A Kinetic Analysis of the Oligonucleotide-modulated ATPase Activity of the Helicase Domain of the NS3 Protein from Hepatitis C Virus

THE FIRST CYCLE OF INTERACTION OF ATP WITH THE ENZYME IS UNIQUE*

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Hepatitis C virus (HCV) helicase (E) formed spectrofluorometrically detectable complexes with a 16-mer and HF16 (a 16-mer with 5'-hexachlorofluorescein moiety). The interaction of helicase with these effectors was investigated by kinetic techniques to determine if the complexes were kinetically competent for ATP hydrolysis. The $k_{cat}$ values for the 16-mer and HF16 were 2.7 and 36 s$^{-1}$, respectively. The maximal value of the rate constant for the approach of an intermediate to the steady-state level has to be at least 4-fold greater than $k_{cat}$ for it to be kinetically competent. This value was 1.2 s$^{-1}$ with HF16 and "E-ATP" and was 1.82 s$^{-1}$ with ATP and E-HF16. These values were too small for formation of these intermediates to be kinetically competent in ATP hydrolysis. Dissociation of "E-HF16-ATP" (0.34 s$^{-1}$) was also too slow to contribute significantly to catalysis. Furthermore, the $K_m$ of E-HF16 for ATP (3 μM) was significantly less than the $K_m$ for ATP hydrolysis at a saturating concentration of HF16 (320 μM). HCV helicase has two nucleotide-binding sites per monomer. If the fluorescence changes observed were associated with structure changes preceding steady-state catalysis (isomerization), pre-steady-state data could be reconciled with the turnover data. Data for the 16-mer yielded similar conclusions.

Even though helicase activity was identified and the associated protein was purified over 20 years ago (1), the kinetic and chemical mechanisms for this class of enzymes have not been elucidated. In particular, the mechanism of coupling ATP hydrolysis to the unwinding of double-stranded DNA is incompletely understood. From extensive studies on the mechanism of action of Escherichia coli Rep helicase, Lohman and coworkers (2, 3–12) have proposed that the catalytically active species of this enzyme is a DNA stabilized dimer. The two DNA-binding sites in the catalytic dimer unwind defined lengths of the DNA duplex by alternatively binding duplex and single-stranded DNA in a process coupled to ATP hydrolysis (2, 5, 6, 12). Recently, it has been shown that E. coli helicase II unwinds approximately 4 base pairs during each catalytic cycle (13). Nucleotide binding also regulates the binding of single-stranded and double-stranded DNA to E. coli Rep helicase. In particular, ATP stimulates the rate of single-stranded DNA exchange by increasing the rate constant for dissociation of DNA (10). Because the active form of this enzyme is a dimer (2, 3, 11), there are minimally two potential sites for ATP binding. Bjornsen et al. (10) have provided kinetic evidence that suggested these two sites communicate. Recently Wong and Lohman (14) made heterodimers of Rep helicase containing ADP-AIF6 tightly bound at the ATP site and covalently cross-linked single-stranded DNA at the DNA-binding site. Through the use of cis- (substrate analogues on the same subunit) and trans (substrate analogues on different subunits)-labeled dimers, they concluded that most of the observed ATPase activity was the result of the cis-labeled subunit. The ATPase activity of the subunit lacking single-stranded DNA was small because of a slow conformational change occurring prior to ADP release and not due to a diminished rate of ATP hydrolysis (14). Even though the kinetic mechanism for ATP hydrolysis by Rep helicase in the presence of single-stranded DNA has been clearly defined, the chemical mechanism for coupling ATP hydrolysis to double-stranded DNA unwinding is still unclear.

Hepatitis C virus (HCV)1 genome encodes for an RNA helicase that presumably is essential for viral replication (15). Recently, a crystal structure of the HCV helicase has been reported (16). However, the protein was crystallized in the absence of a divalent metal cofactor such as Mg$^{2+}$, which is required for ATP hydrolysis and nucleic acid unwinding, and in the absence of nucleic acid or nucleotide. Crystal structures of Rep helicase from E. coli (17) and a helicase from Bacillus stearothermophilus have also been reported. Co-crystals of Rep helicase with nucleic acid and ADP in the absence of Mg$^{2+}$ implicated motifs Ia, V, and III for single-stranded DNA binding, motifs I and IV for nucleotide binding, and motifs II and IV possibly functioned in the coupling of nucleotide and single-stranded DNA binding (17). These structures suggested a single nucleotide-binding site per monomer.

