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RecA Protein Has Extremely High Cooperativity for Substrate in Its ATPase Activity[1]

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The single-stranded DNA-dependent ATPase activity of *Escherichia coli* RecA protein, especially its cooperativity for ATP, was investigated. To measure the ATPase activity in detail, the methods and reaction conditions for the ATPase assay were reexamined. Under conditions where RecA protein always showed a maximal rate of ATP hydrolysis, its poly(dT)-dependent ATPase activity was measured. At 25°C, increasing the concentration of RecA protein from 0.3 to 1.0 μM increased the turnover number (k_{cat}) from 0.16 to 0.19 s^{-1} and the Hill coefficient (n_H) for ATP from 9.3 to 11.6. At 0.5 μM RecA protein, increasing the temperature from 25 to 37°C increased k_{cat} from 0.18 to 0.35 s^{-1} but decreased n_H from 9.8 to 6.6. Interestingly, the ATPase activity of RecA protein measured in this study showed much higher cooperativity for ATP than those reported to date. Furthermore, the n_H value of 11.6 for ATP obtained here was the highest of any ATPase reported so far. These results suggest that the binding of an ATP molecule to a RecA molecule within a nucleoprotein helical filament causes structural change of many other neighboring RecA molecules. This implies that ATP binding induces structural change of the whole nucleoprotein helical filament. Finally, we demonstrated that analysis of cooperativity is useful for revealing how a protein composed of many subunits functions as a whole.

Key words: ATPase, cooperativity, DNA-binding, RecA protein, recombination.

RecA protein is essential for homologous genetic recombination, recombinational DNA repair and induction of the SOS response to DNA damage (*1-3*). These functions are related to the multiple activities of RecA protein: it promotes a strand exchange reaction between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) or between two dsDNAs, catalyzes DNA-dependent ATP hydrolysis and facilitates the autocatalytic cleavage of LexA and related repressors. *Escherichia coli* RecA protein is relatively small, with a molecular weight of approximately 37,800 (*4, 5*). However, the active form in RecA protein-mediated reactions is a nucleoprotein filament composed of a helical protein array that binds to ssDNA or dsDNA.

The recombination reaction of RecA protein is accompanied by ATP hydrolysis, which is DNA-dependent. However, it has been demonstrated that a non-hydrolyzable ATP analogue, ATPγS or ADP·AlF_4, can initiate the recombination reaction under certain conditions (*6-8*). Since the same conformational changes in the nucleoprotein filament are induced by the binding of either ATP or ATPγS (*9*), it has been suggested that the RecA protein-mediated recombination reaction requires the allosteric transition induced by ATP binding, but not the energy released by ATP hydrolysis (*8*). This suggestion is well consistent with the observation that a mutant RecA protein in which lysine at position 72 is substituted by arginine retains the ability to promote exchange of DNA strands despite a drastic decrease of ATPase activity (*10*). For these reasons, it has been suggested that one of the functions of ATP hydrolysis is to facilitate the dissociation of RecA protein from the products of DNA strand exchange and to recycle RecA protein. Studies on the differences between the recombination reactions with ATPγS and ATP have suggested that one of the functions of RecA-mediated ATP hydrolysis is to bypass structural barriers in one or both DNA substrates and to facilitate reciprocal DNA strand exchange (*11, 12*). In the same way, it has been suggested that hydrolysis of ATP induces unidirectional DNA strand exchange and extends the formation of hybrid DNA (*13*). However, these suggestions were made only on the basis of functional aspects of the RecA-mediated ATP hydrolysis. Therefore, it has not yet been elucidated conclusively at the molecular level how RecA protein uses the energy released by ATP hydrolysis.

Detailed kinetic studies of ATPase activity may be useful for clarifying this problem. Although many groups have carried out kinetic studies of the ATPase activity of the RecA protein, the kinetic parameters obtained, such as K_m and k_{cat}, differed (*3, 14*). This was due to differences in the

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Abbreviations: CTssDNA, ssDNA prepared from calf thymus dsDNA by thermal denaturation; dsDNA, double-stranded DNA; ϵDNA, etheno-modified ssDNA; ssDNA, single-stranded DNA.

conditions of measurement, such as the concentrations of protein, salt and DNA, kinds of DNA, temperature and pH, since these factors are known to affect the catalytic activity of RecA protein (*15-17*). Since it is thus inappropriate to compare the data of different studies, it is necessary to reexamine the effects of these factors in detail and carry out measurements under unified conditions.

Another noteworthy feature of kinetic analyses reported so far is their disregard for ATP cooperativity. The RecA protein has been reported to show relatively high cooperativity for ATP (*16, 18*), as often represented by the Hill coefficient (designated n_H). Since the active form is the nucleoprotein filament, study of the cooperativity should help to clarify the RecA-mediated reaction. However, in most of the studies reported so far, Hill coefficients were not determined.

In this study, we established conditions for measurement of the ATPase activity of *E. coli* RecA protein and studied its kinetic reaction, focusing especially on its cooperativity for ATP.

