in studies of ATPases and kinases, and recently beryllofluoride has been shown to be an effective acyl phosphate analog (14, 17, 18). The tetrahedral geometry of the beryllofluoride complex suggests that [BeF₃]ADP mimics the ground-state structure, such as $E$-ATP or $E·P$-ADP, rather than the transi-
tion-state structure of the intermediate in the ATPase reaction (14). Studies of myosin indicate that [BeF₃]ADP mimics the ATP-bound state of the protein, whereas studies of kinesin indicate that it mimics the ADP/Pᵢ-bound state (12, 13, 19). Our studies indicate that [BeF₃]ADP is unlikely to mimic the E/Pᵢ/ADP state because this complex appears to be unstable, as evidenced by [¹⁸O]Pᵢ exchange and ADP inhibition studies. ¹⁸O isotope exchange between inorganic phosphate and water was not observed with the HCV helicase (data not shown), which indicates that either ATP synthesis is negligible or Pᵢ binding is weak. As shown in this article, ADP is not an efficient inhibitor of the ATPase activity, indicating that its binding is weak. On the basis of these results, we suggest that [BeF₃]ADP is an ATP analog of HCV helicase.

[BeF₃]ADP was used as an unhydrolyzable ATP analog to determine the ssDNA affinity of NS3h in the ATP-bound state. Using the fluorescent mantADP compound and FRET, we determined that the apparent $K_d$ of NS3h/[BeF₃]mantADP complex is close to 80 nM. Assuming that NS3h is saturated with BeF₃⁻, this approximates the $K_d$ of NS3h/ATP complex. Note that $K_I$ value of [BeF₃]ADP is much higher than the $K_d$. This may be because the $K_I$ value was obtained by fitting the data to a competitive inhibition model, which assumes that inhibitor and substrate binding are rapid equilibrium steps, and this may not be the case. Therefore, the direct binding assay provides a more accurate estimation of the binding affinity of [BeF₃]ADP.

The tight affinity of NS3h for [BeF₃]ADP facilitated the measurement of the stoichiometry of NS3h/ATP analog complex, which indicated that NS3h monomer has only one ATP binding site. This is in contrast with a report that proposes that the HCV helicase monomer has two ATP binding sites (10). The stoichiometry of 1:1 in our studies was obtained by direct equilibrium binding measurements, whereas the reported studies used the inhibition of ATPase activity as a function of ADP concentration in the presence of NaF to obtain the 1:2 stoichiometry. Thus it appears that the ATPase reaction at the single nucleotide binding site of NS3h controls the conformation of the nucleic acid binding site.

We measured the effect of nucleotides on NS3h binding to DNA. The experiments showed that the presence of ATP analog leads to a 100-fold reduction of NS3h affinity for ss or ss/ds nucleic acid, whereas ADP has no effect. The ATPase cycle of any helicase involves a number of intermediates, some of which potentially have an allosteric effect on the nucleic acid binding site (Scheme I). The key question is how the reactions at the ATP binding site that have allosteric effects on the nucleic acid binding site lead to translocation of the helicase along the length of the nucleic acid. In the case of NS3h, the nucleotides ADP, ADP-Pᵢ, and their analogs do not show high energy interactions with NS3h. Thus, the allosteric effects in NS3h are expected mainly from two steps, ATP binding and product release. Translocation driven by two conformational changes can be accomplished if the helicase utilizes both directional

### Table 1

| Name          | Structure                                                                 | $K_d$, nM no nucleotide | $K_d$, nM ADP[BeF₃] |
|---------------|--------------------------------------------------------------------------|-------------------------|---------------------|
| HP7           | AGATGGTTACTGTTGTTGTCCTCAATG                                      | 0.6 ± 0.1               | 40.5 ± 6           |
| R10           | 5' CACCTCACAAGATAGCTAGACCAGAGAGAATAGCAGCG | 330 ± 30                | 580 ± 60           |
| dT₁₂          | TTTTTTTTTTTTTTTTTTTTTTTTTTTT                                         | 0.4 ± 0.2               | 39 ± 1.3           |
| etheno-20-mer | ATCEGTAGCEGAGTECGT                                                  | <1                      | 57 ± 10            |
| U₁₅           | UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUU
The Brownian motor mechanism of unidirectional translocation is represented in Fig. 8. The mechanism is illustrated in terms of its possible mechanics (A) and energetics (B). The helicase in A is represented by the shaded area. The helicase-nucleic acid binding free energy is plotted along the length of the nucleic acid in B. Tight binding of NS3h helicase to ss nucleic acid occurs without ATP, and most binding events are associated with a forward motion of the helicase, which may be achieved through a possible mechanical feature of the nucleic acid binding site shown in A (transition from 2 to 3). From the binding free energy point of view, this is shown as a downward slope in the energy profile of the complex (transition from position 2 to 3 in B, solid line). ATP binding changes the property of the nucleic acid binding site and makes the "grip" of NS3h on the nucleic acid weaker. This new property of the NS3h-nucleic acid interface corresponds to the horizontal dotted line in B. ATP binding rescues the helicase from an energetic well and allows it to diffuse along the length of the nucleic acid. This period is very short because ATP is rapidly hydrolyzed on the helicase active site. Upon product release, the helicase restores its nucleic acid binding properties along with its asymmetric sawtooth energy profile along the nucleic acid length (B, solid line). Rebinding of the helicase to the nucleic acid can result in a forward motion if the stochastic events during position 4 are favorable; otherwise, after rebinding, the helicase moves to the same place on the nucleic acid. This mechanism illustrates how repeated ATP binding and hydrolysis events lead to unidirectional translocation of the helicase along the length of the nucleic acid.

