Characterization of the Oligomeric States of RecA Protein: Monomeric RecA Protein Can Form a Nucleoprotein Filament†

Ryoji Masui, Tsutomu Mikawa, Ryuichi Kato, and Seiki Kuramitsu*

Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

Received June 1, 1998; Revised Manuscript Received August 3, 1998

ABSTRACT: Self-assembly of RecA protein in solution and on single-stranded DNA exerts a significant effect on the catalytic activities of this protein. To manipulate the self-association reaction, we examined the effects of various salts on the self-association of RecA from *Thermus thermophilus* (ttRecA) by circular dichroism spectroscopy and gel-filtration analysis. We showed that the self-association of ttRecA strongly depends on the kind and concentration of the salt, as well as on the protein concentration. Chaotropic ions were especially useful for obtaining RecA in its hexameric and monomeric states. On the basis of these observations, we were able to regulate the oligomeric states of ttRecA and we then examined the activity of RecA in various oligomeric states. Monomeric ttRecA bound to ssDNA and formed a nucleoprotein filament, which showed ssDNA-dependent ATPase activity. These results suggest that the monomeric form of RecA is an intermediate in filament formation on ssDNA.

Escherichia coli RecA (ecRecA)[1] has been characterized by the numerous functional properties of its active form (*1*−*3*). These actions include homologous recombination of DNA strands, DNA-dependent hydrolysis of ATP, and stimulation of autocleavage of LexA and certain other repressor proteins. RecA is active only when it forms a nucleoprotein filament, which consists of a helical array of RecA monomers bound to single-stranded DNA (ssDNA). This highly ordered structure is sustained predominantly by protein−protein interactions between contiguous monomers (*4*). Thus, analysis of the interaction between RecA and DNA must also consider the self-association reaction (*5*).

Even in the absence of DNA, RecA can self-assemble into a variety of multimeric forms, including rings, rods, filaments, and highly aggregated structures. Light scattering, sedimentation, and electron microscopy have shown that the self-association of RecA strongly depends on the protein concentration, solvent ionic strength, and nucleotide cofactors (*6*−*9*). The complex nature of RecA oligomers has hampered the study of the effect of the self-association properties on the catalytic activity of the enzyme.

One approach to this problem is the manipulation of the self-association reaction of RecA, which can be achieved by judicious choice of experimental conditions. For physical studies under a variety of conditions, a more stable protein is preferred. We have already cloned the *recA* gene of *Thermus thermophilus* HB8 (*10*). This Gram-negative bacterium can grow at temperatures over 75 °C (*11*). In general, proteins isolated from *T. thermophilus* are heat-stable and suitable for physicochemical examination. Complementation experiments showed that *T. thermophilus* RecA (ttRecA) plays the same role as ecRecA (*10*). The conclusion that ecRecA-like function is conserved in thermophilic organisms has also been supported by the study of the RecA of *Thermus aquaticus*, which is a thermophilic bacterium evolutionarily related to *T. thermophilus* (*12*).

Another obstacle to the study of self-association of RecA relates to the technique for assessment of the oligomeric states. Recently, we reported that the circular dichroism (CD) spectrum shows a decrease in the α-helical content of ecRecA upon dissociation of the oligomer to the monomer (*13*). This phenomenon suggests that local folding of the N-terminal domain is coupled to the protein−protein interactions of monomeric ecRecA (*13*, *14*). These studies show that CD spectroscopy is useful for the study of the self-association process of RecA.

In this study, we examined the effects of various salts on the self-association of ttRecA by CD spectroscopy and gel-filtration analysis. The results show that the self-association of ttRecA strongly depends on the kind and concentration of the salt, as well as on the protein concentration. From these investigations the physical nature of the protein−protein interactions responsible for the RecA self-association process was determined. Furthermore, we were able to regulate the oligomeric states of ttRecA, and we examined the activity of RecA in various oligomeric states.

EXPERIMENTAL PROCEDURES

Materials. *T. thermophilus* RecA was overproduced in the *E. coli* strain, BL21(DE3)pLysE, that we transformed using the pET-3a vector carrying the *T. thermophilus recA* gene (*10*). After induction by IPTG, the cells were harvested

† This work was supported in part by Grants-in-Aid for Scientific Research (Nos. 10780385, 10179102, and 08280104) from the Ministry of Education, Science, Sports, and Culture of Japan, and by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists to T.M.

* To whom correspondence should be addressed: tel, +81-6-850-5433; fax, +81-6-850-5442; e-mail, kuramitu@bio.sci.osaka-u.ac.jp.

[1] Abbreviations: ttRecA, *Thermus thermophilus* HB8 RecA protein; ecRecA, *Escherichia coli* RecA protein; CD, circular dichroism; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ϵDNA, etheno-modified calf thymus single-stranded DNA.

10.1021/bi981296c CCC: $15.00
Published on Web 09/30/1998

and stored at −80 °C. Frozen cells were suspended in a buffer containing 1.5 M KCl and disrupted by sonications. (When the cells were disrupted in a buffer containing no KCl, ttRecA was recovered in the precipitated fraction.) The resultant supernatant was heated for 60 min at 60 °C to remove endogenous *E. coli* proteins. After centrifugation, ttRecA was recovered in the supernatant and then purified by several chromatographic procedures using DEAE-cellulose, phenyl-Toyopearl, and phospho-cellulose columns. Before applying to DEAE- or phospho-cellulose columns, the protein solution had to be dialyzed in a buffer containing no salt. Although ttRecA was precipitated during dialysis due to aggregation, we directly applied the dialyzate (suspension) to the columns. The protein was eluted with a linear gradient of NaCl or KCl (0−1.5 M). In the case of phenyl-Toyopearl, the sample was solubilized in a buffer containing a high concentration of ammonium sulfate. Details of the overproduction and purification of ttRecA will be reported elsewhere.

The concentration of RecA was determined using an ϵ_{277} value of 1.46×10^4 M^{-1} cm^{-1}. Etheno-modified calf thymus ssDNA (ϵDNA) was prepared as described before (*14*). Reagents and enzymes were purchased as follows: poly(dC) from Pharmacia; adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) from Boehringer Mannheim; rabbit muscle pyruvate kinase (type II) from Sigma; and pig heart lactate dehydrogenase (grade II) from Toyobo. All of the other chemicals and reagents were purchased from commercial sources.

CD Measurements. CD measurements were carried out in a 1-mm or 10-mm cell at 25 °C using a Jasco spectropolarimeter, model J-720W (Tokyo, Japan). For denaturation experiments, protein samples were incubated for at least 1 h at each urea concentration prior to carrying out spectroscopic measurements.