EXPERIMENTAL PROCEDURES

Materials—The following enzymes, reagents, and chromatographic materials were employed: poly(dT) with an average length of 3,600 bases, calf thymus DNA (type II) and pyruvate kinase (type II) from Sigma; lactate dehydrogenase (grade II) from Toyobo; poly(dT) with an average length of 170 bases, poly(dC) and poly(dA) from Pharmacia Fine Chemicals. All other chemicals and reagents used were purchased from commercial sources.

E. coli wild-type RecA protein was purified and stored as described previously and its concentration was determined using a molar extinction coefficient at 278 nm, ε_{278}, of 2.15×10^4 $M^{-1}\cdot cm^{-1}$ (*19*). Etheno-modified calf thymus ssDNA (εDNA) was prepared as described in the literature (*20, 21*), with minor modifications, and its concentration was determined using an ε_{260} value of 8.33×10^3 $M^{-1}\cdot cm^{-1}$. M13mp19 ssDNA was prepared as described previously (*20*). The concentrations of M13mp19 ssDNA and calf thymus dsDNA were determined by absorbance at 260 nm, with $A_{1cm}^{1mg/ml}=27.8$ and 20 as conversion factors, respectively (*22*). Heat-denatured calf thymus DNA (CTssDNA) was prepared as described previously and its concentration was calculated from the intact dsDNA (*23*). The residue concentration of synthetic deoxyribonucleic acids was determined from the molar extinction coefficients at the absorption maximum near 260 nm at neutral pH: 8,600 $M^{-1}\cdot cm^{-1}$ at 257 nm for poly(dA) (*24*), 7400 $M^{-1}\cdot cm^{-1}$ at 268 nm for poly(dC) (*25*), and 8,520 $M^{-1}\cdot cm^{-1}$ at 264 nm for poly(dT) (*25*). Their average lengths were about 300 bases for poly(dA), 320 bases for poly(dC), and 170 or 3,600 bases for poly(dT).

ATPase Assay—Hydrolysis of ATP by RecA protein was measured by an enzyme-coupled spectrophotometric assay (*26*), with some modifications. The assay was performed in 50 mM Tris-HCl containing 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 2 mM phosphoenolpyruvate, 0.32 mM NADH, and 25 units/ml each of pyruvate kinase and lactate dehydrogenase, pH 7.5, at 25°C. The activity was measured as the change in absorbance in a 1-cm path length cell at steady state, and rates of ATP hydrolysis were calculated from $-A_{340}$ per second data using an extinction coefficient of ε_{340} value of 6.22 $mM^{-1}\cdot cm^{-1}$ for NADH.

Spectroscopy—Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, model MPF-4. The absorbance was measured with a Hitachi spectrophotometer, model U-3000.

Analysis of Enzymatic Kinetics of ATPase Activity—The hydrolysis of ATP by RecA protein would be expected to obey Eq. 1,

$$RecA_m + n\text{ATP} \overset{K_m{}^n}{\rightleftarrows} RecA_m\cdot ATP_n \xrightarrow{k_{cat}} RecA_m + n\text{ADP} + nP_i \quad (1)$$

where $RecA_m$ represents the nucleoprotein filament of RecA protein and $K_m{}^n(M^n)$ is the apparent dissociation constant for the fast binding step of nATP. k_{cat} is the rate constant (s^{-1}) for the rate-determining step of conversion of $RecA_m\cdot ATP_n$ into $RecA_m$, nADP and nP_i. The rate constant per monomeric RecA protein (k_{app}) at a given concentration of ATP is expressed by Eq. 2 (Hill equation):

$$k_{app} = \frac{k_{cat}[\text{ATP}]^n}{K_m{}^n + [\text{ATP}]^n}. \quad (2)$$

All kinetic parameters, K_m, k_{cat}, and n_H, were determined by fitting Eq. 2 to the data. A computer program, Igor (WaveMetrics), was used for calculation of parameters.

RESULTS

Examination of ssDNA Concentration on ATPase Assay—To analyze the ATPase activity of RecA protein in detail, a new ATPase assay method was devised as follows: (1) In order to monitor the ATP hydrolysis spectrophotometrically in real time and to keep the ATP concentration constant, an enzyme-coupled spectrophotometric assay was employed. (2) Since salt concentration greatly affects the ATPase activity of RecA protein, 100 mM KCl was added to the reaction mixture in order to rule out any influence of DNA and ATP as salts. (3) In order to observe ATP hydrolysis with high precision, the measurement temperature was 25°C, where the rate of hydrolysis is slower than that at 37°C. In the presence of excess ATP (1 mM), the DNA concentration required to reach the maximal rate of ATP hydrolysis by RecA protein (1 μM) was examined for each ssDNA (Fig. 1). Each point in Fig. 1 was measured at steady state, where the rate of ATP hydrolysis was kept constant (see Fig. 2). When synthetic homopolymers were used, their residue concentrations required to reach maximum ATPase activity were about 16 times that of the RecA protein for poly(dA), 8 times for poly(dC), and 12 times for poly(dT) (Fig. 1a). Further addition of homopolymer produced no further change in their activities. When natural deoxyribonucleic acids, M13ssDNA and CTssDNA, were used, both rates of hydrolysis were saturated above a DNA concentration 160 times that of the protein (Fig. 1b). These results indicated that the DNA concentration necessary for the maximum rate of ATP hydrolysis by RecA protein varied according to the kind of ssDNA. In the following experiments, the concentrations of ssDNA were set at 20 times that of the protein for poly(dC) and poly(dT), 40 times for poly(dA), and 200 times for CTssDNA and M13ssDNA, at which RecA protein showed maximum ATPase activity.