HCV helicase has a large intrinsic ATPase activity ($k_{cat} = 3$ s$^{-1}$ (19)). Single-stranded DNA or RNA increases the $k_{cat}$ value up to 30-fold (19). In contrast, the $k_{cat}$ values for ATPase activities of bacterial helicases such as Rep helicase and heli-

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1 The abbreviations used are: HCV, hepatitis C virus; MOPS, 3-(N-morpholino)propanesulfonic acid; HF, hexachlorofluorescein moiety linked to a phosphoramidite through aminohexanyl group as defined in the Oligos Etc. catalogue; 16-mer, 5'-TTT TTT ACA ACG TCG T; HF16, 5'-HF-TTT TTT ACA ACG TCG T; ATPase, ATP-hydrolyzing activity associated with the HCV NS3 helicase domain encompassing amino acids 1193–1657 of the HCV type 1b polyprotein; E, free HCV helicase in the presence of Mg$^{2+}$; "E-ATP," complex formed by addition of ATP to E that could be a mixture of E-ATP, E-ADP, and E-P; E-DNA, complex between E and DNA; E-DNAATP, ternary complex formed upon addition of E, DNA, and ATP that could be a mixture of E-DNA-ATP and E-DNAADP; E-DNAADP, E-DNAADP, and E-P, where DNA was HF16 or the 16-mer; (dU)$_{16}$, homo-octamer of deoxyuridine monophosphate.

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case II are increased over 1000-fold by single-stranded DNA (11). The turnover numbers for ATP hydrolysis by HCV helicase and Rep helicase in the presence of nucleic acid are similar. The differential DNA stimulation of these enzymes is the result of the large intrinsic ATPase activity of HCV helicase in the absence of single-stranded nucleic acid. This observation suggested that either HCV helicase ATPase activity was associated with a single site that was not tightly coupled to nucleic acid binding or HCV helicase may have ATPase activity associated with two separate sites on the enzyme. Further support for the latter suggestion was the finding that stoichiometry for ADP binding to the enzyme in the presence of excess Mg$^{2+}$, F$^{-}$, and poly(U) was two nucleotides per nucleic acid-binding site (30).

HCV helicase forms spectrofluorometrically detectable intermediates with DNA (19) that react with ATP. The kinetic competence of the intermediates formed from the reaction of E-DNA with ATP or "E-ATP" with DNA was evaluated from pre-steady-state and steady-state data. In summary, the intermediates associated with these fluorescent changes were catalytically incompetent. These results suggested either the fluorescent changes were monitoring a catalytically inactive ATP-binding site with a fluorescently silent catalytically active site or the first cycle of interaction of enzyme with ATP was unique relative to subsequent cycles of ATP hydrolysis.

**EXPERIMENTAL PROCEDURES**

**Materials—** Rabbit muscle pyruvate kinase, rabbit muscle lactate dehydrogenase, NADH, ATP, phosphoenolpyruvate, and MOPS were from Sigma. HF16 and the 16-mer from Oligo Therapeutics, Inc., were purified by electrophoresis through 20% polyacrylamide gels in 6 M urea. Oligomer concentrations were calculated from the absorbance at 280 nm and the extinction coefficients provided by Oligo Therapeutics. The standard buffer was 0.5 M MOPS K$^+$ and 3.5 mM MgCl$_2$ at pH 7.0. HCV helicase was purified as described previously (19). The concentration of monomer was calculated with $\varepsilon_{280} = 88$ mmol$^{-1}$ cm$^{-1}$.

**Assay of ATPase Activity—** ATPase was assayed spectrophotometrically by the absorbance change at 340 nm resulting from the oxidation of NADH that was coupled to the phosphorylation of ADP through pyruvate kinase and lactate dehydrogenase (20). Rates were calculated using $\Delta A_{240} = 6.22$ mmol$^{-1}$ cm$^{-1}$, a solution of the coupling component (40 units/ml pyruvate kinase, 40 units of lactate dehydrogenase, 8.0 mM phosphoenolpyruvate, and 800 $\mu$M NADH) was prepared in the standard buffer at 5°C. This solution was diluted 4-fold with the standard buffer containing the selected concentrations of DNA and ATP. The reactions were initiated with HCV helicase. The $t_{1/2}$ for phosphorylation of 5 $\mu$M ADP in this assay was 3 s. The measured rate of ATP hydrolysis was not affected by decreasing the standard concentration of the coupling enzyme NADH and phosphoenolpyruvate by 50%.