Fluorescence Measurements. Fluorescence measurements were carried out with a Hitachi spectrofluorometer, model F-4500. The emission spectra were measured in a 5 mm × 5 mm cell at 25 °C after incubation at 25 °C for 1 h.

Gel Filtration. Gel-filtration analysis was performed using a Superdex 200 HR column (1 cm × 30 cm; Pharmacia Biotech Inc.) in a Gilson HPLC chromatography system. For denaturation experiments, the samples were incubated at 25 °C for at least 1 h at each concentration of urea and were eluted with buffer containing the same concentration of urea at a flow rate of 0.5 mL/min. The column was calibrated as described previously (*13*). The elution profile was monitored by recording the absorbance at 220 or 230 nm. All of the measurements were performed at approximately 25 °C.

ATPase Assay. ATPase activity was measured by an enzyme-coupled method (*15*), and the obtained data were analyzed by the Hill equation. Kinetic parameters were determined by fitting the data to the equation.

Electron Microscopy. The ttRecA (2 μM) was incubated with 20 μM ϵDNA in 25 mM Tris-HCl, 10 mM magnesium acetate, 0.2 mM ATPγS, and 50 mM salt, pH 7.5, at 25 °C for 30 min. To this reaction mixture were added NaF and $Al(NO_3)_3$ to a final concentration of 2.5 mM. After additional incubation at 37 °C for 20 min, the sample was negatively stained with 2% (w/v) uranyl acetate for 2 min on carbon-coated copper grids which had been glow-discharged. A JEOL 1010 electron microscope operating at an accelerating voltage of 80 kV was used for all microscopy.

RESULTS

The gene for ttRecA has been cloned and sequenced (*10*). We overproduced ttRecA in *E. coli* and purified it to homogeneity. The ttRecA showed strand exchange activity at 65 °C, but not at 37 °C, although nucleoprotein filament complex formation was observed at both temperatures. The protein showed ssDNA-dependent ATPase activity, and its activity was maximal at 65 °C. A full description of this study will be presented elsewhere.

CD Spectra in Urea. During the preparation of ttRecA, we noticed that ttRecA tends to aggregate in the absence of a high concentration of salts such as 1.5 M KCl. This observation prompted us to think that a high concentration of a salt may affect the oligomeric state of ttRecA. In the case of ecRecA, the oligomeric and monomeric states that occur during its denaturation process can be distinguished by their far-UV CD spectra (*13*). To investigate the effects of salts on the oligomeric states of ttRecA, we first examined the effect of KCl on urea denaturation of ttRecA by observing the far-UV CD spectra (Figure 1A).

In the presence of 0.1 M KCl, the ellipticity of ttRecA in the absence of urea could not be measured because of aggregation of the protein. Above 1.0 M urea, the solution was clear and suitable for CD spectrum measurements; its ellipticity was almost constant up to 3.5 M urea. At around 4.1 M urea a large cooperative transition was observed. In contrast, addition of 1.0 M KCl resulted in the disappearance of the aggregate, even in the absence of urea, indicating repression of aggregation by KCl. Although the ellipticity was constant up to 1.0 M urea, a small transition occurred at 1.5−2.0 M urea, which was not observed in the presence of 0.1 M KCl. Then the ellipticity was almost unaltered up to 4.5 M urea, and a large cooperative transition occurred with a midpoint of 6.0 M urea, higher than that for 0.1 M KCl.

A spectral change typical of the small transition can be seen in the inset in Figure 1A. Compared with the spectrum at 3.0 M urea in the presence of 0.1 M KCl, that in 1.0 M KCl showed reduced ellipticity centered at around 220 nm. A similar decrease in the ellipticity was observed for ecRecA in the presence of about 1.0 M urea; at this urea concentration ecRecA exists in an almost completely monomeric state and local unfolding occurs in its N-terminal domain (*13*). Therefore, it was probable that the small transition observed for ttRecA reflected the dissociation of ttRecA oligomers. Furthermore, this notion suggested that the large transition corresponded to the unfolding of the monomer.

In the case of 0.5 M KCl, the small transition was less cooperative and was difficult to recognize because the intensity was gradually decreased, whereas in the range from 1.0 to 2.0 M KCl, no significant shift of the midpoint of about 1.5 M urea was observed (Figure 1A). As for the large transition, its midpoint increased as the KCl concentrations increased from 0.1 to 2.0 M. These results suggested that KCl affected the stability of both the ttRecA oligomer and monomer in the presence of urea.

The effect of KCl on the emission maximum during urea denaturation was also examined by fluorescence spectroscopy

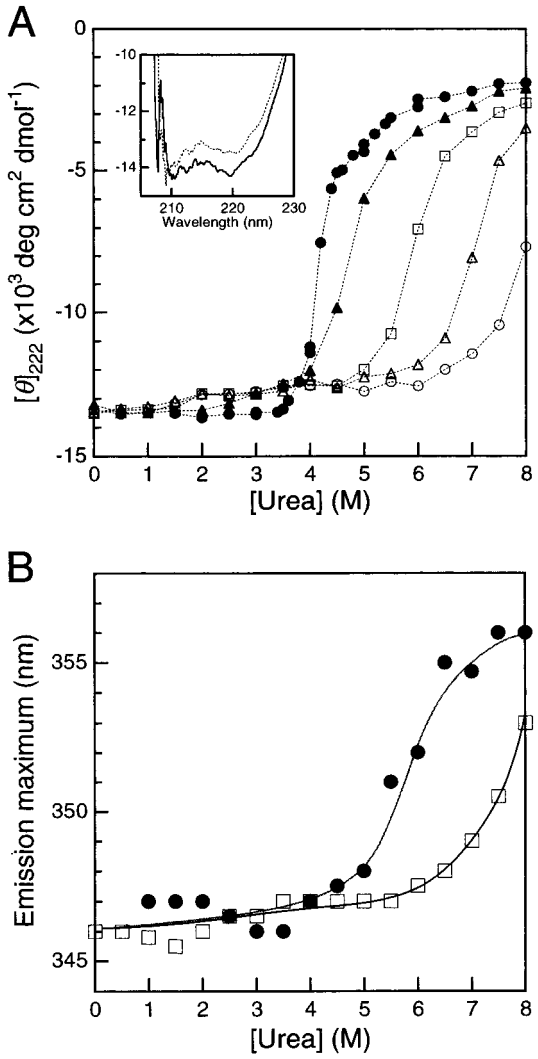

FIGURE 1: Effect of the KCl concentration on urea denaturation of ttRecA. (A) dependence of the mean residue ellipticity at 222 nm on the urea concentration in the presence of various concentrations of KCl. Far-UV CD spectra of 10 μM RecA were measured in a 1-mm light path cell at 25 °C in 25 mM Tris-HCl, 1 mM DTT, and KCl, pH 7.5, in the presence of various concentrations of urea. The samples were incubated at 25 °C for 1 h at each urea concentration prior to measurement. The concentrations of KCl were as follows: closed circles, 0.1 M; closed triangles, 0.5 M; open squares, 1.0 M; open triangles, 1.5 M; and open circles, 2.0 M. In the presence of 0.1 M KCl, the ellipticity at 0, 0.5, or 1.0 M urea could not be measured due to aggregation of the protein. The inset shows the far-UV CD spectra of ttRecA in the presence of 3.0 M urea and either 0.1 M KCl (solid line) or 1.0 M KCl (dotted line). (B) Dependence of the emission maximum of protein fluorescence on the urea concentration in the presence of 0.1 M KCl (circles) or 1.0 M KCl (squares). Measurements were performed at an excitation wavelength of 295 nm in a 5 mm × 5 mm cell at 25 °C in 25 mM Tris-HCl and KCl, pH 7.5. The samples were incubated at 25 °C for 1 h prior to measurement.