Figure 1 also shows that the maximal rates of ATP hydrolysis with the respective ssDNAs differed from one another. The order of stimulating ability was: poly(dC)>

poly(dT) > CTssDNA > M13ssDNA > poly(dA). This result suggests that the kind of DNA affects the catalytic efficiency of ATP hydrolysis by RecA protein. Since these DNAs differed from one another not only in composition but also in length, as described in "EXPERIMENTAL PROCEDURES," the dependence of ATPase activity on concentration and the kind of DNA may have been due to the length of DNA employed. Therefore, measurements were carried out using two poly(dT)s with average lengths of 170 and 3,600 bases (Fig. 1). Since both DNAs gave almost the same results, the above differences among the various DNAs were not due to the differences in their length, at least within the range examined.

Examination of the Kind of ssDNA on ATPase Assay— In the presence of ssDNA at the concentration described above and with 1 mM ATP, the ATPase activity of RecA protein with each ssDNA was measured until it reached steady state (Fig. 2). The activity varied with the kind of ssDNA. With regard to the ATPase activity with poly(dC) or poly(dT), the lag phase until the rate of ATP hydrolysis reached steady state was less than 30 s, whereas a long lag was observed when ssDNAs other than poly(dC) and poly(dT) were used (Fig. 2b). In particular, it took about 3 h to reach a steady state with poly(dA) (data not shown).

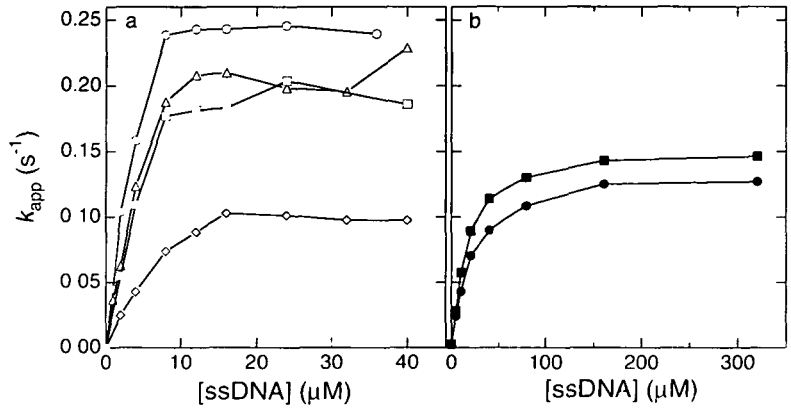

Fig. 1. **Concentration of DNA required to reach the maximum rate of ATP hydrolysis by RecA protein.** a: For synthetic homopolymers. b: For natural deoxyribonucleic acids. ATPase assays were performed in 50 mM Tris-HCl containing 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 2 mM phosphoenolpyruvate, 0.32 mM NADH, and 25 units/ml each of pyruvate kinase and lactate dehydrogenase, pH 7.5, at 25°C as described in "EXPERIMENTAL PROCEDURES." The reaction mixture contained 1 mM ATP, 1 μM RecA protein, and the indicated concentration of ssDNA. Symbols: open circles, poly(dC) with average length of 320 bases; open triangles, poly(dT) with average length of 3,600 bases; open squares, poly(dT) with average length of 170 bases; open diamonds, poly(dA) with average length of 300 bases; closed squares, CTssDNA; closed circles, M13ssDNA.

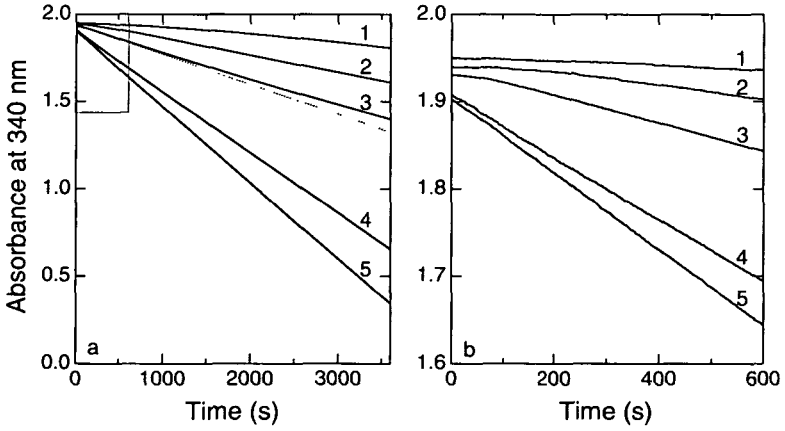

Fig. 2. **ATPase activities of the RecA protein with each ssDNA.** a: The reaction was started by addition of RecA protein to the reaction mixture, and after 30 s ATP hydrolysis was monitored at 340 nm. The concentration of ssDNA was 6 μM for poly(dC) and poly(dT), 12 μM for poly(dA), and 60 μM for CTssDNA and M13ssDNA. The reaction mixture contained 1 mM ATP, 0.3 μM RecA protein, and each ssDNA. The ssDNAs used for ATPase assays were: 1, poly(dA); 2, M13ssDNA; 3, CTssDNA; 4, poly(dT); 5, poly(dC). The dotted line is the initial slope for CTssDNA between 300 and 600 s. b: Enlargement of the boxed area in a.

