# Interdependence of the Kinetics of NTP Hydrolysis and the Stability of the RecA−ssDNA Complex†

Francine S. Katz and Floyd R. Bryant*

*Department of Biochemistry and Molecular Biology, Bloomberg School of Public Health, The Johns Hopkins University, Baltimore, Maryland 21205*

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ABSTRACT: The ssDNA-dependent NTP hydrolysis activity of the RecA protein was examined using a series of $dT_n$ oligomers ranging in size from $dT_{10}$ to $dT_{2000}$ as the ssDNA effector. There were three distinct manifestations of the $dT_n$-dependent NTP hydrolysis reaction, depending on the length of the $dT_n$ effector that was used. With longer $dT_n$ oligomers, NTP hydrolysis occurred with a turnover number of 20−25 $min^{-1}$ and the observed $S_{0.5}$ value for the NTP was independent of the concentration of the $dT_n$ oligomer (DNA concentration-independent hydrolysis). With $dT_n$ oligomers of intermediate length, NTP hydrolysis still occurred with a turnover number of 20−25 $min^{-1}$, but the observed $S_{0.5}$ for the NTP decreased with increasing $dT_n$ concentration until reaching a value similar to that obtained with the longer $dT_n$ oligomers (DNA concentration-dependent hydrolysis). With shorter $dT_n$ oligomers, the NTP hydrolysis activity was effectively eliminated. Although this general progression of kinetic behavior was observed for the three structurally related NTPs (dATP, ATP, and GTP), the $dT_n$ oligomer length at which DNA concentration-independent, DNA concentration-dependent, and no NTP hydrolysis was observed depended on the NTP being considered. For example, dATP ($S_{0.5}$ = 35 $\mu$M) was hydrolyzed in the presence of $dT_{20}$, whereas ATP ($S_{0.5}$ = 70 $\mu$M) and GTP ($S_{0.5}$ = 1200 $\mu$M) required at least $dT_{50}$ and $dT_{200}$ for hydrolysis, respectively. These results are discussed in terms of a kinetic model in which the stability of the RecA−ssDNA−NTP complex is dependent on the intrinsic $S_{0.5}$ value of the NTP being hydrolyzed.

The RecA protein of *Escherichia coli* ($M_r$ = 37 842; 352 amino acids) is essential for homologous genetic recombination and for the postreplicative repair of damaged DNA. The purified RecA protein binds cooperatively to ssDNA,[1] forming a helical filament-like structure with 1 RecA monomer per 3.5 nucleotides of ssDNA and 6 RecA monomers per turn of the filament. This RecA−ssDNA complex catalyzes the hydrolysis of ATP to ADP and $P_i$. In addition, the RecA protein will promote a variety of ATP-dependent DNA pairing reactions that presumably reflect in vivo recombination functions. These reactions include the assimilation of a linear ssDNA into a homologous dsDNA (D-loop formation) and the exchange of strands between a circular ssDNA and a homologous linear dsDNA (three-strand exchange). The complex formed between the RecA protein and ssDNA, known as the presynaptic complex, is the active recombinational entity in these DNA pairing reactions (*1*, *2*).

The mechanistic role of ATP binding and hydrolysis in the RecA protein-promoted DNA strand exchange reaction has not yet been determined (*1*, *2*). The elucidation of the mechanism of DNA strand exchange will require a detailed understanding of the interaction of the RecA−ssDNA presynaptic complex with ATP. We have examined the effect of ssDNA effector length on the RecA protein-catalyzed hydrolysis of ATP, as well as on the hydrolysis of the structurally related purine nucleoside triphosphates, dATP and GTP (*3*). The results of this investigation, which reveal an interdependence of the kinetics of NTP hydrolysis and the stability of the RecA−ssDNA complex, are described in this report.

## EXPERIMENTAL PROCEDURES

*Materials.* [H163W]RecA protein was prepared as described previously (*4*). ATP, [α-$^{32}$P]ATP, dATP, [α-$^{32}$P]-dATP, GTP, [α-$^{32}$P]GTP, and $dT_{200}$ were from Amersham Pharmacia Biotech. $dT_{10}$, $dT_{20}$, $dT_{50}$, and $dT_{100}$ were from BRL Life Technology, and $dT_{2000}$ was from Sigma. All DNA concentrations are expressed as total nucleotides.

*NTP Hydrolysis Assay*. ATP, dATP, and GTP hydrolysis reactions were measured using a thin-layer chromatography method as previously described (*5*). The specific conditions that were used for each set of reactions are given in the relevant figure legends. For each reaction, a time course of NTP hydrolysis was generated and the initial rate of NTP hydrolysis was derived from the initial linear phase of the time course. Each of the data points shown in Figures 1−5 (representing an initial rate of NTP hydrolysis) was the result of a single experimental determination, and all of the data points in a given figure were generated in a single experimental session; this protocol was followed so that all of the velocity curves in a given figure could be directly compared with each other. Each of the velocity curves in Figures 1−5

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* To whom correspondence should be addressed. Phone: (410) 955-3895. Fax: (410) 472-3378. E-mail: fbryant@jhsph.edu.

[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; NTP, nucleoside triphosphate.

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Table 1: Kinetic Parameters for $dT_n$-Dependent NTP Hydrolysis by the [H163W]RecA Protein[a]

| | dATP | | ATP | | GTP | |
|---|---|---|---|---|---|---|
| ssDNA | $V_{max}$ ($min^{-1}$) | $S_{0.5}$ ($\mu$M) | $V_{max}$ ($min^{-1}$) | $S_{0.5}$ ($\mu$M) | $V_{max}$ ($min^{-1}$) | $S_{0.5}$ ($\mu$M) |
| $dT_{10}$ | – | – | – | – | | |
| $dT_{20}$ | 23 | 80 | – | – | | |
| $dT_{50}$ | 25 | 40 | 21 | 90 | – | – |
| $dT_{100}$ | 22 | 40 | 20 | 60 | – | – |
| $dT_{200}$ | 22 | 50 | 22 | 95 | 20 | 1600 |

[a] The NTP hydrolysis reaction solutions contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 3 $\mu$M [H163W]RecA protein, 75 $\mu$M $dT_n$, and various concentrations of the indicated NTP. The reactions were initiated by the addition of [H163W]RecA protein and carried out at 37 °C. The initial rates of NTP hydrolysis were plotted vs NTP concentration and then fit by the standard Hill equation to yield values of $V_{max}$ and $S_{0.5}$ for each NTP with each $dT_n$ oligomer. The designation "–" indicates that the initial rate of NTP hydrolysis was less than 2 $min^{-1}$ at the highest NTP concentration that was examined (2 mM).

was repeated at least three times, however, with equivalent results ($V_{max}$ and $S_{0.5}$ values typically varied by less than ±10% between runs). The solid lines in Figures 1−5 represent fits of the Hill equation to the data points shown in the figures and were generated using the graphing program KaleidaGraph.