(Figure 1B). The transition in the emission maximum was observed with a midpoint of 5.5 M urea in the presence of 0.1 M KCl. In the case of 1.0 M KCl, the midpoint for the transition was higher than that for 0.1 M KCl. The midpoints for these concentrations of KCl were larger than those for the large transitions observed by far-UV CD spectroscopy. These differences may be due to the localization of the sole tryptophan residue in the C-terminal domain of ttRecA (*10*). Together with the results of the CD measurements, these results suggest that the large transition observed at high

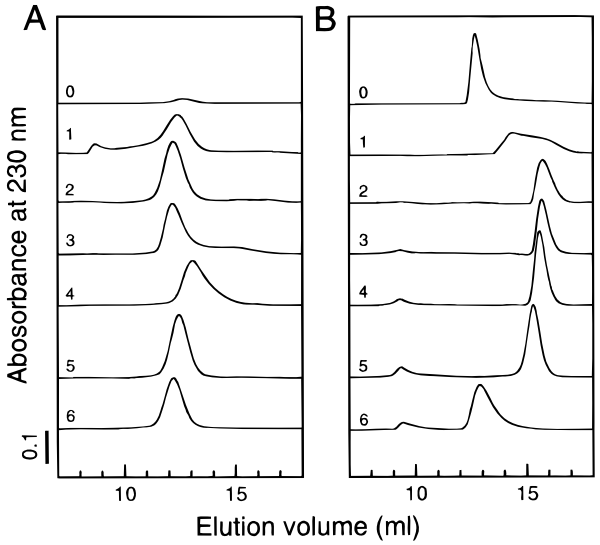

FIGURE 2: Effect of the KCl concentration on gel-filtration elution profiles of ttRecA in the presence of various concentrations of urea. ttRecA was eluted from a Superdex S-200 HR column with 25 mM Tris-HCl, 1 mM DTT, and 0.1 M KCl (A) or 1.0 M KCl (B) in the presence of the following concentrations of urea: 0, 0 M; 1, 1.0 M; 2, 2.0 M; 3, 3.0 M; 4, 4.0 M; 5, 5.0 M; and 6, 6.0 M. The solution of 10 μM RecA and the indicated concentration of urea was incubated in elution buffer prior to elution at 25 °C for 1 h. ttRecA was not completely soluble in 0 and 1.0 M urea in the presence of 0.1 M KCl, so for these samples, the supernatant of the sample, obtained by centrifugation, was applied to the column. The small peak that eluted at 8.6 mL was considered to be the aggregate.

concentrations of urea corresponds to unfolding of ttRecA molecules and that the presence of KCl also affects the unfolding process.

Gel Filtration in Urea. Next, we examined by gel filtration the effect of the KCl concentration on the oligomeric state and the compactness of ttRecA during denaturation (Figure 2). In the presence of 1.0 M KCl, ttRecA with 0 M urea was eluted at 12.2 mL (Figure 2B). On the basis of the elution volumes of molecular mass standards in native conformations, this elution volume was estimated to correspond to a relative molecular mass of approximately 220 000. This value is close to that of a hexamer of ttRecA. When the concentration of urea was increased to 2.0 M, ttRecA predominantly eluted at 15.6 mL, which was the largest elution volume among the samples examined in this study. This elution volume corresponded to a relative molecular mass of approximately 50 000, which is slightly larger than that of the ttRecA monomer calculated from its amino acid sequence. These behaviors suggested that the hexameric ttRecA dissociated to monomers in this concentration range of urea.

When the urea concentration was increased from 5.0 to 6.0 M in the presence of 1.0 M KCl, the elution volume of ttRecA drastically decreased. This reduction of the elution volume at a high urea concentration seemed to correspond to the unfolding of the monomer. After all, the gel-filtration analysis in the presence of 1.0 M KCl suggested that there are two phases in the denaturation process of ttRecA: dissociation of the hexamer to the monomer and unfolding of the monomer.

In contrast, the denaturation process of ttRecA in the presence of 0.1 M KCl was different from that in 1.0 M

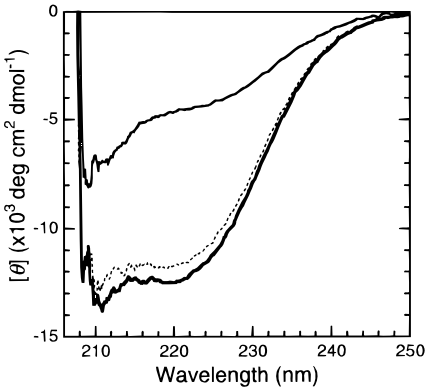

FIGURE 3: Refolding of denatured ttRecA by addition of KCl in the presence of urea. A solution of 10 μM RecA, 5.0 M urea, and 0.1 M KCl was incubated at 25 °C for 1 h and then diluted with an equal volume of 5.0 M urea solution containing 0.1 M KCl (thin line) or 2.0 M KCl (dotted line). After a 1-h incubation, the far-UV CD spectra were measured. Independently, the spectrum of 5 μM RecA in the presence of 5.0 M urea and 1.0 M KCl (thick line) was measured. The measurements were performed in 25 mM Tris-HCl, 1 mM DTT, pH 7.5, at 25 °C.

KCl. ttRecA in the presence of 1.0−3.0 M urea and 0.1 M KCl was eluted at 12.2 mL, which appeared to correspond to the hexamer (Figure 2A). With 4.0 M urea, the peak was shifted to 13.6 mL, but this shift was not observed in the presence of 1.0 M KCl. As its apparent molecular mass was much larger than that of the ttRecA monomer, calculated from its sequence, this peak was thought to correspond to a partially unfolded molecule. With 5.0 and 6.0 M urea, ttRecA eluted at 12.4 mL, which probably corresponded to a completely unfolded monomer. A peak at 15.6 mL, which was observed for 1.0 M KCl, was not observed during denaturation in the presence of 0.1 M KCl. Therefore, these results suggest that, in the presence of 0.1 M KCl, ttRecA denatures from a folded hexamer to an unfolded monomer without existing as a folded monomer. This model was consistent with that based on CD spectral changes during the denaturation of ttRecA.