**Titration of HCV Helicase—** Titration of helicase (E) with a ligand (L) was monitored by quenching of intrinsic protein fluorescence or by quenching of fluorescence of hexachlorofluoresceinyl-labeled DNA upon formation of the enzyme-ligand complex (E-L). It was assumed that this process could be described by the modified mechanism of Equation 1, where $K$ was the dissociation constant of E for L, $[L]$ was the concentration of enzyme sites binding ligand, and $[L]_0$ was the concentration of ligand.

$$E + L \rightleftharpoons E[L]$$

(Eq. 1)

Furthermore, it was assumed that the fractional fluorescence ($F([L])$) remaining was related to the total concentration of added ligand sites ([L]) by Equation 2 in which $\Delta F_w$ was the fractional fluorescence decrease resulting from conversion of E to E-L.

$$F([L]) = 1 - \Delta F_w \frac{[E - L]}{[E]}$$

(Eq. 2)

Because the concentration of enzyme was in many cases comparable to the dissociation constant of the enzyme for L, the concentration of E-L for the simple scheme of Equation 1 was related to [L] by Equation 3.

$$[E - L] = \frac{[L]_0 + [E]_0 + K}{2} - \frac{1}{2} \sqrt{([L]_0 + [E]_0 + K)^2 - 4[E][L]_0}$$

(Eq. 3)

During the titration of E with the 16-mer, which was monitored by the quenching of the intrinsic protein fluorescence ($\lambda_{ex} = 280$ nm and $\lambda_{em} = 340$ nm), there was significant drift in the fluorescence signal. This was corrected for by a blank titration in which the experimental signal obtained after addition of the 16-mer to the enzyme was normalized to the respective amplitude of the phase associated with the fluorescence mode. The fluorescence of the hexachlorofluoresceinyl moiety was monitored on the stopped-flow spectrophotometer with $\lambda_{ex} = 500$ nm and $\lambda_{em} > 530$ nm. Intrinsic protein fluorescence was monitored with $\lambda_{ex} = 280$–290 nm and $\lambda_{em} > 305$ nm. Stopped-flow time courses were an average of 4–6 experiments. Fluorescence data from the stopped-flow spectrophotometer were presented as the voltage...
change from the beginning of the reaction. These changes were not normalized to the initial fluorescence of the solution because the solutions contained varying amounts of HF16 that were in excess of enzyme. For a group of experiments associated with a particular figure, the photomultiplier voltage was held constant. However, the magnitude of the voltage changes should not be compared between groups of experiments, only the voltage changes associated with a single group of experiments should be compared. The appropriate equations were fitted to the data by nonlinear least squares using SigmaPlot from Jandel Scientific (Corte Madera, CA).

RESULTS

$k_{\text{cat}}$ for ATP Hydrolysis by HCV Helicase with HF16 and the 16-mer as Single-stranded Nucleic Acid Effectors—The goal of the studies described herein was to determine if the formation of spectrally observed intermediates was a step in the steady-state catalytic cycle of ATP hydrolysis by HCV helicase. The criteria for kinetic competence of an intermediate in the catalytic cycle was that the value of the limiting first-order rate constant for the approach to the steady-state was at least 4-fold greater than the $k_{\text{cat}}$ for ATP hydrolysis. Thus, the value of $k_{\text{cat}}$ was the number of importance determined from steady-state rate data. The value for $k_{\text{cat}}$ for ATP hydrolysis by HCV helicase is dependent on the single-stranded DNA effector. For example (dU)18 increased the value of $k_{\text{cat}}$ by 1.4 fold (19). With HF16 as the DNA effector, the ATPase activity of the enzyme was inhibited at small ATP concentrations and enhanced at large ATP concentrations (Fig. 1). The complex concentration dependence of the initial rate data was adequately described by Equation 4 with $K_D = 2.73 \pm 0.09 \text{ nM}$ for HF16. The $k_{\text{cat}}$ for the 16-mer was calculated to be $36 \pm 7 \text{ s}^{-1}$ from an analogous data set. The rate of hydrolysis of 1.6 mM ATP with the 16-mer was not inhibited at concentrations between 1 and 60 nM. This result indicated that the value of $k_{\text{cat}}$ was independent of enzyme concentration so that it was valid to compare $k_{\text{cat}}$ values with kinetic parameters determined at different enzyme concentrations. The values for the $K_d$ of the enzyme for ATP at near-saturating concentrations of HF16 (1600 nM) and the 16-mer (6000 nM) were determined to be 320 ± 50 and 410 ± 20 μM, respectively.

Affinity of HCV Helicase for HF16 and the 16-mer—The binding of HCV helicase to HF16 was monitored by the quenching of HF16 fluorescence upon formation of E/HF16. Titration of 22.4 nM HF16 with HCV helicase (Fig. 2) was described by Equation 2.