## RESULTS

### *Dependence of NTP Hydrolysis on ssDNA Effector Length*

The RecA protein-catalyzed ssDNA-dependent hydrolysis of dATP, ATP, and GTP was examined under standard reaction conditions (pH 7.5 and 37 °C), using a series of $dT_n$ oligomers ranging in size from $dT_{10}$ to $dT_{200}$ as the ssDNA effector. In the initial set of reactions, the RecA protein concentration was fixed at 3 $\mu$M and the $dT_n$ oligomer concentration was 75 $\mu$M [total nucleotide; since each RecA monomer binds to approximately 3.5 nucleotides of ssDNA (*1*, *2*), this corresponds to an approximately 7-fold excess of $dT_n$ relative to the RecA protein]. The rates of NTP hydrolysis (defined here as moles of NTP hydrolyzed per minute per mole of RecA) were measured over a range of NTP concentrations. Turnover numbers (equivalent here to the maximal rate of NTP hydrolysis, or $V_{max}$) and $S_{0.5}$ values (the NTP concentration required for $0.5V_{max}$) were determined for each NTP with each $dT_n$ oligomer. The results are summarized in Table 1 (the velocity curves for the reactions with $dT_{200}$ are shown in Figure 1; other curves not shown).[2]

As shown in Figure 1, dATP, ATP, and GTP were each hydrolyzed by the RecA protein when $dT_{200}$ was provided as the ssDNA effector. The turnover numbers for the hydrolysis of dATP (22 $min^{-1}$), ATP (22 $min^{-1}$), and GTP (20 $min^{-1}$) were similar. The $S_{0.5}$ values for dATP (50 $\mu$M), ATP (95 $\mu$M), and GTP (1600 $\mu$M) varied widely, however.

[2] The kinetic experiments reported in this paper were carried out with our mutant [H163W]RecA protein so that the results could be related directly to our ongoing transient fluorescence studies with this protein; the [H163W]RecA protein is functionally identical to the wild-type RecA protein, and the tryptophan residue at position 163 allows us to monitor NTP-mediated conformational changes in the RecA−ssDNA complex (*4*). In all cases, equivalent kinetic results were obtained with the wild-type RecA protein (data not shown).

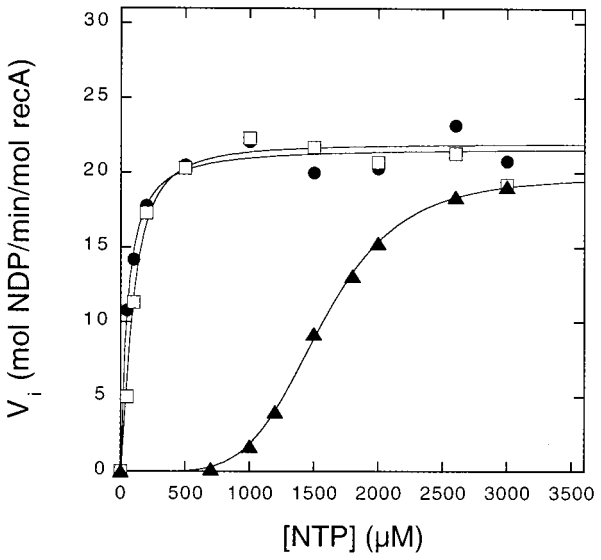


FIGURE 1: $dT_{200}$-dependent NTP hydrolysis by the [H163W]RecA protein. The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 3 $\mu$M [H163W]RecA protein, 75 $\mu$M $dT_{200}$, and the indicated concentrations of dATP, ATP, or GTP. The reactions were carried out at 37 °C. The points represent the initial rates of dATP (●), ATP (□), and GTP (▲) hydrolysis that were measured at the indicated concentrations of NTP. The solid lines were generated using the standard Hill equation and the following parameters: $V_{max}$ = 22 $min^{-1}$, $S_{0.5}$ = 50 $\mu$M, and $n$ = 1.1 (dATP); $V_{max}$ = 22 $min^{-1}$, $S_{0.5}$ = 95 $\mu$M, and $n$ = 1.5 (ATP); and $V_{max}$ = 20 $min^{-1}$, $S_{0.5}$ = 1600 $\mu$M, and $n$ = 5.1 (GTP).

Although dATP, ATP, and GTP were each hydrolyzed by the RecA protein in the presence of $dT_{200}$, the hydrolysis of the three NTPs varied greatly when shorter $dT_n$ oligomers were used as the ssDNA effector (Table 1). For example, although GTP hydrolysis occurred with a turnover number of approximately 20 $min^{-1}$ with $dT_{200}$, no GTP hydrolysis was detected when $dT_{10}$, $dT_{20}$, $dT_{50}$, or $dT_{100}$ was provided as the ssDNA effector [Table 1; "no hydrolysis" is defined here as an observed rate of NTP hydrolysis of less than 10% of the expected maximal rate (i.e., <2 $min^{-1}$) at an NTP concentration of 2 mM]. Similarly, ATP hydrolysis occurred with a turnover number of approximately 21−22 $min^{-1}$ in the presence of $dT_{50}$, $dT_{100}$, and $dT_{200}$, but no ATP hydrolysis was detected when $dT_{10}$ or $dT_{20}$ was used as the ssDNA effector. Finally, dATP hydrolysis occurred with a turnover number of approximately 22−25 $min^{-1}$ in the presence of $dT_{20}$, $dT_{50}$, $dT_{100}$, and $dT_{200}$, but no dATP hydrolysis was detected when $dT_{10}$ was used as the ssDNA effector. These results indicate that the minimal size $dT_n$ oligomer that is able to serve as an effector for the RecA protein-catalyzed NTP hydrolysis reaction depends on the NTP being hydrolyzed, with those NTPs with higher $S_{0.5}$ values requiring longer $dT_n$ oligomers as effectors for hydrolysis (Table 1).

### *Dependence of NTP Hydrolysis on ssDNA Effector Concentration*

The NTP hydrolysis reactions that were used to generate the kinetic parameters in Table 1 were carried out at a fixed concentration of RecA protein (3 $\mu$M) and $dT_n$ oligomer (75 $\mu$M). Next, the effect of $dT_n$ oligomer concentration on the hydrolysis of each NTP was determined.