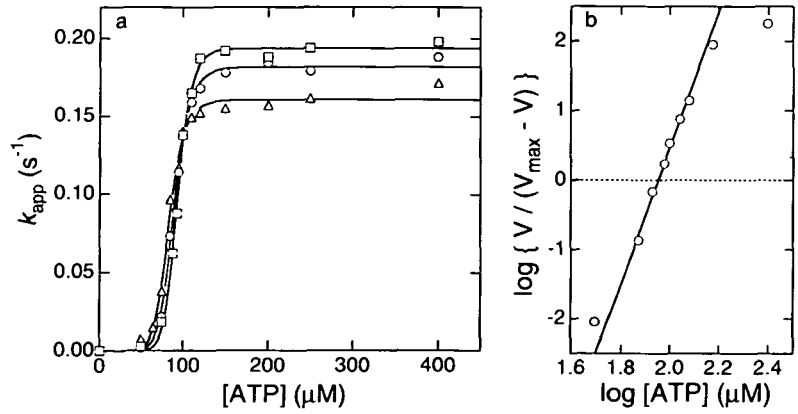

Fig. 3. **Dependence of ATPase activity on ATP concentration.** a: The reaction mixture contained poly(dT), RecA protein, and the indicated concentrations of ATP. The concentrations of RecA protein are represented as follows: squares, 1.0 μM; circles, 0.5 μM; triangles, 0.3 μM. The concentration of poly(dT) was 20 times that of RecA protein. b: Hill plot on 0.5 μM RecA protein. The n_H value was determined to be 9.8 from this plot.

Furthermore, when CTssDNA was used, the activity decreased gradually after a time lag of about 4 min (Fig. 2a). These results indicated that poly(dC) and poly(dT) would be useful for ssDNA-dependent ATPase measurement of RecA protein, since they showed neither a substantial lag nor a subsequent decrease in activity. To allow comparison with previous work (*27*), poly(dT) was used as ssDNA in the following experiments.

Effect of Protein Concentration on ATPase Activity—At a protein concentration of 0.3, 0.5, or 1.0 μM, the ATPase activity of RecA protein was measured at various concentrations of ATP (Fig. 3). The plot of k_{app} against ATP concentration showed a highly sigmoidal curve at all protein concentrations examined, suggesting that RecA protein has extremely high cooperativity in its ATPase activity. Since the RecA protein has only one site for ATP binding (*28*), this cooperativity for ATP is considered to result from the nucleoprotein filament as a whole. To evaluate the cooperativity, the Hill coefficient (n_H), which indicates the cooperativity of a binding site for a ligand, was determined by curve fitting Eq. 2. As shown in Fig. 3, the obtained data were well fitted by the theoretical curves, indicating that the nucleoprotein filament of RecA protein as a whole has cooperativity for ATP like an allosteric enzyme. The determined n_H values were 11.6, 9.8, and 9.3 for 1.0, 0.5, and 0.3 μM RecA protein, respectively (Table I). As these high n_H values indicate high cooperativity among the binding sites, it is suggested that structural changes of many neighboring RecA molecules are induced by the binding of an ATP molecule to a RecA molecule in a helical filament.

To investigate the effect of the salt concentration on cooperativity, the ATPase activity was measured in the absence of KCl (Fig. 4). The lack of KCl led to an increase in the k_{cat} (0.18 to 0.22 s^{-1}) and a decrease in the K_m (90 to 64 μM). Although the specificity constant (k_{cat}/K_m, 2,000 to 3,400 $s^{-1} \cdot M^{-1}$) was increased, the n_H value (9.8 to 9.3) was almost unaltered.

With regard to the ATPase activity of RecA protein, the K_m and k_{cat} values were also determined for different protein concentrations (Table I). Since the k_{cat} value also increased with an increase in the concentration of RecA protein, the dependence of the k_{cat} value on protein concentration was investigated at concentrations of RecA protein ranging from 0.15 to 1.0 μM (Fig. 5). The k_{cat} values were almost constant within the range 0.5-1.0 μM RecA protein, but significantly decreased below 0.3 μM. These results imply that below a protein concentration of 0.3 μM, RecA protein is defective in binding to DNA or in formation of an

TABLE I. **Steady state kinetic parameters for ATP hydrolysis catalyzed by RecA protein.**

	Temperature (°C)	[RecA] (μM)	K_m (μM)	k_{cat} (s^{-1})	n_H
Poly(dT)	25	0.3	83	0.16	9.3
	25	0.5	90	0.18	9.8
	25	1.0	94	0.19	11.6
	30	0.5	84	0.25	8.2
	37	0.5	103	0.35	6.6
Poly(dC)	25	0.5	70	0.20	7.8
Poly(dA)	25	1.0	315	0.085	6.9