At saturating substrate concentration, the pseudo first-order rate constant for approach to the steady-state ($k_{\text{obs}}$) and $k_{\text{cat}}$ are given by Equations 11 and 12.

$$k_{\text{obs}} = k + a h$$ (Eq. 11)

$$k_{\text{cat}} = \frac{a h}{(a + 1)}$$ (Eq. 12)

The expression of $k_{\text{obs}}$ in terms of $a$ and $k_{\text{cat}}$ is given by Equation 13.

$$k_{\text{obs}} = \frac{(a^2 + 2a + 1)k_{\text{cat}}}{a}$$ (Eq. 13)

The minimal value of $k_{\text{obs}}$ for a given value of $k_{\text{cat}}$ can be shown by elementary calculus to occur with $a = 1$. Substituting this value for $a$ into Equation 13 demonstrated that the minimal value of $k_{\text{obs}}$ was $4 \times k_{\text{cat}}$. The first-order rate constant for the approach of an enzymatic intermediate on the catalytic pathway to a steady-state value must be minimally 4 times larger than $k_{\text{cat}}$. Considering the simple scheme of Equation 10 in which formation and breakdown of the catalytic intermediate (EP) is described by $k$ and $a k$, respectively.

$$E + S \rightarrow ES \rightarrow k \rightarrow EP$$ (Eq. 10)

$K_D$ values for $K_D$ for dissociation of $E/DN$A, the calculated values for $K_D$ were associated with considerable error. Nonetheless, these estimates for the values of $K_E$ were similar to the estimate values calculated from bimolecular association rate constants and dissociation rate constants (see below).

Kinetics for Equilibration of HCV Helicase with HF16 and the 16-mer—The time course for quenching of the fluorescence of HF16 with HCV helicase was a first-order process (Fig. 3, inset). The pseudo first-order rate constant for this process ($k_{\text{obs}}$) was linearly dependent on the concentration of HF16 with [HF16] > [E] (Fig. 3). These data indicated that the $K_D$ for formation of the initial complex between $E$ and $HF16$ (Equation 6) was much greater than the highest concentration of HF16 tested. Consequently, Equation 9 was fitted to these data to

$$k_{\text{cat}}$$

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Equations 2 and 3 (L = HF16) with $K = 0.48 \pm 0.02 \text{ nM}$ and $\Delta F_o = 0.457 \pm 0.006$ Quenching of intrinsic protein fluorescence monitored the binding of HCV helicase to the 16-mer. Titration of 33.5 nM E (Fig. 2, inset) was described by Equations 2 and 3 with $K = 2.2 \pm 0.2 \text{ nM}$ and $\Delta F_o = 0.377$.

Fig. 1. Steady-state hydrolysis of ATP with HF16 as the single-stranded DNA effector. The initial rate of ATP hydrolysis by HCV helicase was determined for selected ATP and HF16 concentrations at 21 °C. The concentration of enzyme was 38.5 nM. The solid lines were calculated by Equation 4. The value of $k_{\text{cat}}$ was 2.73 s$^{-1}$.

Fig. 2. Titration of helicase by HF16 and the 16-mer. The binding of HF16 to enzyme was monitored by the quenching of the fluorescence of HF16 ($\lambda_{oex} = 510 \text{ nm and } \lambda_{oem} = 552 \text{ nm})$. The fluorescence of HF16 (22.4 nM) was titrated with helicase. The solid line was calculated with Equations 2 and 3 and the following values for the parameters: $K = 0.48 \pm 0.02 \text{ nM and } \Delta F_o = 0.457$. The binding of enzyme to the 16-mer (inset) was monitored by the quenching of the protein fluorescence ($\lambda_{oex} = 280 \text{ nm and } \lambda_{oem} = 340 \text{ nm})$. The fluorescence of helicase (33.5 nM) was titrated with the 16-mer. The solid line was calculated by Equations 2 and 3 with $K = 2.2 \pm 0.2 \text{ nM and } \Delta F_o = 0.377$.
estimate a value for the apparent bimolecular rate constant for association of HCV helicase with HF16 ($k_{a,b}$ in Equation 9) with $k_{-2} = 0$ of $640 \pm 20 \mu M^{-1} s^{-1}$. The reaction of the 16-mer with HCV helicase was monitored by the quenching of the intrinsic protein fluorescence upon formation of E-16. The pseudo first-order rate constant for this reaction was also linearly dependent on the concentration of the 16-mer yielding an analogous bimolecular rate constant of $99 \pm 2 \mu M^{-1} s^{-1}$.