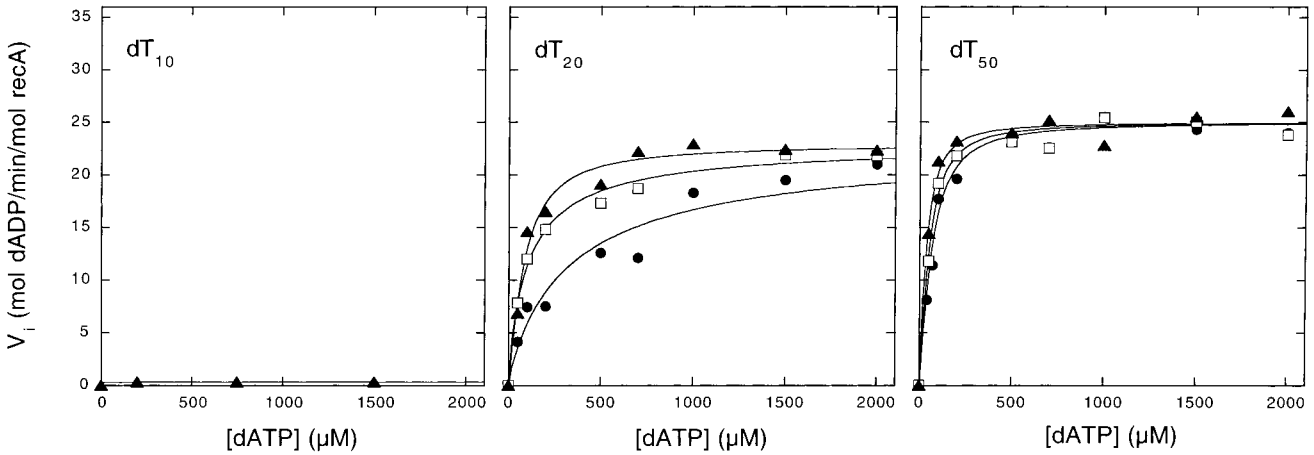


FIGURE 2: Effect of DNA length and concentration on the kinetics of dATP hydrolysis. The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 3 $\mu$M [H163W]RecA protein, the indicated concentrations of dATP, and 15 (●), 40 (□), or 75 $\mu$M (▲) $dT_{10}$, $dT_{20}$, or $dT_{50}$, as indicated. The reactions were carried out at 37 °C. The points represent the initial rates of dATP hydrolysis that were measured at the indicated concentrations of dATP. The solid lines were generated using the standard Hill equation and the following parameters: $V_{max} = 23$ min$^{-1}$, $S_{0.5} = 340$ $\mu$M, and $n = 0.9$ (15 $\mu$M $dT_{20}$); $V_{max} = 23$ min$^{-1}$, $S_{0.5} = 105$ $\mu$M, and $n = 0.9$ (40 $\mu$M $dT_{20}$); $V_{max} = 23$ min$^{-1}$, $S_{0.5} = 80$ $\mu$M, and $n = 1.3$ (75 $\mu$M $dT_{20}$); $V_{max} = 25$ min$^{-1}$, $S_{0.5} = 60$ $\mu$M, and $n = 1.4$ (15 $\mu$M $dT_{50}$); $V_{max} = 25$ min$^{-1}$, $S_{0.5} = 50$ $\mu$M, and $n = 1.4$ (40 $\mu$M $dT_{50}$); and $V_{max} = 25$ min$^{-1}$, $S_{0.5} = 40$ $\mu$M, and $n = 1.4$ (75 $\mu$M $dT_{50}$).

*dATP Hydrolysis.* The shortest $dT_n$ oligomer that could support dATP hydrolysis was $dT_{20}$ (Table 1). The dependence of dATP hydrolysis on dATP concentration was determined at a fixed concentration of RecA protein (3 $\mu$M) and concentrations of $dT_{20}$ ranging from 15 $\mu$M (1.5-fold excess relative to the RecA protein concentration) to 75 $\mu$M (7-fold excess relative to the RecA protein concentration). As shown in Figure 2, dATP hydrolysis reached the same maximal rate of approximately 23 min$^{-1}$ at each concentration of $dT_{20}$ examined, thus indicating that all of the RecA protein was bound to $dT_{20}$ and activated for dATP hydrolysis, even at the lowest concentration of $dT_{20}$. The $S_{0.5}$ for dATP varied with $dT_{20}$ concentration, however, ranging from a value of 340 $\mu$M at 15 $\mu$M $dT_{20}$ to 80 $\mu$M at 75 $\mu$M $dT_{20}$ (Figure 2). The Hill coefficients for the velocity curves ranged from 0.9 to 1.3, indicating that the dependence of the dATP hydrolysis reaction on dATP concentration was not significantly cooperative under these conditions. Under the same conditions, there was no dATP hydrolysis when $dT_{10}$ (75 $\mu$M) was used as the ssDNA effector (Figure 2).

The dependence of dATP hydrolysis on dATP concentration was also determined at a fixed concentration of RecA protein (3 $\mu$M) and concentrations of $dT_{50}$ ranging from 15 to 75 $\mu$M (Figure 2). In contrast to the results that were obtained with $dT_{20}$, both the maximal rate of dATP hydrolysis (25 min$^{-1}$) and the $S_{0.5}$ for dATP (40−60 $\mu$M) appeared to be independent of DNA concentration when $dT_{50}$ was used as the ssDNA effector. The Hill coefficients for the velocity curves were approximately 1.4, again indicating that the dependence of the dATP hydrolysis reaction on dATP concentration was not significantly cooperative under these reaction conditions. Similar results were obtained when either $dT_{100}$ or $dT_{200}$ was used as the ssDNA effector (data not shown).

*ATP Hydrolysis*. The shortest $dT_n$ oligomer that could support ATP hydrolysis was $dT_{50}$ (Table 1). The dependence of ATP hydrolysis on ATP concentration was determined at a fixed concentration of RecA protein (3 $\mu$M) and concentrations of $dT_{50}$ ranging from 15 to 75 $\mu$M. As shown in Figure 3, the rate of ATP hydrolysis reached the same maximal rate of approximately 21 min$^{-1}$ at each concentration of $dT_{50}$ that was examined, thus indicating that all of the RecA protein was bound to $dT_{50}$ and activated for ATP hydrolysis, even at the lowest concentration of $dT_{50}$. The $S_{0.5}$ for ATP varied with $dT_{50}$ concentration, however, ranging from a value of 220 $\mu$M at 15 $\mu$M $dT_{50}$ to 90 $\mu$M at 75 $\mu$M $dT_{50}$ (Figure 3). The Hill coefficients for the velocity curves ranged from 0.8 to 1.5, indicating that the dependence of the ATP hydrolysis reaction on ATP concentration was not significantly cooperative under these reaction conditions. Under the same conditions, there was no ATP hydrolysis when $dT_{20}$ (75 $\mu$M) was used as the ssDNA effector (Figure 3).

The dependence of ATP hydrolysis on ATP concentration was also determined at a fixed concentration of RecA protein (3 $\mu$M) and concentrations of $dT_{200}$ ranging from 15 to 75 $\mu$M. As shown in Figure 3, both the maximal rate of ATP hydrolysis (22 min$^{-1}$) and the $S_{0.5}$ for ATP (80−95 $\mu$M) appeared to be independent of DNA concentration when $dT_{200}$ was used as the ssDNA effector. The Hill coefficients for the velocity curves ranged from 1.2 to 1.5, again indicating that the dependence of the ATP hydrolysis reaction on ATP concentration was not significantly cooperative under these reaction conditions. Similar results were obtained when $dT_{100}$ was used as the ssDNA effector (data not shown).

Interestingly, the pattern of $dT_n$-dependent NTP hydrolysis kinetics that was obtained for ATP (Figure 3) was similar to that obtained for dATP (Figure 2), except that the $dT_n$ oligomer lengths at which no hydrolysis, DNA concentration-dependent hydrolysis, and DNA concentration-independent hydrolysis occurred were longer for ATP than for dATP.