Effect of KCl on ttRecA Stability. The results obtained by CD spectroscopy and gel-filtration analyses indicated that high concentrations of KCl have two effects on the denaturation of ttRecA. One effect is that a high concentration of KCl stimulates the dissociation of the ttRecA oligomers, including the dissociation of the high molecular mass aggregate and the hexamer. In the absence of urea, the addition of 1.0 M KCl led to dissociation of the aggregate to the hexamers. Furthermore, in the presence of 2.0 M urea, for example, the addition of 1.0 M KCl led to the dissociation of the hexamer to monomers.

The other effect is that a high concentration of KCl stabilizes the monomeric state of ttRecA. ttRecA in the presence of KCl above 1.0 M is thought to exist as an almost folded monomer, in the range between the small transition and the large transition. The unfolding of the monomer occurred at higher concentrations of urea as the KCl concentrations increased (Figure 1A). Therefore, we concluded that high concentrations of KCl stabilize the ttRecA monomer.

To confirm the stabilization effect of KCl to monomeric ttRecA, refolding of the unfolded ttRecA by addition of KCl was examined (Figure 3). In the presence of 0.1 M KCl, ttRecA was almost unfolded with 5.0 M urea. When 1.0 M KCl (final concentration) was added, while the urea concentration was kept at 5.0 M, the intensity of its CD spectrum (dotted line) was restored to that of the almost folded monomeric state (thick line). No refolding was observed when 0.1 M KCl was added (thin line). These results indicate that KCl directly affected the stability of the monomeric ttRecA.

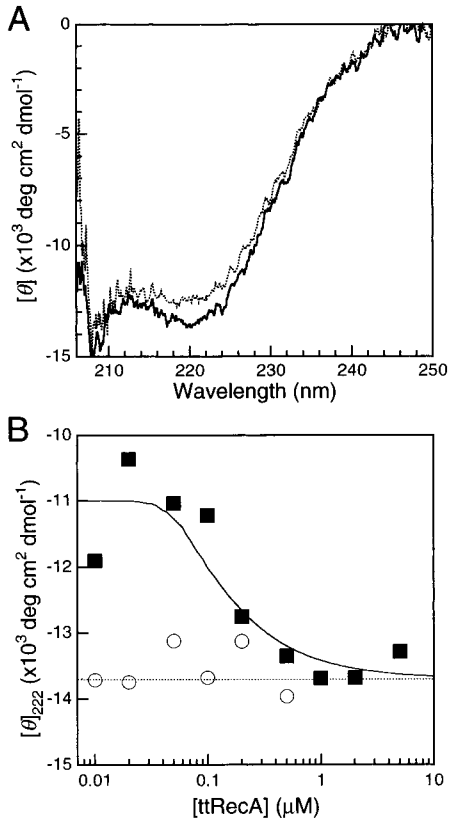

FIGURE 4: Far-UV CD spectra of ttRecA at low protein concentrations. (A) spectra of 0.1 μM ttRecA in the presence of 0.1 M KCl (solid line) or 1.0 M KCl (dotted line). Measurements were performed with a 1-cm-path-length cell in 25 mM Tris-HCl, 1 mM DTT, pH 7.5, at 25 °C. (B) Dependence of the mean residue ellipticity at 222 nm on the protein concentration. The measurement conditions were the same as those described for (A). The KCl concentrations examined were 0.1 M (open circles) and 1.0 M (closed squares). The ellipticity at relatively high protein concentrations, especially in the presence of 0.1 M KCl, could not be measured due to aggregation of the protein. The solid line indicates the theoretical curve for an equilibrium between monomers and hexamers with a dissociation constant of 0.1 nM (see Results for details).

Effect of KCl at Low Protein Concentrations. If the oligomeric states of ttRecA are dependent on the protein concentration, as is ecRecA, a decrease in the ttRecA concentration should lead to dissociation of the oligomer. To examine the effect of KCl on the dissociation of ttRecA oligomers, the far-UV CD spectra of ttRecA at low protein concentrations were measured in the presence of KCl.

At the protein concentration of 0.1 μM, the aggregate disappeared even in the presence of 0.1 M KCl. Under these conditions, ttRecA had a CD spectrum similar to that of the hexamer (Figure 4A, solid line). In contrast, in the presence of 1.0 M KCl, the CD spectrum showed reduced ellipticity centered at around 220 nm (dotted line), which was similar to that of the monomer observed during urea denaturation

(see Figure 1A inset). The ellipticity of ttRecA at 222 nm in 1.0 M KCl showed dependence on the protein concentration (Figure 4B). Increasing the KCl concentration to 3.0 M resulted in no further decrease in the ellipticity compared to that in 1.0 M KCl (data not shown). At 0.1 M KCl, however, no dependence on the protein concentration was found between 0.01 and 0.5 μM ttRecA.

Next, the oligomeric states of ttRecA at low protein concentrations were analyzed by gel filtration. In the presence of 1.0 M KCl, 10 or 5 μM ttRecA eluted at the elution volume corresponding to the hexamer (Figure 5C). At and below 0.5 μM ttRecA, the protein predominantly eluted at the elution volume corresponding to the monomer (Figure 5D). In contrast, ttRecA in the presence of 0.1 M KCl eluted at the elution volume corresponding to the hexamer at protein concentrations from 0.05 to 10 μM (Figure 5A,B). These behaviors were consistent with the dependence of the ellipticity on the protein concentration. Thus, we concluded that a high concentration of KCl stimulates the dissociation of the ttRecA oligomer even in the absence of a denaturant.

According to the above observations, ellipticity at 222 nm can be considered to be an indication of an oligomeric state of RecA. Therefore, an equilibrium constant for the dissociation reaction of the hexamer to monomers can be estimated from the data shown in Figure 4B. If the monomers (A) and hexamers (A_6) are populated states in the transition zone, the overall reaction will be described as a concerted reaction in the following manner.

$$6A \overset{K_d}{\rightleftharpoons} A_6$$

The dissociation constant (K_d) is defined as shown in eq 1.

$$K_d = [A]^6/[A_6] \quad (1)$$

The brackets denote concentration. The total concentration ($[A]_0$) of monomers and hexamers is related to the respective concentrations, as shown in eq 2.

$$[A]_0 = [A] + 6[A_6] \quad (2)$$

The observed ellipticity (θ_{obs}) is expressed as shown in eq 3.

$$\theta_{obs} = \theta_A[A] + \theta_{A6}[A_6] \quad (3)$$

θ_A and θ_{A6} are the molar ellipticities of the monomers and hexamers, respectively. The values of θ_A and θ_{A6} were determined from the data in Figure 4B. The parameter K_d was determined by fitting eq 3 to the observed ellipticities for various values of $[A]_0$. The determined value of K_d was approximately 0.1 nM. This value was much smaller than that for ecRecA, which is approximately 0.1 μM (*13*).