The dependence of the pseudo first-order rate constant for the reaction of HF16 with HCV helicase appeared to extrapolate to a small value (Fig. 3, ordinate intercept) at zero concentration of HF16. This result suggested that the rate constant describing the dissociation of E-HF16 ($k_{2}$ in Equation 9) was very small. A direct measure of the dissociation of E-HF16 was made by monitoring the associated increase in fluorescence of HF16 ($\lambda_{em} = 500$ nm, $\lambda_{em} \sim 530$ nm) upon release of the enzyme in the presence of excess dextran sulfate that trapped free HCV helicase (Equation 14).

$$k_{ob} \times 10^{-9}$$

The fluorescence ($\lambda_{em} = 500$ nm, $\lambda_{em} \sim 530$ nm) of a solution of 13.2 mM E and 18.8 mM HF16 increased in a first-order process ($k_{obs}$) upon addition of 2.0 mM dextran sulfate. $k_{obs}$ had a value of $0.087 \pm 0.001$ s$^{-1}$ with 2.0 mM dextran sulfate and a similar value ($0.109 \pm 0.001$ s$^{-1}$) with 0.4 mM dextran sulfate. Because $k_{obs}$ was similar for two dextran sulfate concentrations, this value was assigned to the dissociation rate constant ($k_{2}$) for EHF16. Similar experiments with E-16 ($\lambda_{em} = 280$ nm, $\lambda_{em} \sim 305$ nm) yielded a value for $k_{-2}$ of $0.96 \pm 0.009$ s$^{-1}$.

The apparent dissociation constants ($K$) of E for HF16 and the 16-mer were calculated from the relationship $K = K_{D,E} \times k_{-2}/k_{2}$, where $k_{-2}$ was the effective rate constant for dissociation of E-HF16, and $k_{2}/K_{D,E}$ was the effective association rate constant for reaction of HCV helicase and HF16 (Equation 6, $L = HF16$). The values of $K$ for HCV helicase and the 16-mer were calculated from the kinetic data to be 0.14 and 1.0 nM, respectively, which were in reasonable agreement with the values estimated from titration data (0.48 and 2.2 nM, respectively).

**Fig. 3.** Kinetics of the reaction of HCV helicase with HF16. The reaction of 13 nM helicase with selected concentrations of HF16 was monitored on the stopped-flow spectrophotometer with $\lambda_{em} = 500$ nm and $\lambda_{em} \sim 530$ nm. Insets: time course for the reaction of 154 nM HF16 with the enzyme. The solid line was calculated with Equation 5 ($i = 1$) and $k_{1} = 80.5$ s$^{-1}$. The value of $k_{obs}$ ($k_{1}$) was linearly dependent on HF16 concentration. The solid line was calculated by Equation 9 with $k_{2}/K_{D,E} = 640 \mu M^{-1} s^{-1}$.

**Fig. 4.** Kinetics of the reaction of E-ATP with HF16 in the presence of 2.0 mM ATP. E-ATP was generated by premixing 26.4 nM enzyme with 2.0 mM ATP. E-ATP was mixed with selected concentrations of HF16 as described in the legend to Fig. 3. The dependence of the observed pseudo first-order rate constant for approach to the steady-state and the amplitudes (inset) were described by Equations 7 and 8. The value of $k_{obs}$ in the absence of HF16 was an independently determined value for the rate constant for dissociation of E-ATP-HF16. The solid line was calculated by these equations with $k_{-2} = 0.37$ s$^{-1}$, $k_{2} = 1.2$ s$^{-1}$, $K = 240$ nM, and $\Delta F = 0.4$.

**Fig. 5.** Time course for dissociation of E-HF16 in the presence of ATP. E-HF16, which was generated from 9.3 nM helicase and 17.7 nM HF16, was reacted with 2.0 mM ATP. Changes in the fluorescence of HF16 were monitored as described in the legend to Fig. 3. The solid line was calculated with Equation 5 ($i = 2$) and $F_{0} = -0.494$ V, $F_{i} = -3.98$ V, $k_{1} = 1.01$ s$^{-1}$, $F_{0} = 4.3$ V, and $k_{2} = 0.403$ s$^{-1}$ if E-HF16 was incubated with 2.0 mM ATP prior to mixing with 1 mM dextran sulfate and 2.0 mM ATP to trap free enzyme as E-HF16 dissociated, the time course was monophasic (inset). The solid line was calculated by Equation 5 ($i = 1$) with $F_{0} = -0.847$ V, $F_{i} = 0.847$ V, and $k_{1} = 0.341$ s$^{-1}$.