The proposed model does not involve NS3h oligomerization. Fig. 8, A and B, shows, respectively, the mechanics and energetics of the Brownian motor mechanism. The binding of NS3h to ssDNA is a high energy event. Some special properties of ssDNA-NS3h interface can utilize this energy for unidirectional translocation by creating a local binding free energy gradient along the ssDNA length. This is represented by the sawtooth binding energy profile in Fig. 8B (solid line). In the absence of ATP, NS3h is trapped on the nucleic acid in its lowest energy state, unable to translocate (Fig. 8, A and B, positions 3 and 5). The binding of ATP switches NS3h to a different conformation with a weaker affinity for nucleic acid (Fig. 8, A and B, positions 2 and 4). In this weak binding state, the binding free energy is constant along the nucleic acid length (Fig. 8B, dotted line), which allows the helicase to slide along the length of the nucleic acid in either direction because of Brownian motion (Fig. 8, A and B, position 4). In the weakly bound state, there is a possibility that NS3h completely dissociates from the nucleic acid and that it is a nonproductive event. The random movement of the helicase along the length of the nucleic acid lasts only a short time because of rapid ATP hydrolysis. After ATP is hydrolyzed and products are released, the helicase binds DNA tightly. If Brownian motion moved the helicase backward, then upon rebinding to the nucleic acid the helicase will end up in the same position in which it started, without net movement (Fig. 8B, position 3). The helicase that diffuses in the forward direction (Fig. 8B, position 5) will end up one step forward from its original position upon tight binding to the nucleic acid. Thus repeated binding and release of the helicase catalyzed by the NTPase cycles results in a net unidirectional translocation of the helicase along the length of the nucleic acid.

According to the mechanism proposed here for NS3h, the energy of ATP binding is used to recover the enzyme from the energetic well of a tightly bound state, whereas ATP hydrolysis allows the protein to assume the tightly binding state and to trigger helicase rebinding linked to forward motion. The proposed mechanism may be generally applicable to other helicases, although they may have phases of their ATPase cycle and DNA binding affinity coordinated in a different manner. For example, ATP binding may switch the helicase into a tight ssDNA binding state rather than the weak binding state observed for the HCV helicase. The proposed model is applicable to helicases that function as monomers or oligomers. With oligomeric helicases, an increased processivity in movement would be observed if the ATPase reaction is coordinated among the subunits.

Acknowledgment—We thank Dr. Dong-Eun Kim for generously providing mantADP and mantATP as well as conducting the water-phosphate 18O exchange NMR experiments.

REFERENCES

1. Gwack, Y., Kim, D. W., Han, J. H., and Choe, J. (1997) Eur. J. Biochem. 250, 47–54.
2. Gwack, Y., Yoo, H., Song, I., Choe, J., and Han, J. H. (1999) J. Virol. 73, 2899–2915.
3. Levin, M. K., and Patel, S. S. (2002) in Molecular motors (Schliwa, M., ed) pp. 179–198. Wiley-VCH Verlag GmbH, Weinheim, Germany.
4. Patel, S. S., and Fica, K. M. (2000) Annu. Rev. Biochem. 69, 651–697.
5. Preuschat, P., Averret, D. R., Clarke, B. E., and Porter, D. J. T. (1996) J. Biol. Chem. 271, 24449–24457.
6. Levin, M. K., and Patel, S. S. (1999) J. Biol. Chem. 274, 31389–31346.
7. Levin, M. K., and Patel, S. S. (2000) J. Biol. Chem. 275, 29377–29385.
8. Yao, N. H., Hessson, T., Cable, M., Hong, Z., Kwong, A. D., Le, H. Y., and Weber, P. C. (1997) Nat. Struct. Biol. 4, 463–467.
9. Kim, J. L., Morgenstern, K. A., Griffith, J. P., Dwyer, M. D., Thomson, J. A., Murecko, M. A., Lin, C., and Caron, P. R. (1998) Structure 6, 89–100.
10. Porter, D. J. T. (1998) J. Biol. Chem. 273, 7390–7396.
11. Kim, D. W., Gwack, Y., Han, J. H., and Choe, J. (1998) Biochem. Biophys. Res. Commun. 253, 169–166.
12. Ponomarev, M. A., Timofeev, V. P., and Levitsky, D. I. (1993) FEBS Lett. 311, 261–263.
13. Fisher, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., and Rayment, I. (1995) Biochemistry 34, 8960–8972.
14. Chalhoub, M. (1990) Trends Biochem. Sci. 15, 6–10.
15. Martin, R. B. (1998) Biochem. Biophys. Res. Commun. 255, 1194–1200.
16. Delagoutte, E., and von Hippel, P. H. (2002) Q. Rev. Biochem. 35, 431–478.
17. Cho, H., Wang, W., Kim, H., Yokota, H., Dano, S., Kim, S. H., Wemmer, D., Kustu, S., and Yan, D. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 8525–8530.
18. Yan, D., Cho, H. S., Hastings, C. A., Igo, M. M., Lee, S. Y., Pelton, J. G., Stewart, V., Wemmer, D. E., and Kustu, S. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14789–14794.
19. Shibuya, H., Kondo, K., Kimura, N., and Maruta, S. (2002) J. Biochem. (Tokyo) 132, 573–579.

2 M. K. Levin and S. S. Patel, unpublished results.