Effects of Various Salts. A high concentration of salts causes both stabilization and destabilization of proteins (*16*). Some kinds of salts cause dissociation of oligomeric proteins, which is referred to as a chaotropic effect. Both potassium and chloride ions, used in the above experiments, are chaotropic ions, although their effects as chaotropic ions are weak. To verify whether the dissociation of ttRecA by KCl is due to the chaotropic effect, the effects of various salts on the ttRecA oligomer were examined.

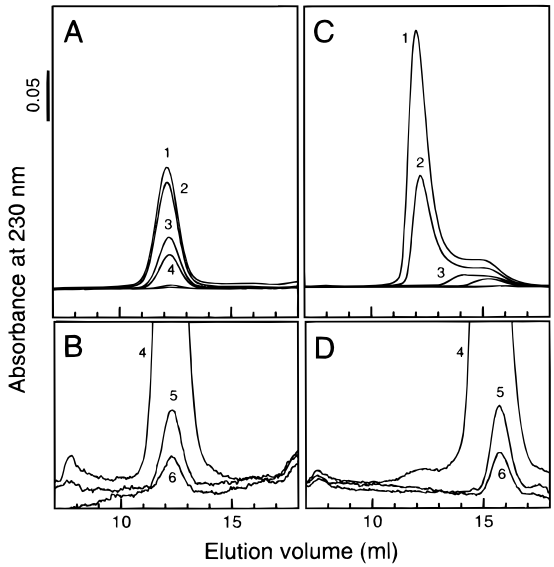

FIGURE 5: Effect of the protein concentration on the elution profiles of gel filtration. ttRecA was eluted from a Superdex S-200 HR column with 25 mM Tris-HCl, 1 mM DTT, and 0.1 M KCl (A and B) or 1.0 M KCl (C and D). The samples were incubated at 25 °C for 1 h in elution buffer prior to elution, and after centrifugation of the sample, the supernatant was injected on the column. The areas of the peaks of ttRecA at 10 and 5.0 μM in panel A are smaller than those in panel C because some of the ttRecA in these samples precipitated in the presence of 0.1 M KCl. Panels B and D show the absorbance scale of the elution profiles magnified 10 times. The protein concentrations applied were the following: 1, 10 μM; 2, 5.0 μM; 3, 1.0 μM; 4, 0.5 μM; 5, 0.1 μM; and 6, 0.05 μM.

Figure 6A shows the effects of various salts on urea denaturation of ttRecA. Three different anions, which were added at 1.0 M as sodium salts, showed different effects on the small transition seen at a low concentrations of urea. Sodium chloride showed a transition midpoint at about 1.5−2.0 M urea, which is similar to that for potassium chloride. The transition midpoint for sodium acetate, 2.5−3.0 M urea, was higher than that seen for the chloride salts. Surprisingly, in the presence of sodium perchlorate, the small transition seen in the presence of the other salts was absent. Instead, the ellipticity intensity in the range from 0 to 3.0 M urea was very close to that seen after the small transition with the other sodium salts. The CD spectrum with 1.0 M sodium perchlorate had a reduced intensity only at around 220 nm compared to that with 1.0 M sodium chloride (Figure 6B). This result suggests that perchlorate ions at 1.0 M caused the dissociation of ttRecA to the monomeric state.

The order of chaotropic strength of the anions was perchlorate > chloride ≫ acetate, although the acetate ion is not usually called a chaotropic ion. Thus, these results indicate that a chaotropic effect is responsible for the dissociation of the ttRecA oligomer. The involvement of chaotropic ions was supported by the observation that the aggregate of 10 μM ttRecA did not completely disappear in the presence of sulfate ions, which are known as antichaotropic ions. The spectrum in the presence of 1.0 M sodium sulfate (Figure 6B) had a different shape and a reduced intensity compared with that of 1.0 M sodium chloride, indicating that the solution was still slightly turbid due to aggregation.

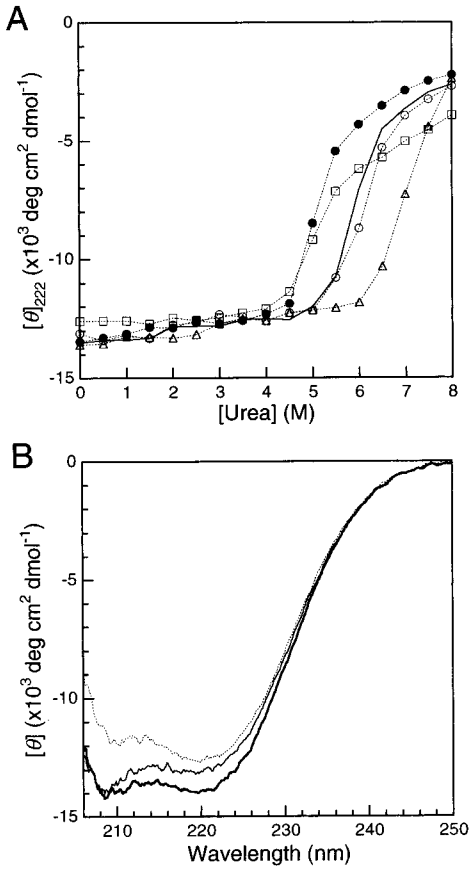

FIGURE 6: Effects of various salts on urea denaturation of ttRecA. (A) Dependence of mean residue ellipticity at 222 nm on urea concentration was examined in the presence of 1.0 M salt. The measurement conditions were the same as those described for Figure 1A except that the various salts were tested: open circles, NaCl; open squares, $NaClO_4$; open triangles, CH_3COONa; and closed circles, LiCl. The solid line represents the denaturation curve in the presence of 1.0 M KCl, which is the same as that in Figure 1A. (B) Far-UV CD spectra of 10 μM ttRecA in the presence of 1.0 M NaCl (thick line), 1.0 M $NaClO_4$ (thin line), or 1.0 M Na_2SO_4 (dotted line). The measurement conditions were the same as those described for Figure 1A, except for the absence of urea. The reduced intensity for Na_2SO_4 was due to the aggregation of the protein.