**Kinetics for the Approach to the Steady-state from E-ATP and HF16 or the 16-mer**—The time courses for the approach to the steady-state starting with 2.0 mM ATP, 13.2 nM E-ATP, and selected concentrations of HF16 were first-order processes ($k_{obs}$). The dependence of $k_{obs}$ on HF16 concentration was described by Equations 7 and 8 with $k_{-2,obs} = 0.37 \pm 0.02$ s$^{-1}$, $k_{2} = 1.2 \pm 0.2$ s$^{-1}$, $K = 240 \pm 70$ nM, and $\Delta F = 0.40 \pm 0.03$ V (Fig. 4). Similarly, the approach to the steady-state starting with 50 nM E-ATP and selected concentrations of the 16-mer was a first-order process. Because the highest concentration of the 16-mer was significantly less than the $K_{D}$ for the reaction, Equation 9 was fitted to the data with $k_{-2,obs} = 2.0 \pm 0.1$ s$^{-1}$, $k_{2}/K_{D,E} = 0.41 \pm 0.01$ $\mu M^{-1}$ s$^{-1}$.

**Dissociation of E-HF16-ATP or E-16-ATP**—The dissociation constant of the enzyme for HF16 was increased over 100-fold by ATP (compare data of Fig. 2 with that of Fig. 4). Consequently, mixing ATP with a solution of E-HF16 (HF16) <
The release of HF16 from the enzyme was monitored by the associated increase in fluorescence of HF16 ($\lambda_{ex} = 500$ nm, $\lambda_{em} > 530$ nm). The approach to the steady-state upon addition of 1.0 mM ATP to 9.3 nM E-HF16 that was generated from 9.3 nM helicase and 17.7 nM HF16 was a biphasic process that indicated that ATP bound to the E-HF16 complex to form an intermediate ("E-HF16-ATP") from which HF16 dissociated (Fig. 5). Equation 5 ($i = 2$) was fitted to these data to give $F_0 = -0.494 \pm 0.003$ V, $F_1 = -3.98 \pm 0.2$ V, $k_1 = 1.01 \pm 0.03$ s$^{-1}$, $F_2 = 4.3 \pm 0.2$ V, and $k_2 = 0.403 \pm 0.008$ s$^{-1}$. The early phase of the reaction was attributed to formation of E-HF16-ATP from E-HF16 and ATP, whereas the late phase of the reaction was attributed to dissociation of HF16 from E-HF16-ATP. When dextran sulfate was added to a steady-state mixture of ATP, HCV helicase, and HF16 to trap free E as it was cycled during catalysis, the time course for formation of free HF16 was monophasic (Fig. 5, inset). Equation 5 ($i = 1$) was fitted to these data with $F_0 = -0.67 \pm 0.002$ V, $F_1 = 0.067 \pm 0.002$ V, and $k_1 = 0.341 \pm 0.002$ s$^{-1}$. The value of the rate constant for the latter process was in good agreement with the value for the late phase of the reaction in the presence of ATP alone (0.403 s$^{-1}$).

ATP also induced the dissociation of E-16. The release of the 16-mer from E-16 was monitored by the associated change in intrinsic protein fluorescence ($\lambda_{ex} = 280$ nm, $\lambda_{em} > 305$ nm) upon formation of (E-ATP) from (E-16-ATP). The addition of 1.0 mM ATP to a solution of 50 nM E-16 generated from 50 nM E and 75 nM 16-mer resulted in a biphasic increase in fluorescence. These data (not shown) were described by Equation 5 ($i = 2$) with $F_0 = -0.129 \pm 0.001$ V, $F_1 = -0.83 \pm 0.07$ V, $k_1 = 2.34 \pm 0.05$ s$^{-1}$, $F_2 = 1.08 \pm 0.07$ V, and $k_2 = 1.70 \pm 0.02$ s$^{-1}$. The early phase of the reaction was attributed to formation of E-16-ATP from E-16 and ATP, whereas the late phase of the reaction was attributed to dissociation of the 16-mer from E-16-ATP.