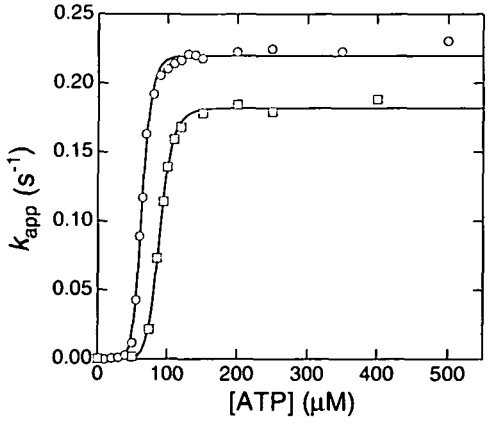

Fig. 4. **Effect of KCl on the dependence of ATPase activity on ATP concentration.** The reaction mixture contained 0.5 μM RecA, 10 μM poly(dT). The measurement was performed in the presence (squares) or absence (circles) of 100 mM KCl.

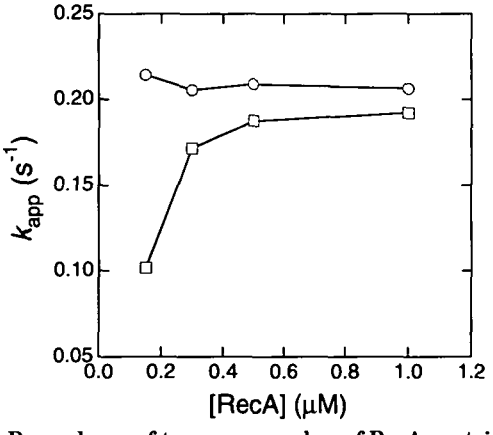

Fig. 5. **Dependence of turnover number of RecA protein on its concentration.** The reaction mixture contained poly(dT) at a concentration 20 times that of RecA protein, 400 μM ATP, and the indicated concentrations of RecA protein. Measurements were performed in the presence (squares) or absence (circles) of 100 mM KCl.

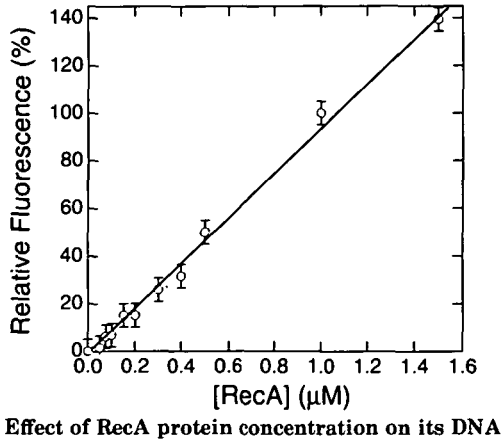

Fig. 6. **Effect of RecA protein concentration on its DNA binding ability.** The reaction conditions were the same as for the ATPase assay, except that εDNA was used as ssDNA. The reaction mixture contained 10 μM εDNA, 1 mM ATP, and the indicated concentration of RecA protein. The εDNA was excited at 305 nm. The ordinate represents the fluorescence intensities at 410 nm relative to the intensity at 1.0 μM RecA protein.

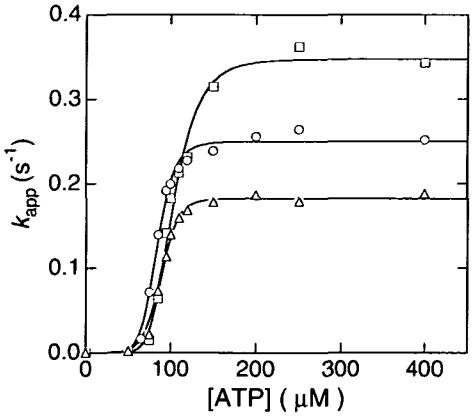

Fig. 7. **Dependence of ATPase activity on temperature.** ATPase assays were performed at 25, 30, and 37°C. The reaction mixture contained 10 μM poly(dT), 0.5 μM RecA protein, and the indicated concentrations of ATP. Temperature: squares, 37°C; circles, 30°C; triangles, 25°C.

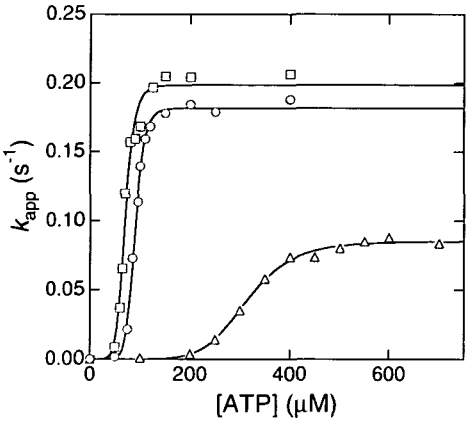

Fig. 8. **Effect of DNA species on ATPase activity.** The reaction mixture contained each ssDNA, RecA protein, and the indicated concentrations of ATP. The concentration of ssDNA was 40 μM poly(dA), 20 μM poly(dC), and 20 μM poly(dT). The concentration of RecA protein was 1.0 μM for poly(dA), 0.5 μM for poly(dC) and poly(dT). DNA species: squares, poly(dC); circles, poly(dT); triangles, poly(dA).

active nucleoprotein filament. However, no such decrease was observed in the absence of KCl (Fig. 5), suggesting that the presence of KCl affects the ability of RecA to bind ssDNA at low protein concentrations.