*GTP Hydrolysis*. The shortest $dT_n$ oligomer that could support GTP hydrolysis was $dT_{200}$ (Table 1). The dependence of GTP hydrolysis on GTP concentration was determined at a fixed concentration of RecA protein (3 $\mu$M) and concentrations of $dT_{200}$ ranging from 15 to 75 $\mu$M. As shown in Figure 4, the rate of GTP hydrolysis approached a maximal rate of approximately 20 min$^{-1}$ with both 30 and 75 $\mu$M $dT_{200}$. The $S_{0.5}$ for GTP varied with $dT_{200}$ concentration, however, ranging from an estimated value of 6000 $\mu$M at 15 $\mu$M $dT_{200}$ (assuming a $V_{max}$ of 20 min$^{-1}$) to 1600 $\mu$M at 75 $\mu$M $dT_{200}$

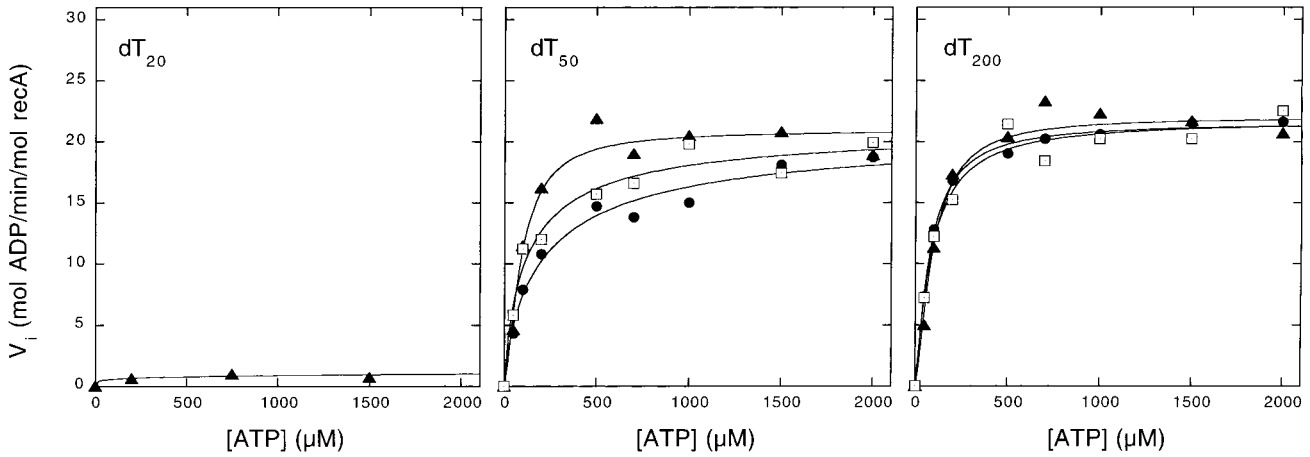


FIGURE 3: Effect of DNA length and concentration on the kinetics of ATP hydrolysis. The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 3 $\mu$M [H163W]RecA protein, the indicated concentrations of ATP, and 15 (●), 30 (□), or 75 $\mu$M (▲) $dT_{20}$, $dT_{50}$, or $dT_{200}$, as indicated. The reactions were carried out at 37 °C. The points represent the initial rates of ATP hydrolysis that were measured at the indicated concentrations of ATP. The solid lines were generated using the standard Hill equation and the following parameters: $V_{max} = 21$ min$^{-1}$, $S_{0.5} = 220$ $\mu$M, and $n = 0.8$ (15 $\mu$M $dT_{50}$); $V_{max} = 21$ min$^{-1}$, $S_{0.5} = 125$ $\mu$M, and $n = 0.8$ (30 $\mu$M $dT_{50}$); $V_{max} = 21$ min$^{-1}$, $S_{0.5} = 90$ $\mu$M, and $n = 1.5$ (75 $\mu$M $dT_{50}$); $V_{max} = 22$ min$^{-1}$, $S_{0.5} = 80$ $\mu$M, and $n = 1.3$ (15 $\mu$M $dT_{200}$); $V_{max} = 22$ min$^{-1}$, $S_{0.5} = 90$ $\mu$M, and $n = 1.2$ (30 $\mu$M $dT_{200}$); and $V_{max} = 22$ min$^{-1}$, $S_{0.5} = 95$ $\mu$M, and $n = 1.5$ (75 $\mu$M $dT_{200}$).

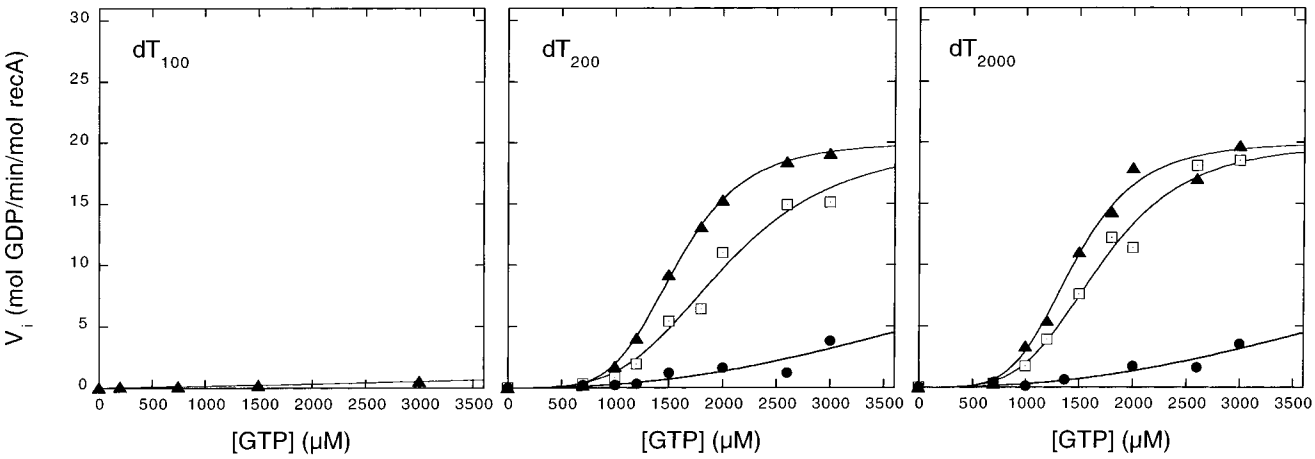


FIGURE 4: Effect of DNA length and concentration on the kinetics of GTP hydrolysis. The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 3 $\mu$M [H163W]RecA protein, the indicated concentrations of GTP, and either 15 (●), 30 (□), or 75 $\mu$M (▲) $dT_{100}$, $dT_{200}$, or $dT_{2000}$, as indicated. The reactions were carried out at 37 °C. The points represent the initial rates of GTP hydrolysis that were measured at the indicated concentrations of GTP. The solid lines were generated using the standard Hill equation and the following parameters: $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 6000$ $\mu$M, and $n = 2.4$ (15 $\mu$M $dT_{200}$); $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 2000$ $\mu$M, and $n = 3.8$ (30 $\mu$M $dT_{200}$); $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 1600$ $\mu$M, and $n = 5.0$ (75 $\mu$M $dT_{200}$); $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 6000$ $\mu$M, and $n = 2.4$ (15 $\mu$M $dT_{2000}$); $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 1700$ $\mu$M, and $n = 4.2$ (30 $\mu$M $dT_{2000}$); and $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 1400$ $\mu$M, and $n = 4.8$ (75 $\mu$M $dT_{2000}$).