**Kinetics for the Approach to the Steady-state from E-HF16 or E-16 and ATP**—The previous results indicated that the reaction of E-HF16 with ATP to form E-HF16-ATP was associated with a fluorescence decrease (Fig. 6A). Because the concentration of HF16 in these experiments was less than the dissociation constant of E-ATP for HF16, E-HF16-ATP partially dissociated to E-ATP and HF16. The dissociation phase of the reaction was eliminated by including high concentrations of HF16 in the reaction. Thus, the reaction of 2.0 nM ATP with 50 nM E-HF16, which was generated by premixing 50 nM E with 492 nM HF16, was a first-order process (Fig. 6A). Equation 5 ($i = 1$) was fitted to these data with $F_0 = 0.262 \pm 0.01$ V, $F_1 = -0.262 \pm 0.001$ V, and $k_1 = 1.67 \pm 0.03$ s$^{-1}$. This rate constant described the approach to the steady-state after mixing E-HF16 with ATP. The values of pseudo first-order rate constant ($k_{obs}$) and the amplitudes of the fluorescence change for this reaction were determined for selected ATP concentrations (Fig. 6B). Equations 7 and 8 were fitted to these data with $K = 121 \pm 7$ nM, $k_2 = 1.82 \pm 0.03$ s$^{-1}$, $k_{2,obs} = 0.05 \pm 0.01$ s$^{-1}$, and $\Delta F = 0.29 \pm 0.01$ V. If E-ATP-HF16 were a catalytic intermediate in ATP steady-state ATP hydrolysis as depicted in Equation 15, the value of $k_{2,obs}$ should have been the sum of $k_2$ and $k_{cat}$. A data set similar to that for E-ATP and HF16 was collected.
for the reaction of E-ATP with the 16-mer. E-16 was generated by premixing 50 nM E with 12 μM 16-mer. Addition of 1.0 mM ATP resulted in a first-order increase in intrinsic protein fluorescence (data not shown). Equation 5 (i = 1) was fitted to these data with \( F_0 = 0.109 \pm 0.002 \text{ V} \), \( F_1 = -0.109 \pm 0.002 \text{ V} \), and \( k_{1} = 2.55 \pm 0.07 \text{ s}^{-1} \). The values of pseudo first-order rate constant \( (k_{\text{cat}}) \) and the amplitudes of the fluorescence change for this reaction were determined for selected ATP concentrations. Equations 7 and 8 were fitted to these data with \( K = 170 \pm 20 \mu \text{M} \), \( k_2 = 2.9 \pm 0.2 \text{ s}^{-1} \), \( k_{2,\text{obs}} = 0.49 \pm 0.06 \text{ s}^{-1} \), and \( \Delta F_{\text{p}} = 0.104 \pm 0.004 \text{ V} \). The kinetic parameters for these reactions are summarized in Table I.

DISCUSSION

Helicases catalyze the ATP-dependent unwinding of double-stranded DNA. The mechanisms for the unwinding reaction are being actively investigated in numerous laboratories (2, 18, 21–24). Recent mechanistic studies on \( E.\ coli \) Rep helicase are forming the basis for a detailed understanding of the kinetic mechanism for DNA unwinding by a helicase (2). Lohman (6) has classified the mechanism for helicase action as being either “active” or “passive.” In a passive type mechanism, the enzyme binds preferentially to single-stranded DNA and unwinds double-stranded DNA by binding to single-stranded DNA that is formed transiently from the duplex as the result of thermal fluctuations. In contrast, an active mechanism requires that the enzyme bind to both double-stranded DNA and single-stranded DNA, and the enzyme destabilizes multiple base pairs during each catalytic step. This mechanism requires that the enzyme have multiple DNA-binding sites either on the same subunit or as an oligomeric protein. During catalysis, one DNA-binding site alternatively binds double-stranded and single-stranded DNA as the enzyme rolls along the double-stranded DNA. Whichever mechanism is operative for unwinding of double-stranded DNA, the coupling of ATP hydrolysis to translocation is crucial for the unwinding process.

The coupling of ATP hydrolysis to conformational transitions of other domains in members of helicase superfamily I is being investigated by site-directed mutagenesis of conserved amino acids in each of the six motifs. For example, \( E.\ coli \) helicase II mutants D248N, K35M, and R284A greatly reduced the single-stranded DNA-stimulated ATPase activity (25–27). Recently, site-specific substitutions in each of the six motifs of HSV helicase (superfamily I) produced large effects when in motifs I and II and small effects when in motifs III–VI on DNA-dependent ATPase activity (28). To supplement these studies, our goal was to characterize the single-stranded DNA-stimulated ATPase activity of HCV helicase by steady-state and pre-steady-state kinetic techniques. In particular, the kinetic competence of spectrofluorometrically observed intermediates was determined. HCV helicase quenched the fluorescence of HF16, whereas HF16 and the 16-mer quenched the intrinsic protein fluorescence. The kinetics for formation of these intermediates with modified fluorescence properties were compared with the values for \( k_{\text{cat}} \) for ATP hydrolysis to decide whether or not the intermediates were kinetically competent for catalysis of ATP hydrolysis.