We also tested cations and found that lithium ions caused a shift of the midpoint of the small transition toward a lower concentration of urea, with less cooperativity, than potassium or sodium ions (Figure 6A). Lithium ions are stronger chaotropic ions than the other two cations examined, so these results confirmed the notion that a chaotropic effect is responsible for the stimulation of dissociation of ttRecA oligomer. These results also show that lithium and perchlorate ions have a strong effect on the dissociation of ttRecA.

In addition to stimulation of oligomer dissociation, the higher concentrations of KCl also stabilized the monomeric state of ttRecA, which was reflected in the shift of the large transition midpoint (Figure 1B). Compared to KCl, $NaClO_4$ and LiCl caused the midpoint of the large transition to shift to the lower concentrations of urea (Figure 6A). These results indicated that strong chaotropic ions destabilize the monomer of ttRecA. This notion was supported by the effect of sodium acetate; the monomeric state was stabilized against urea by acetate ions. Thus, the stabilization by KCl may be a solvent effect, not a chaotropic effect.

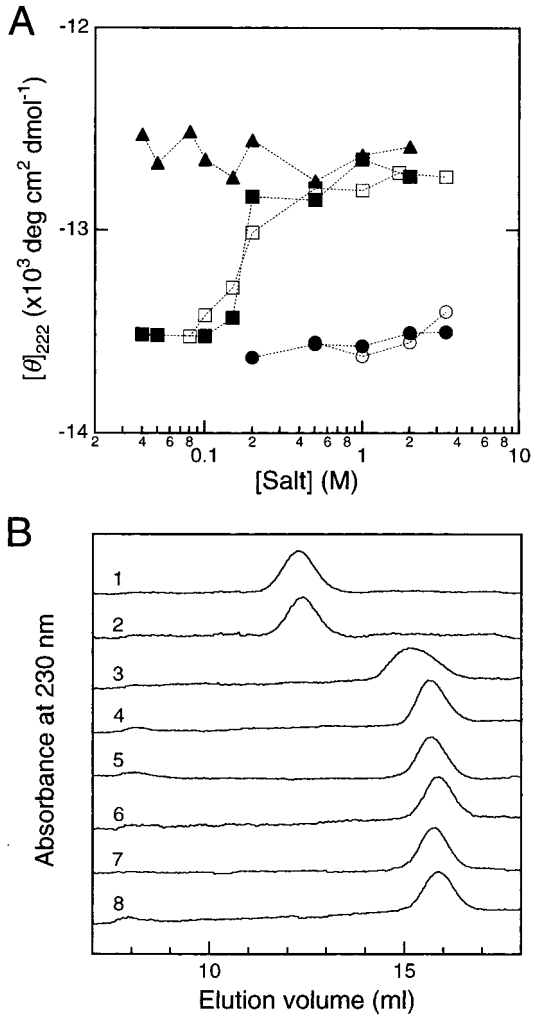

FIGURE 7: Effects of chaotropic ions on the oligomeric states of ttRecA. (A) Dependence of the mean residue ellipticity at 222 nm on various concentrations of NaCl (circles), $NaClO_4$ (squares), or $LiClO_4$ (triangles). The closed and open symbols represent the data for 10 and 2 μM ttRecA. (B) Elution profiles of gel filtration of 1 μM ttRecA in the presence of various concentrations of $NaClO_4$. ttRecA was eluted from a Superdex S-200 HR column with 25 mM Tris-HCl, 1 mM DTT, and $NaClO_4$. The concentrations of $NaClO_4$ were the following: 1, 50 mM; 2, 100 mM; 3, 200 mM; 4, 300 mM; 5, 400 mM; 6, 1.0 M; 7, 1.5 M; and 8, 3.0 M. The other conditions were the same as those for Figure 2.

The dissociation of the ttRecA oligomer at low protein concentrations was also investigated in the presence of various salts. Figure 7A shows the dependence of the ellipticity at 222 nm on salt concentrations. In the presence of $NaClO_4$ the ellipticity of ttRecA, both at 2 and 10 μM, varied depending on the salt concentration; below 0.1 M the ellipticity corresponded to the hexamer, from 0.1 to 0.5 M it decreased depending on the salt concentration, and above 0.5 M it corresponded to the monomer. Note that at 2 and 10 μM ttRecA aggregated at $NaClO_4$ below 40 and 80 mM, respectively. The assessment of the oligomeric state based on the CD spectra was confirmed by gel-filtration analysis (Figure 7B).

In the presence of 0.2 M NaCl, the 2 μM ttRecA was present as the hexamer and similar behavior was observed for 10 μM ttRecA. Below 0.2 and 0.5 M NaCl, ttRecA aggregated at 2 and 10 μM, respectively. In contrast, ttRecA existed as a monomer in the presence of 40 mM to 2.0 M

$LiClO_4$. This strong stimulation of oligomer dissociation was not unexpected because $LiClO_4$ is a very strong chaotrope. These results confirmed that chaotropic ions stimulate the dissociation of the ttRecA oligomer.

Binding to ssDNA. On the basis of the results described above, the monomeric, hexameric, and aggregated states of ttRecA at 2 μM could be achieved by the addition of 50 mM $LiClO_4$, $NaClO_4$, and KCl, respectively. This enabled us to study the functional properties of ttRecA in different oligomeric states. First, we investigated the ability of ttRecA in different oligomeric states to bind ssDNA by fluorescence analysis using ϵDNA, which increases its fluorescence upon binding to proteins, including RecA.

When the ttRecA was titrated with ϵDNA in the presence of ATPγS and magnesium acetate, the fluorescence intensity was increased in the presence of each of the salts examined. The titration curves were hyperbolic, which reflected specific binding of the protein to ϵDNA (Figure 8A). The absence of ATPγS resulted in no significant change in the fluorescence spectra (data not shown). This observation implied that the affinity of ttRecA to ssDNA is lower than that of ecRecA since ecRecA binds ssDNA in the absence of ATPγS. The oligomeric states of ttRecA were not altered by the addition of ATP and magnesium acetate; the 2 μM ttRecA existed as the hexamer and the monomer in 50 mM $NaClO_4$ and 50 mM $LiClO_4$, respectively (Figure 8C). These results indicated that ttRecA in the monomeric or hexameric states can bind ssDNA. Near the level of saturation, however, the extent of the increase of the fluorescence in the presence of KCl was smaller than those with the other two salts. These differences implied differences in the mode of ttRecA binding to ssDNA.

The far-UV CD spectrum was also measured for the ttRecA bound to ϵDNA in the presence of $NaClO_4$ and $LiClO_4$ (Figure 8B). Upon binding to ϵDNA, the ellipticity at around 220 nm was unaltered for each salt. These results suggest, that in the presence of $LiClO_4$, the N-terminal region of the protein remains unfolded even in the complex with ssDNA. Furthermore, the ellipticity at around 208 nm slightly but significantly decreased upon binding to ϵDNA in both cases. Whether this spectral change was due to the conformational change of the protein or the ssDNA is not clear.