The effect of RecA protein concentration on its DNA binding ability was examined in terms of the change in εDNA fluorescence that occurs upon binding to RecA protein (Fig. 6). The intensity of fluorescence was proportional to the protein concentration even at low protein concentration, where the rate of ATP hydrolysis by RecA protein decreased (Fig. 5). These results suggest that at such a low protein concentration the RecA protein can bind DNA but cannot form an active nucleoprotein filament in the presence of 100 mM KCl.

Effect of Temperature on ATPase Activity—The effect of temperature on the ATPase activity of RecA protein was then examined (Fig. 7). High cooperativity for ATP was also observed over the range of temperature examined. The k_{cat} value increased with temperature, and at 37°C (0.35 s^{-1}) was about twofold that at 25°C (0.18 s^{-1}) (Table I). The activation energy calculated from the Arrhenius plot of k_{cat} was 11 kcal·mol^{-1}. However, the n_H value decreased with increasing temperature, and at 37°C (n_H=6.6) was about 0.7 times that at 25°C (n_H=9.8) (Table I). The K_m value was almost constant over the temperature range examined. These results indicate that a rise in temperature promotes the ATPase activity of RecA protein but suppresses its cooperativity for ATP.

Effect of ssDNA Type on ATPase Activity—The plot of k_{cat} against ATP concentration with poly(dC) was similar to that with poly(dT), whereas the plot with poly(dA) was significantly different from the others (Fig. 8). However, all three curves showed cooperativity and their n_H values were also high (Table I). The n_H values were 7.8 and 6.9 for poly(dC) and poly(dA), respectively; much smaller than for poly(dT). The K_m values obtained with each homopolymer differed from one another: 70 μM for poly(dC), 315 μM for poly(dA), and 90 μM for poly(dT) (Table I). The k_{cat} value with poly(dA) was 0.085 s^{-1}, which was less than half that with the others (Table I). These results suggest that the ATPase activity of RecA protein depends on the type of ssDNA employed.

DISCUSSION

In this study, we have demonstrated that RecA protein has a surprisingly higher cooperativity for substrate in its ATPase activity than any other enzyme reported so far. The maximal value of n_H obtained was 11.6, whereas the highest values reported to date have been less than 3.3 (*16, 29-31*). This difference may be due to methodological variations in the ATPase assay. Except for Lee and Cox (*30*), the other researchers employed thin-layer chromatography in the absence of an ATP regeneration system, where it was not possible to keep the ATP concentration constant during measurement. Furthermore, since thin-layer chromatography cannot monitor ATPase activity in real time, it is difficult to confirm whether the measurement represents a steady state. In contrast, the enzyme-coupled spectrophotometric assay used in this study is able to both monitor ATPase activity in real time and keep the ATP concentration constant during measurement. This enzyme-coupled spectrophotometric assay is thus capable of measuring the ATPase activity of the RecA protein with greater precision than thin-layer chromatography. This difference in accuracy may account for the difference in the n_H value.

Although Lee and Cox employed an enzyme-coupled spectrophotometric assay at 25°C, the n_H value indicated in their paper was 3.3 (*27*). In their measurement, however, the concentrations of RecA protein and poly(dT) were 5 and 10 μM, respectively. Since the site size of RecA protein for ssDNA is thought to be above three (*3*), their concentration of poly(dT) must be lower than that for the maximal ATPase activity. Also, in the cases where the thin-layer chromatography was employed, the ssDNA/RecA ratios were smaller than 3 (*16, 29, 31*). We determined the DNA concentration required for maximal ATPase activity of RecA protein for each ssDNA (Fig. 1). Then, all measurements were performed under conditions in which RecA protein showed maximal ATPase activity. Therefore, it is reasonable to think that the difference in the n_H value is due

to the difference in the ssDNA/RecA ratio between this study and other studies. To confirm this notion, the ATPase activity was measured at the ssDNA/RecA ratio of 3 (Fig. 9). The result showed that the n_H value was 4.0, which appears to agree with the results from the other studies performed with the ssDNA/RecA ratio smaller than 3.

The salt concentration during measurement of ATPase activity may affect the ssDNA/RecA ratio at which the maximal activity was measured. However, this possibility was excluded by the observation that the dependence of ATPase activity on poly(dT) concentration was unaltered in the absence of KCl (Fig. 10). Alternatively, the salt concentration may cause a difference in n_H value, because the activity of RecA protein is sensitive to salt concentration (*15, 17*). We added 100 mM KCl to the reaction mixture in order to reduce the influence of DNA and ATP added to the reaction mixture as salts. Furthermore, the salt concentration used in this study was closer to the physiological salt concentration in the cell (*32, 33*). Previous studies employing low n_H values used salt concentrations lower than that in this study. However, our study demonstrated that the difference in salt concentration had little effect on the cooperativity (Fig. 4).