(Figure 4). The Hill coefficients for the velocity curves that were obtained at 30 and 75 $\mu$M $dT_{200}$ were approximately 4−5, clearly indicating that the GTP hydrolysis reaction was cooperatively dependent on GTP concentration. Similar results were obtained when $dT_{2000}$ was used as the ssDNA effector (Figure 4), indicating that increasing the length of the ssDNA effector 10-fold does not eliminate the apparent dependence of the $S_{0.5}$ for GTP on ssDNA effector concentration. Under the same conditions, there was no GTP hydrolysis when $dT_{100}$ (75 $\mu$M) was used as the ssDNA effector (Figure 4).

### *GTP Hydrolysis by the [D100N]RecA Protein*

The results presented above suggested that the minimal size $dT_n$ oligomer that is able to serve as an effector for the RecA protein-catalyzed NTP hydrolysis reaction is related to the $S_{0.5}$ value of the NTP. Thus, GTP, which has the highest $S_{0.5}$ value of the three NTPs that were examined, was hydrolyzed in the presence of $dT_{200}$, but not in the presence of $dT_{100}$ (Table 1). ATP and dATP, in contrast, were hydrolyzed in the presence of either $dT_{100}$ or $dT_{200}$ (Table 1). To further explore the apparent correlation between the $S_{0.5}$ value of an NTP and the minimal ssDNA effector size, we examined the ssDNA-dependent GTP hydrolysis activity of our mutant [D100N]RecA protein (*6*). In the active site of the RecA protein, the negatively charged carboxylate side chain of Asp100 forms a hydrogen bond with the exocyclic 6-amino group of ATP (*7*). This same Asp100 side chain presumably interacts unfavorably with the 6-carbonyl group of GTP, and this unfavorable interaction is likely responsible for the elevated $S_{0.5}$ of GTP relative to that of ATP (Table 1). In the [D100N]RecA protein, however, the Asp100 group has been replaced with an uncharged Asn residue (*6*). This mutation significantly lowers the $S_{0.5}$ value

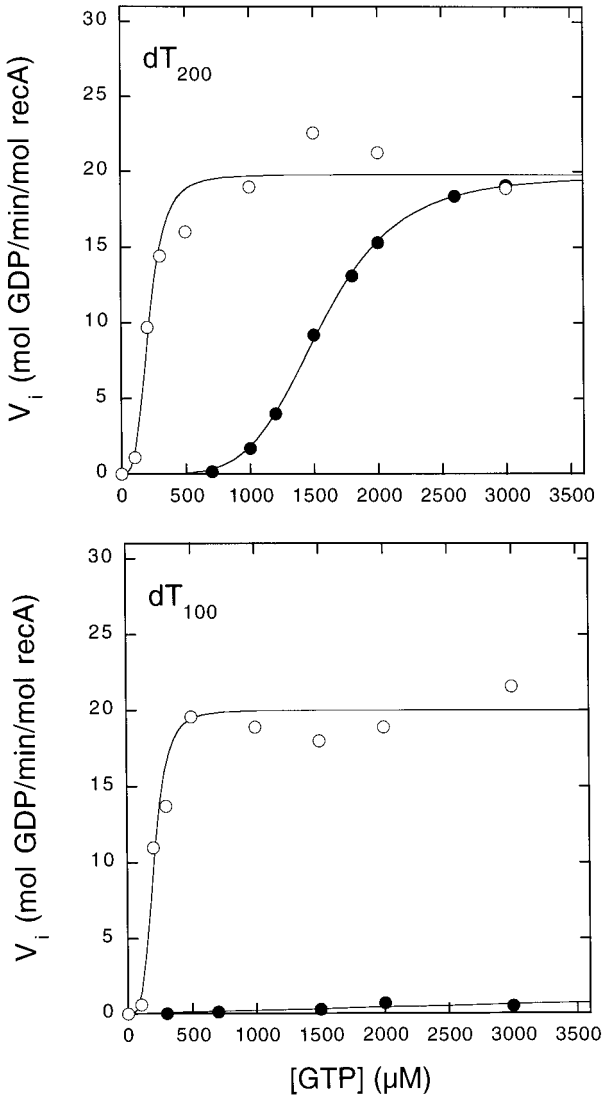


FIGURE 5: Effect of the D100N mutation on the DNA length dependence of the GTP hydrolysis reaction. The reaction solutions contained 25 mM Tris-HCl (pH 7.5), 5% glycerol, 1 mM DTT, 10 mM $MgCl_2$, 3 $\mu$M [H163W]RecA protein (●) or [D100N]RecA protein (○), the indicated concentrations of GTP, and 75 $\mu$M $dT_{100}$ or $dT_{200}$, as indicated. The reactions were carried out at 37 °C. The points represent the initial rates of GTP hydrolysis that were measured at the indicated concentrations of GTP. The solid lines were generated using the standard Hill equation and the following parameters: $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 215$ $\mu$M, and $n = 3.5$ ($dT_{200}$/[D100N]RecA protein); $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 1600$ $\mu$M, and $n = 5.1$ ($dT_{200}$/[H163W]RecA protein); $V_{max} = 20$ min$^{-1}$, $S_{0.5} = 205$ $\mu$M, and $n = 4.0$ ($dT_{100}$/[D100N]RecA protein); and no reaction ($dT_{100}$/[H163W]RecA protein).

for GTP. For example, the $S_{0.5}$ that was obtained for GTP with $dT_{200}$ (75 $\mu$M) as the ssDNA effector was 1600 $\mu$M for the RecA protein, but only 215 $\mu$M for the [D100N]RecA protein (Figure 5; note that the mutation has no effect on the $V_{max}$ for GTP hydrolysis).

As shown in Figure 5, the RecA protein catalyzed GTP hydrolysis with a maximal rate of approximately 20 min$^{-1}$ in the presence of $dT_{200}$, but exhibited no GTP hydrolysis activity in the presence of $dT_{100}$. The [D100N]RecA protein, in contrast, catalyzed GTP hydrolysis with a maximal rate of 20 min$^{-1}$ in the presence of either $dT_{100}$ or $dT_{200}$ (Figure 5). These results support the conclusion that the minimal size $dT_n$ oligomer that is able to serve as an effector for the RecA protein-catalyzed NTP hydrolysis reaction is determined by the $S_{0.5}$ value of the NTP being hydrolyzed.