To further investigate RecA binding to ssDNA, the ssDNA-dependent ATPase activity was measured (Figure 9A). When the hydrolysis reaction was started by the addition of 2 μM ttRecA to ϵDNA in the presence of each salt at 50 mM, the ATPase activity was observed, using an enzyme-coupled assay (see Experimental Procedures), for each of the salts examined. A steady state was quickly achieved in the presence of KCl, but in the presence of $NaClO_4$ or $LiClO_4$, a much longer time, more than 1 h, was required to achieve a steady state. These results suggest that the type of salt present affects the initial binding reaction of ttRecA to DNA.

Based on the steady-state observed for each salt, the kinetic parameters were obtained by fitting the data to the Hill equation (Figure 9B). The kinetic parameters are summarized in Table 1. The larger turnover number (k_{cat}) was observed in the presence of a more strongly chaotropic ion. The Michaelis constant (K_m) for KCl was smaller than those for the other two. These results suggest that these salts exert different effects on the ATP hydrolysis reaction catalyzed by ttRecA in nucleoprotein filaments. In contrast to those parameters, the Hill coefficients were approximately 3.0 in all cases, which indicated that the cooperativity for ATP binding was not affected by the salts.

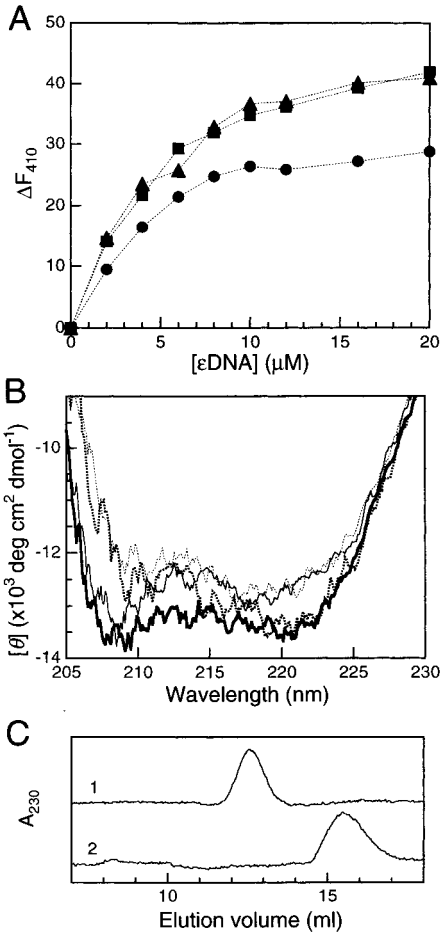

FIGURE 8: Binding of ttRecA to ϵDNA in the presence of various salts. (A) Fluorescence spectral changes. ttRecA (2 μM) was incubated at 25 °C for 1 h with the indicated concentration of ϵDNA in 25 mM Tris-HCl, 10 mM magnesium acetate, 0.2 mM ATPγS in the presence of each of the salts (50 mM). The emission spectra excited by 305-nm light were measured using a 5 mm × 5 mm cell at 25 °C. The ordinate represents the difference of the fluorescence emission intensity at 410 nm between ϵDNA in the presence and absence of ttRecA. The symbols are the following: circles, KCl; squares, $NaClO_4$; triangles, $LiClO_4$. (B) CD spectral changes of 2 μM ttRecA. The samples were prepared as described in (A). The measurements were carried out using a 1-mm cell at 25 °C. The thick and thin lines represent the spectra for $NaClO_4$ and $LiClO_4$, respectively. The solid and dotted lines represent the spectra in the absence and presence of 20 μM ϵDNA, respectively. The spectra displayed in the presence of ϵDNA were corrected for that of ϵDNA alone. (C) Elution profiles of gel filtration of 2 μM RecA in the presence of 50 mM $NaClO_4$ (1) or 50 mM $LiClO_4$ (2). ttRecA was eluted from a Superdex S-200 HR column with 25 mM Tris-HCl, 0.2 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, and 50 mM each salt. Each sample was incubated at 25 °C for 15 min in the same buffer before injection onto the column.

To confirm the formation of ttRecA−ssDNA complexes in the presence of chaotropic salts, we examined the nucleoprotein filaments by electron microscopy. Typical nucleoprotein filaments were observed in the presence of $NaClO_4$ or $LiClO_4$ (Figure 10). These results indicate that nucleoprotein filaments are formed in the presence of chaotropic salts.

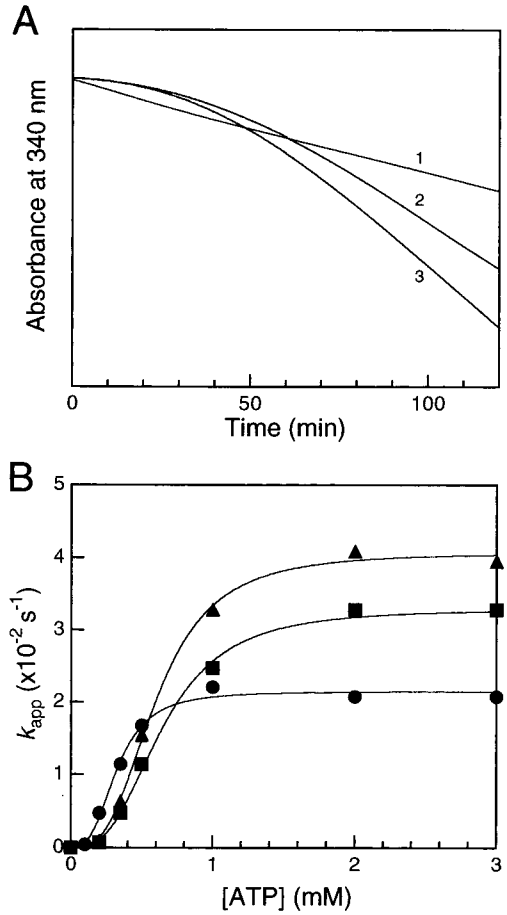

FIGURE 9: ssDNA-dependent ATPase activity of ttRecA in the presence of various salts. (A) Dependence of ATP hydrolysis on time. In addition to the enzymes and reagents required for the enzyme-coupled assay (see Experimental Procedures), the reaction mixture contained 2 μM ttRecA, 100 μM ϵDNA, 25 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 5 mM ATP, and 50 mM KCl (1), 50 mM $NaClO_4$ (2), or 50 mM $LiClO_4$ (3). (B) Dependence of ATPase activity on the ATP concentration. The ordinate (k_{app}) represents the apparent rate of ATP hydrolysis per ttRecA molecule. The solid lines represent the theoretical curves obtained by fitting the data to the Hill equation. The symbols are the following: circles, KCl; squares, $NaClO_4$; triangles, $LiClO_4$.