We were able to determine the n_H value of the nucleoprotein filament of RecA protein for ATP like an allosteric enzyme. The maximal value of n_H obtained here was 11.6. Such a high n_H value suggests that the binding of an ATP molecule to a RecA molecule within a nucleoprotein filament causes structural change in many neighboring RecA molecules (Fig. 11). This suggestion is consistent with electron microscopic observations indicating that the binding of ATP or ATPγS to RecA protein causes conformational change in the nucleoprotein filament (*9*). A similar conformational change was also suggested by the fluorescent property of a mutant RecA protein whose histidine at position 163 was substituted by a tryptophan residue (*34*). In studies on the affinity of ATP for RecA protein by circular dichroism (CD) spectroscopic analysis, we have also suggested that ATP binding causes a conformational change in the RecA protein (*35, 36*). In addition, the conformational change in the nucleoprotein filament of RecA protein caused by ATP or ATPγS is known to be necessary to initiate the recombination reaction (*37*). This well explains the high n_H values obtained here, and accords with the idea that the allosteric transition of RecA protein requires only ATP binding, not ATP hydrolysis (*8*).

The maximal n_H value, 11.6, also suggested that the functional unit of RecA protein is more than 11 sub-multiple. Since there are approximately 6 RecA protein monomers and 18 to 19 nucleotides per helical turn (*38*), the functional unit needs at least 2 turns and the ssDNA needed for a functional unit is above 30 bases. This suggestion is consistent with the observation that RecA protein requires ssDNA at least 30 bases long to hydrolyze ATP efficiently (*39*).

Increasing the protein concentration increased the values of k_{cat} and n_H (Table I). RecA protein can bind DNA but

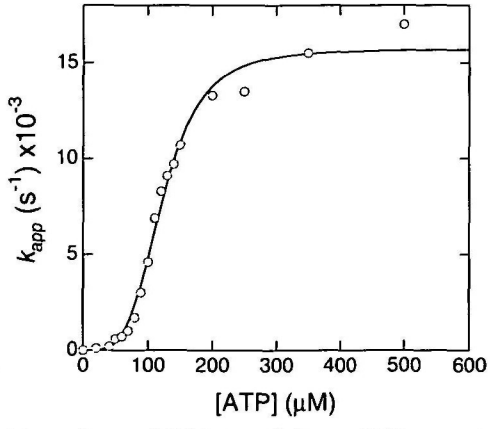

Fig. 9. **Dependence of ATPase activity on ATP concentration at a low ratio of poly(dT) to RecA.** The reaction mixture contained 0.5 μM RecA, 1.5 μM poly(dT), and the indicated concentrations of ATP.

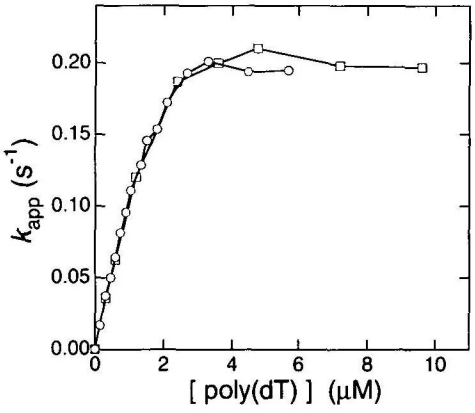

Fig. 10. **Effect of KCl on the dependence of ATPase activity on poly(dT) concentration.** The reaction mixture contained 0.3 μM RecA, 500 μM ATP, and the indicated concentrations of poly(dT). The measurement was performed in the presence (squares) or absence (circles) of 100 mM KCl.

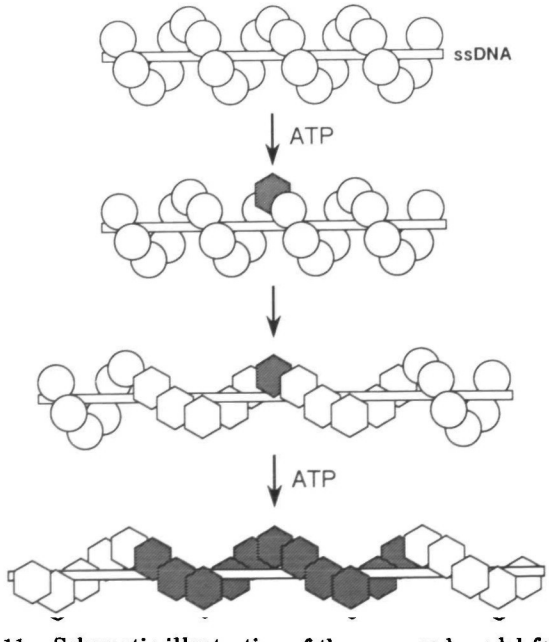

Fig. 11. **Schematic illustration of the proposed model for activation of RecA-ssDNA complex.** Inactive and activated RecA molecules are shown as circles and hexagons, respectively. RecA molecules bound to ATP are shown as filled symbols. Rods represent ssDNA molecules. See "DISCUSSION" for details.

cannot hydrolyze ATP efficiently below a protein concentration of 0.3 μM in the presence of 100 mM KCl (Figs. 5 and 6). These results suggest that at such a low protein concentration, RecA protein cannot form an active nucleoprotein filament. It is probable that a low RecA protein concentration leads to the dominant formation of short nucleoprotein filaments. Such a short nucleoprotein filament may contain a smaller number of RecA molecules than a long one, and therefore give a small n_H value.