## DISCUSSION

Our results indicate that there are three distinct manifestations of the NTP hydrolysis activity of the RecA protein, depending on the length of the $dT_n$ oligomer that is provided as the ssDNA effector. With longer $dT_n$ oligomers, NTP hydrolysis occurs with a turnover number of 20−25 min$^{-1}$ and the observed $S_{0.5}$ value for the NTP is independent of the concentration of the $dT_n$ oligomer (DNA concentration-independent hydrolysis). With $dT_n$ oligomers of intermediate length, NTP hydrolysis still occurs with a turnover number of 20−25 min$^{-1}$, but the observed $S_{0.5}$ for the NTP decreases with increasing $dT_n$ concentration until reaching a value similar to that obtained with the longer $dT_n$ oligomers (DNA concentration-dependent hydrolysis). With shorter $dT_n$ oligomers, the NTP hydrolysis activity is effectively eliminated. Although this general progression of kinetic behavior was observed for the three structurally related NTPs (dATP, ATP, and GTP), the $dT_n$ oligomer length at which DNA concentration-independent, DNA concentration-dependent, and no NTP hydrolysis was observed depended on the NTP being considered.

An inspection of our results suggests that the minimal size $dT_n$ oligomer that is able to serve as an effector for the RecA protein-catalyzed NTP hydrolysis reaction depends on the $S_{0.5}$ value of the NTP being hydrolyzed (Table 1). Thus, dATP (which has the lowest $S_{0.5}$ value of the NTPs that were examined) is hydrolyzed in the presence of $dT_{20}$; ATP (which has the intermediate $S_{0.5}$ value) is hydrolyzed in the presence of $dT_{50}$, and GTP (which has the highest $S_{0.5}$ value) requires $dT_{200}$ for hydrolysis. These results indicate that the higher the $S_{0.5}$ value is for a given NTP, the longer the $dT_n$ oligomer has to be to function as an effector for the hydrolysis of that NTP. This conclusion is supported by our results which showed that when the $S_{0.5}$ for GTP was reduced by replacing Asp100 in the active site of the RecA protein with Asn (Figure 5), the miminal $dT_n$ oligomer size required for GTP hydrolysis was reduced from $dT_{200}$ to $dT_{100}$.

To determine the basis for the apparent correlation between the $S_{0.5}$ of an NTP and the minimal ssDNA effector size, it is useful to first consider the NTP hydrolysis reactions that occur under DNA concentration-dependent conditions. A minimal kinetic mechanism which can account for the dependence of the $S_{0.5}$ for an NTP on the ssDNA effector concentration is shown in Scheme 1, where E represents the RecA protein, $dT_n$ represents the ssDNA effector, $K_{DNA}$ is the dissociation constant for the RecA−$dT_n$ complex, $k_p$ is the rate constant for NTP hydrolysis, and $S_{0.5}$ is the intrinsic dissociation constant for NTP from the RecA−DNA−NTP complex.

Scheme 1

$$E + dT_n \underset{K_{DNA}}{\rightleftharpoons} E \cdot dT_n + NTP \underset{S_{0.5}}{\rightleftharpoons} E \cdot dT_n \cdot NTP \xrightarrow{k_p} NDP + P_i$$

In this mechanism, the observed $S_{0.5}$ value [$S_{0.5}$(obs)] for an NTP will vary with DNA concentration because the concentration of DNA determines the fraction of the RecA protein that will be in a form of a RecA−$dT_n$ complex (and

capable of combining with NTP and catalyzing NTP hydrolysis). The general steady-state expression for the dependence of $S_{0.5}$(obs) on DNA concentration according to the mechanism in Scheme 1 is given by eq 1:

$$S_{0.5}(\text{obs}) = S_{0.5}(1 + K_{\text{DNA}}/[\text{DNA}]) \quad (1)$$

To more rigorously evaluate the compatability of this minimal model with our experimental results, however, we used the kinetics simulation program KINSIM (*8*) to generate the time courses of NTP hydrolysis that would be predicted by Scheme 1 at different DNA and NTP concentrations, and with various values of $k_p$, $K_{\text{DNA}}$, and $S_{0.5}$. The rates of each of the simulated NTP hydrolysis reactions were then plotted as a function of NTP concentration to generate a series of simulated velocity curves analogous to the experimental velocity curves shown in Figures 2−4. These simulated velocity curves were then used to calculate the $S_{0.5}$(obs) values that would be predicted by the mechanism in Scheme 1 for each NTP at different concentrations of DNA.

The $S_{0.5}$(obs) values that were calculated from Scheme 1 for the $dT_{20}$-dependent dATP hydrolysis reaction are shown in Figure 6. The close agreement between the calculated values, which were generated using an intrinsic $S_{0.5}$ for dATP of 35 $\mu$M and a $K_{\text{DNA}}$ for $dT_{20}$ of 70 $\mu$M, and the experimentally determined $S_{0.5}$(obs) values indicates that the mechanism in Scheme 1 is sufficient to account for the dependence of the $S_{0.5}$(obs) for dATP on $dT_{20}$ concentration. The $S_{0.5}$(obs) values that were calculated for the $dT_{50}$-dependent dATP hydrolysis reaction are also shown in Figure 6. Again, the close agreement between the calculated and experimental $S_{0.5}$ values indicates that the mechanism in Scheme 1 is sufficient to account for the dependence of the $S_{0.5}$(obs) for dATP on $dT_{50}$ concentration. In this case, however, although the intrinsic $S_{0.5}$ for dATP of 35 $\mu$M was identical to that used for the $dT_{20}$-dependent reaction, the best fits were obtained with a $K_{\text{DNA}}$ for $dT_{50}$ of approximately $\leq$1 $\mu$M. Because the $K_{\text{DNA}}$ for $dT_{50}$ was much lower than that for $dT_{20}$ (such that the RecA protein is completely in the form of a RecA−$dT_{50}$ complex even at the lowest $dT_{50}$ concentration examined), the $S_{0.5}$(obs) for dATP appears to be independent of DNA concentration when $dT_{50}$ (or a longer $dT_n$ oligomer) is used as the ssDNA effector (and is approximately equal to the intrinsic $S_{0.5}$ for dATP). Conversely, the $K_{\text{DNA}}$ for the shorter oligomer, $dT_{10}$, may be much larger than that for $dT_{20}$ (such that an insignificant amount of the RecA−$dT_{10}$ complex exists under our reaction conditions). In this case, little or no dATP hydrolysis would be detected, even at the highest dATP concentration that was examined, when $dT_{10}$ is used as the ssDNA effector (Figure 2).