The dissociation of DNA from the enzyme in the presence of ATP was too slow for this process to be an obligatory step in the catalytic cycle. The rate constants for dissociation of \( E\)-HF16 and \( E\)-16 in the presence of 2.0 mM ATP were 0.341 s\(^{-1}\) and 1.70 s\(^{-1}\), respectively, whereas the values of \( k_{\text{cat}} \) were 2.73 and 36 s\(^{-1}\), respectively. Because DNA dissociated from E-DNA infrequently during the catalytic cycle, the enzyme cycled primarily between E-DNA and E-DNA-ATP during ATP hydrolysis. If E-DNA-ATP were on the pathway for hydrolysis of ATP, the pseudo-first-order rate constant for the approach to the steady-state starting with E-DNA and a saturating concentration of ATP must be at least 4-fold greater than the value of \( k_{\text{cat}} \)\(^{-2}\). Furthermore, the concentration of ATP that resulted in 50% of the maximal amount of the intermediate must be greater than or equal to the \( K_{m} \) for ATP. These conditions were not met by the pre-steady-state intermediate formed from E-DNA and ATP. For instance, the maximal value for the first-order rate constant for the approach to the steady-state concentration of HF16-ATP starting with HF16 and ATP was 1.82 s\(^{-1}\), whereas \( k_{\text{cat}} \) was 2.73 s\(^{-1}\). The minimal value for the rate constant for approach to the steady state at saturating concentration of ATP has to be 10.9 s\(^{-1}\) for this process to be on the catalytic cycle. Furthermore, the concentration of ATP that yielded 50% of the maximal amount of the intermediate for a saturating concentration of HF16 should be equal to the \( K_{m} \) for the steady-state rate of ATP hydrolysis. The value of \( K_{m} \) for the ATP at saturating concentrations of HF16 was 320 μM, whereas the concentration of ATP that yield 50% of the intermediate was only 3 μM. Thus, formation of this intermediate was not a competent step in the catalytic cycle of ATP hydrolysis.

These results suggested either that the formation of E-DNA-ATP occurred independently of and was unrelated to the catalytic process or that formation of this species introduced the active form of the enzyme into the catalytic cycle. If the latter were the case, an isomerization step could be included in the kinetic mechanism such that reaction of enzyme with the first molecule of ATP was different than its reaction with subsequent molecules of ATP (Scheme I). In this model, steady-state ATP hydrolysis was mediated principally through cycling of \( E\)-HF16 (Scheme I), whereas the reaction monitored on the stopped-flow spectrofluorometer was conversion of \( E\)-HF16 to \( E\)-HF16\(^{-1}\). These data have not addressed the fate of ATP in the conversion of \( E\)-HF16 to \( E\)-HF16\(^{-1}\). Possibly a structural isomerization dependent on phosphorylation of the enzyme was involved that transformed inactive enzyme into a catalytically active form. If this were the case, formation of \( E\)-HF16\(^{-1}\) and its reversion to \( E\)-HF16 (\( <0.05 \text{ s}^{-1}\) in Scheme I) could involve net hydrolysis of ATP.

The data presented herein for hydrolysis of ATP by \( E\)-HF31 suggested that the first cycle of interaction of ATP with \( E\)-HF31 was unique relative to subsequent cycles of interaction of the enzyme with ATP. This concept has been suggested for other DNA unwinding proteins such as the \( E.\ coli \) Rep helicase and the \( E.\ coli \) Rec A protein. For Rep helicase, the approach to the steady state for the reaction of 1.0 mM ATP with enzyme preloaded with 2-amino-purine-labeled d(pT)\(^{-15}\) appeared to be too slow for the formation of ternary complex to be a kinetically competent step during ATP hydrolysis (10). Furthermore, Wong and Lohman (14) have suggested from studies on the K28I mutant of Rep helicase that a global conformation change of the protein occurs prior to the first turnover. Similar results have been described for a mutant \( E.\ coli \) Rec A protein (H163W) that catalyzed single-stranded DNA-dependent ATPase. The fluorescence of the H163W protein was used to monitor confor-
national changes occurring during ATP hydrolysis (29). The first cycle of interaction of the mutant protein with ATP was too slow to be on the catalytic pathway. Consequently, Stole and Bryant (29) proposed an isomerization model similar to that described in Scheme I. The steady-state hydrolysis of ATP by these DNA unwinding proteins was preceded by a unique isomerization of the initial ternary enzyme-DNA-ATP complex that may be a general phenomenon for all DNA unwinding proteins.

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