Increasing the temperature also increased the k_{cat} value but decreased the n_H value (Table I). If ATP hydrolysis is necessary to facilitate the dissociation of RecA protein from DNA, as mentioned in some studies (*7, 8*), the average size of the nucleoprotein filament in a steady state at higher temperature is presumably shorter than that at lower temperature because of rapid dissociation of RecA protein from the DNA. The increase in the proportion of short nucleoprotein filaments may be the reason for the decrease in the n_H value with increasing temperature. Alternatively, increasing the temperature may weaken the protein-protein interaction of RecA protein within the nucleoprotein filament. This weakening of the interaction may affect the n_H value. If this is the case, protein-protein interaction between RecA protein molecules on ssDNA would depend on electrostatic rather than hydrophobic energy, because the former is generally weakened, but the latter strengthened, by an increase in temperature. In the absence of ssDNA, the RecA protein molecules participate in various interactions in their crystalline form (*40*). In a study of RecA protein using affinity chromatography, it was suggested that hydrophobic interactions contribute significantly to the subunit recognition of RecA protein in solution (*41*). However, the crystal structure of the complex of the RecA protein with DNA or ATP has not yet been clarified. Therefore, the main contributing force to the protein-protein interaction of RecA protein molecules on ssDNA remains to be elucidated. The idea that protein-protein interaction between RecA protein molecules depends on electrostatic force may help in considering the structure of the complex of the RecA protein with DNA and ATP.

The higher the apparent K_m value for ssDNA, the longer the time lag and the lower the k_{cat} value (Figs. 1, 2, and 8 and Table I). Among synthetic homopolymers, poly(dA) had the highest apparent K_m value and showed the longest time lag and lowest k_{cat} value. Poly(dT) and poly(dC) gave similar results, and M13 and CTssDNA, which contain both purines and pyrimidines, gave values intermediate between those of purine and pyrimidine homopolymers. Therefore, the difference in k_{cat} value for each homopolymer may depend on whether it contains purines or pyrimidines. The difference in K_m, k_{cat}, or n_H for each homopolymer suggests that the shape of the nucleoprotein filament on each homopolymer differs. This may be related to base recognition by RecA protein, which may be required for homologous pairing.

The reported time lag for dsDNA-dependent ATPase activity is considered to be a lag phase involving transient denaturation of the dsDNA to form regions of ssDNA (*42*). In this study a similar time lag was observed when natural ssDNA or poly(dA) was used (Fig. 2). In particular, it took about 3 h to reach a steady state with poly(dA) (data not shown). The reason for this long lag may be related to the secondary structure of poly(dA), since poly(dA) is well known to form a single-stranded helical structure by strong stacking between adenine residues (*43*). This view is consistent with the fact that natural ssDNAs with complex secondary structures also showed a time lag. A time lag for M13ssDNA has also been reported only at 25°C or in the presence of 200 mM NaCl (*42*). This may be due to the secondary structure of DNA, because a decrease in temperature or an increase in salt concentration is known to increase the rigidity of DNA (*44*). In other words, the lag may be the period needed for RecA protein to dissolve the secondary structure of DNA. However, RecA protein probably cannot dissolve some complex secondary structures of DNA, because RecA protein has no helicase activity. Therefore, when DNA with such a complex structure is used, RecA protein may bind only to the region of DNA where the secondary structure can be dissolved. For this reason, RecA protein may need a high concentration of DNA for maximal ATPase activity when DNA with a complex secondary structure is used.

When CTssDNA was used as the ssDNA, the ATPase activity of the RecA decreased gradually after a lag of about 4 min (Fig. 2a). It is known that the addition of dsDNA containing a homologous sequence decreases the rate of ssDNA-dependent ATP hydrolysis by RecA protein (*22*). As CTssDNA was prepared by heat denaturation of dsDNA, small amounts of the dsDNA region may have remained in the CTssDNA solution. Alternatively, the complementarity between CTssDNA molecules may form a partial dsDNA region. These small dsDNA regions may cause the decrease in ATPase activity.

To understand proteins which function as polymers, it is important to know how the subunits communicate with one another. In this study, we demonstrated that precise analysis of cooperativity is useful for clarifying how RecA protein polymer functions as a whole. The maximal n_H value on ATP obtained in this study was the highest reported for any ATPase to our knowledge. Substrate cooperativity has been studied in many proteins and enzymes (*45*), such as hemoglobin and aspartate carbamoyltransferase. An example with a relatively high cooperativity is the giant molecule of earthworm hemoglobin, which contains 100-200 hemes. Since its n_H value is sensitive to divalent cations, the role of these cations in the function of the protein has been discussed (*46*). Precise kinetic studies of enzyme cooperativity as well as structural biological studies (including X-ray crystallography, NMR, and electron microscopy) will also be powerful methods for clarifying the function of other polymer proteins, including tubulin, myosin, and actin.

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