The $S_{0.5}$(obs) values that were calculated from Scheme 1 for the $dT_{50}$-dependent ATP hydrolysis reaction are shown in Figure 6. The close agreement between the calculated values, which were generated using an intrinsic $S_{0.5}$ for ATP of 70 $\mu$M and a $K_{\text{DNA}}$ for $dT_{50}$ of 25 $\mu$M, and the experimentally determined $S_{0.5}$(obs) values indicates that the mechanism in Scheme 1 is sufficient to account for the dependence of the $S_{0.5}$(obs) for ATP on $dT_{50}$ concentration. The $S_{0.5}$(obs) values that were calculated for the $dT_{200}$-dependent ATP hydrolysis reaction are also shown in Figure 6. Again, the close agreement between the calculated and

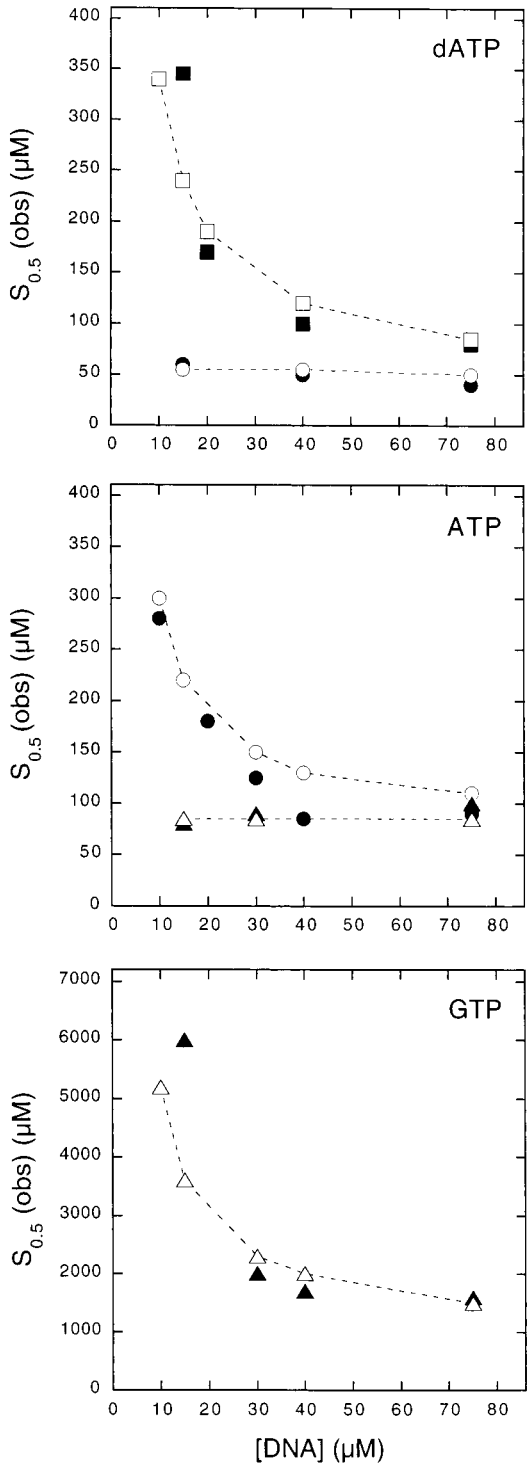


FIGURE 6: Dependence of the $S_{0.5}$(obs) for NTP hydrolysis on DNA length and concentration. The $S_{0.5}$(obs) values that were derived from the velocity curves shown in Figures 2−4 and additional curves not shown (black symbols) and $S_{0.5}$(obs) values that were calculated from the kinetic mechanism depicted in Scheme 1 (white symbols) are plotted as a function of $dT_n$ concentration: $dT_{20}$ (□ and ■), $dT_{50}$ (○ and ●), and $dT_{200}$ (△ and ▲). The calculated $S_{0.5}$(obs) values were generated using the following parameters: $S_{0.5}$ = 35 $\mu$M and $K_{\text{DNA}}$ = 70 $\mu$M (dATP/$dT_{20}$); $S_{0.5}$ = 35 $\mu$M and $K_{\text{DNA}}$ = 1 $\mu$M (dATP/$dT_{50}$); $S_{0.5}$ = 70 $\mu$M and $K_{\text{DNA}}$ = 25 $\mu$M (ATP/$dT_{50}$); $S_{0.5}$ = 70 $\mu$M and $K_{\text{DNA}}$ = 1 $\mu$M (ATP/$dT_{200}$); and $S_{0.5}$ = 1200 $\mu$M and $K_{\text{DNA}}$ = 30 $\mu$M (GTP/$dT_{200}$).

experimental $S_{0.5}$ values indicates that the mechanism in Scheme 1 is sufficient to account for the dependence of the

$S_{0.5}$(obs) for ATP on $dT_{200}$ concentration. In this case, however, although the intrinsic $S_{0.5}$ for ATP of 70 $\mu$M was identical to that used for the $dT_{50}$-dependent reaction, the best fits were obtained with a $K_{DNA}$ for $dT_{200}$ of approximately $\leq 1$ $\mu$M. Because the $K_{DNA}$ for $dT_{200}$ was much lower than that for $dT_{50}$ (such that the RecA protein is completely in the form of a RecA−$dT_{200}$ complex even at the lowest $dT_{200}$ concentration that was examined), the $S_{0.5}$-(obs) for ATP appears to be independent of DNA concentration when $dT_{200}$ is used as the ssDNA effector (and is approximately equal to the intrinsic $S_{0.5}$ for ATP). Conversely, the $K_{DNA}$ for the shorter oligomer, $dT_{20}$, may be much larger than that for $dT_{50}$ (such that an insignificant amount of the RecA−$dT_{20}$ complex exists under our reaction conditions). In this case, little or no ATP hydrolysis would be detected, even at the highest ATP concentration that was examined, when $dT_{20}$ is used as the ssDNA effector (Figure 3).

It is significant that the $K_{DNA}$ that was determined for $dT_{50}$ from the kinetics of the ATP hydrolysis reaction was approximately 25 $\mu$M, whereas the kinetics of the dATP hydrolysis reaction indicated that the $K_{DNA}$ for $dT_{50}$ was $\leq 1$ $\mu$M. These results imply that the $K_{DNA}$ for a given $dT_n$ oligomer depends on the specific NTP whose hydrolysis is being measured. This finding indicates that although the minimal mechanism in Scheme 1 is sufficient to account for the kinetic patterns that were observed for the NTP hydrolysis reactions, it must be overly simplistic in mechanistic detail. In particular, although the RecA protein is depicted in Scheme 1 as an isolated monomer, it actually binds to ssDNA as a polymeric filament of interacting monomers. Thus, to more clearly understand the molecular basis for our kinetic results, it is necessary to consider the mechanism by which the RecA protein dissociates from the polymeric RecA−ssDNA complex and how this might affect the observed value of the apparent dissociation constant, $K_{DNA}$. Cox and co-workers have shown that RecA protein dissociates from ssDNA by an end-dependent process in which RecA monomers dissociate preferentially from the 5′-end of the RecA−ssDNA filament. RecA protein monomers can reassemble onto the ssDNA by a process that involves a rate-limiting nucleation and a subsequent rapid polymerization of additional monomers in a 5′ → 3′ direction on the ssDNA (*9*, *10*). These investigators have further shown that although RecA dissociation occurs in the presence of ATP, no RecA dissociation is observed in the presence of dATP (*9*, *10*). Although the mechanistic basis for the differential effects of ATP and dATP on filament disassembly was not determined, their observations are consistent with our results which indicate that the affinity of the RecA protein for $dT_{50}$ is much lower in the presence of ATP (apparent $K_{DNA} = 25$ $\mu$M) than in the presence of dATP (apparent $K_{DNA} \leq 1$ $\mu$M).

The end-dependent nature of RecA protein filament assembly and disassembly may also account for the effect of ssDNA length on the kinetics of the NTP hydrolysis reaction. To maintain a steady-state rate of NTP hydrolysis, it is presumably necessary that any dissociation of RecA monomers from the 5′-end of the filament be countered by the rebinding of RecA monomers to ssDNA. The relative efficiencies of RecA protein dissociation and rebinding may vary depending on the length of the ssDNA effector. Dissociation may be less favored when a longer ssDNA is used as an effector because there will potentially be fewer filament ends from which dissociation can occur. Moreover, the dissociation of RecA monomers may be more easily offset by RecA reassociation when a longer ssDNA effector is used because each rate-limiting RecA nucleation step can potentially result in the rapid polymerization of a larger number of RecA monomers onto the ssDNA. Because the rate of end-dependent RecA dissociation depends on the identity of the NTP (*9*, *10*), the actual length of the ssDNA effector that would be required to maintain a stable rate of NTP hydrolysis will depend on the NTP being hydrolyzed. Thus, dATP (which suppresses end-dependent dissociation) is hydrolyzed with $dT_{20}$ as the ssDNA effector, whereas ATP (which permits end-dependent dissociation) is not hydrolyzed at an appreciable rate in the presence of $dT_{20}$. Alternatively, although our discussion thus far assumes that RecA monomers assemble onto separate individual $dT_n$ molecules, it is conceivable that multiple $dT_n$ molecules may bind to a single filament of RecA protein. For example, although dATP is hydrolyzed more efficiently with $dT_{50}$ as the ssDNA effector (DNA concentration-independent conditions; apparent $K_{DNA} < 1$ $\mu$M), it is possible that at high concentrations multiple $dT_{20}$ molecules could align end to end in a single RecA filament and thereby activate the dATP hydrolysis activity of the monomers within that filament (DNA concentration-dependent conditions; apparent $K_{DNA} = 70$ $\mu$M). However, since $dT_{20}$ is unable to activate the RecA protein for ATP hydrolysis, it is clear that the implementation of an end-to-end $dT_n$ alignment mechanism (if such a mechanism does exist) must somehow depend on the identity of the NTP being hydrolyzed. A definitive molecular explanation for the DNA length dependence of the NTP hydrolysis activity of the RecA protein will require a comprehensive structural analysis of the complexes that are formed by the RecA protein with different $dT_n$ oligomers in the presence of various nucleotide cofactors.

Because of the pronounced cooperative dependence on GTP concentration, the GTP hydrolysis reaction could not be fit as closely by the minimal mechanism in Scheme 1 as were the dATP and ATP hydrolysis reactions (which did not exhibit a significantly cooperative dependence on NTP concentration). However, the agreement between the calculated $S_{0.5}$(obs) values, which were generated from Scheme 1 using an intrinsic $S_{0.5}$ for GTP of 1200 $\mu$M and a $K_{DNA}$ for $dT_{200}$ of 30 $\mu$M, and the experimentally determined $S_{0.5}$(obs) values indicates that the mechanism depicted in Scheme 1 is sufficient (at least to a first approximation) to account for the general dependence of the $S_{0.5}$(obs) for GTP on $dT_{200}$ concentration (Figure 6). As noted above, however, the $K_{DNA}$ that was determined for $dT_{200}$ in the presence of ATP was approximately $\leq 1$ $\mu$M. This difference in $K_{DNA}$ values suggests that the rate of RecA protein dissociation in the presence of GTP may be greater than that which occurs in the presence of ATP. This conclusion is consistent with the observation that full ATP hydrolysis activity is observed with $dT_{50}$ as the ssDNA effector, whereas GTP hydrolysis is not detected with $dT_n$ oligomers shorter than $dT_{200}$ (Table 1).

The results discussed above suggest that the rate of end-dependent RecA protein dissociation and the minimal ssDNA effector length for the NTP hydrolysis reaction will depend on the intrinsic $S_{0.5}$ value of the NTP being hydrolyzed. The proposed correlation between the $S_{0.5}$ value and ssDNA

effector length is further supported by preliminary studies in which we examined the effect of pH on the kinetics of the NTP hydrolysis reaction. We have shown previously that the $S_{0.5}$ value of a given NTP can decrease as much as 2−3-fold when the pH of the reaction solution is reduced from pH 7.5 (standard conditions) to pH 6.5 (*3*, *11*). Accordingly, although GTP is not hydrolyzed by the RecA protein in the presence of $dT_{100}$ at pH 7.5 (Figure 4), we have found that it is hydrolyzed with a maximal rate of 20 $min^{-1}$ in the presence of $dT_{100}$ at pH 6.5 (unpublished results). Similarly, although ATP is not hydrolyzed by the RecA protein in the presence of $dT_{20}$ at pH 7.5 (Figure 3), we have found that it is hydrolyzed in the presence of $dT_{20}$ at pH 6.5 (unpublished results). We are currently exploring the effect of pH on the RecA protein-catalyzed NTP hydrolysis reaction in more detail to more clearly elucidate the nature of the correlation between the $S_{0.5}$ value and filament stability. Interestingly, Cox and co-workers have noted that end-dependent RecA filament disassembly can be suppressed by either (i) substituting dATP for ATP (as discussed above) or (ii) decreasing the pH from 7.5 to 6.5 (*9*, *10*). Our results suggest that in both cases, the observed suppression of disassembly may occur because the altered experimental conditions serve to decrease the $S_{0.5}$ value of the NTP in the reaction system.

Although the minimal kinetic mechanism in Scheme 1 provides a useful framework for correlating the kinetics of NTP hydrolysis with the apparent stability of the RecA−ssDNA complex, it does not take into account the complexities of the RecA protein-catalyzed NTP hydrolysis reaction that arises as a result of cooperative interactions between individual RecA monomers within the polymeric RecA−ssDNA complex. Furthermore, in previous studies, we identified a linkage between the $S_{0.5}$ value of an NTP and the conformational state of the RecA−ssDNA complex (*3*, *6*, *11*). These studies indicated that an NTP must have an $S_{0.5}$ value of approximately $\leq 100$ $\mu$M to stabilize the strand exchange-active conformation of the RecA−ssDNA complex. For example, although ATP and GTP are hydrolyzed by the RecA protein with essentially identical turnover numbers (20 $min^{-1}$), ATP ($S_{0.5} = 70$ $\mu$M) functions as a cofactor for the strand exchange reaction, whereas GTP ($S_{0.5} = 1200$ $\mu$M) is inactive as a strand exchange cofactor (*3*). Thus, the pronounced difference in the minimal length of the ssDNA effector that is required for the GTP hydrolysis reaction compared to those required for the ATP and dATP hydrolysis reactions may not be due simply to the higher $S_{0.5}$ value of GTP, but also to an inability of GTP to support the isomerization of the RecA−ssDNA complex to a more stable strand exchange-active conformational state. Studies to further define the relationship between the $S_{0.5}$ of an NTP and the conformational state and stability of the RecA−ssDNA complex are in progress.

## NOTE ADDED AFTER ASAP POSTING

As a result of an error during processing of the manuscript, many italic characters were made boldface in the version released ASAP on 8/23/01. The correct version was posted 09/11/01.

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