Table 1. Kinetic Parameters for ssDNA-Dependent ATPase Activity

	k_{cat} (s^{-1})	K_m (μM)	n^a	k_{cat}/K_m (s^{-1} M^{-1})
KCl	0.022	326	2.9	68
$NaClO_4$	0.033	647	2.9	51
$LiClO_4$	0.040	601	3.0	67

[a] *n*, Hill coefficient.

DISCUSSION

In this study we have shown that the monomeric ttRecA binds to ssDNA and forms active nucleoprotein filaments. We examined whether using various kinds and concentrations of salts would allow us to regulate the oligomeric stability of ttRecA. We found that chaotropic ions, even at relatively low concentrations, strongly affected the oligomeric state of ttRecA. When the ttRecA aggregate disappeared after the addition of some kinds of salts, the hexamer was predominantly present as the smallest oligomeric form. Similar behavior by ecRecA was not observed in a gel-filtration study (*13*). Thus, this difference suggests that the hexameric

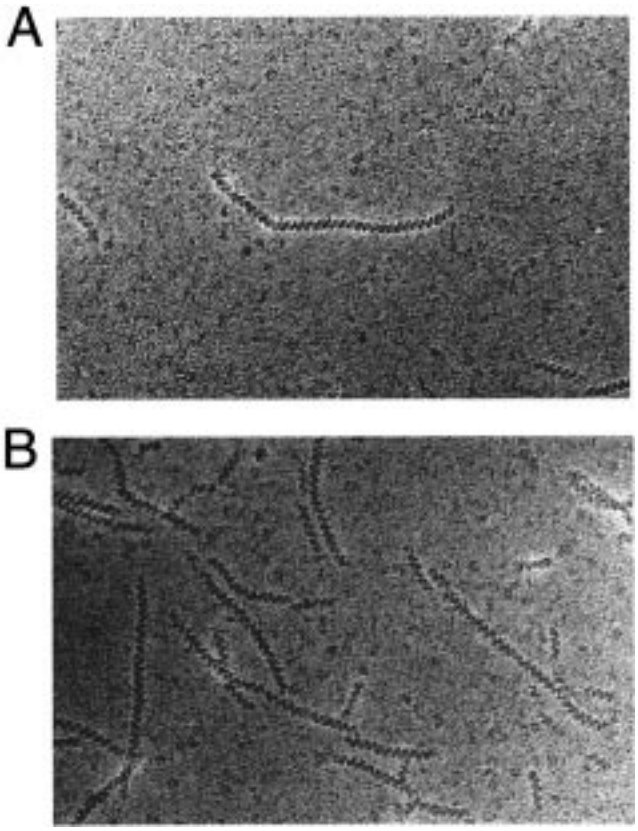

FIGURE 10: Electron micrographs of ttRecA−ϵDNA nucleoprotein complexes. ttRecA (2 μM) was incubated with 20 μM ϵDNA in the presence of 50 mM $NaClO_4$ (A) or 50 mM $LiClO_4$ (B). The reaction mixture contained 25 mM Tris-HCl, 10 mM magnesium acetate, and 0.2 mM ATPγS, pH 7.5.

structure of ttRecA is especially stable. The stronger interaction between the monomers in the ttRecA hexamer was indicated by its smaller value of K_d for the dissociation reaction of the hexamer, compared to that for ecRecA. Electron microscopic analysis has also shown that the RecA from *T. aquaticus* forms particularly stable hexameric rings (*17*, *18*). The high stability of the hexameric structures of thermophilic RecA proteins may be related to their thermostability. A detailed comparison of the stabilities of ecRecA and ttRecA will be presented elsewhere.

One of the most interesting results of this study is that a monomeric state of ttRecA can be achieved, especially by the use of chaotropic ions. As chaotropic ions are known to weaken hydrophobic interactions via destruction of the water structure, this result suggests that hydrophobic interactions contribute to the protein−protein interactions in the ttRecA hexamer. However, ttRecA dissociation during urea denaturation was also stimulated by adding sodium acetate, which is not considered to be a chaotrope, and by increasing the concentration of KCl from 0.1 to 1.0 M. Therefore, electrostatic interaction may also be involved in the protein−protein interactions. It seems likely that these forces contribute differently to the stability of ttRecA and ecRecA hexamers. Such differences may lead to the successful dissociation of the hexamer of ttRecA with chaotropic ions. Note that the monomeric state, which occurred during urea denaturation, was destabilized by LiCl and $NaClO_4$, but was stabilized by sodium acetate. These results suggest that hydrophobic interactions also contribute to the stability of ttRecA monomers.

Upon the dissociation of the hexamer to the monomers, a concomitant reduction of the ellipticity at around 220 nm was observed. This structural change is very similar to that observed for ecRecA upon dissociation of the oligomer to the monomer (*13*). The slightly larger size of the ecRecA monomer on gel filtration can be rationalized by local unfolding of the N-terminal region upon disruption of the protein−protein interactions (*13*). Therefore, these results suggest that local unfolding upon dissociation of the oligomer to the monomer is common to the RecA protein.

The most important finding of this study is that monomeric ttRecA can bind to ssDNA. Previous analyses showed that free filament formation and ssDNA binding by ecRecA are competing reactions and not directly interconvertible (*8, 19*). However, it has not been clear which species represents the fundamental unit from which the filaments are assembled on DNA. Hexameric rings and short rods are predominant forms under the conditions commonly used to monitor RecA reactions in vitro (*6*). In this study, we showed that the nucleoprotein filament can be formed either from the hexamer or from the monomer of ttRecA. Thus, both species are the candidates for the intermediate in the self-association pathway of RecA. Recently, it was reported that the hexameric ring of RecA is structurally similar to the hexameric F_1-ATPase and hexameric helicases (*18*). Such a hexameric ring structure may have an important role in the reactions catalyzed by RecA. However, it is unlikely that the hexamer is an intermediate in filament assembly, since the contacts at the interfaces between the subunits in the hexamer are very different from those in the filament (*18*). It is possible that the hexamer first dissociates into monomers prior to filament formation. Therefore, we conclude that the monomeric form of RecA is an intermediate in filament formation.

As mentioned above, the N-terminal region of ttRecA is partially unfolded in the monomeric state. The CD spectra indicated that the locally unfolded state was preserved even in the nucleoprotein filament formed from the monomer. Nevertheless, this filament was active in ssDNA-binding and ATP-hydrolysis reactions. Previously, we suggested that the folding of the N-terminal region of ecRecA plays an important role in protein−protein interactions (*14*). This apparent discrepancy can be explained by the stronger interactions between monomers of ttRecA compared to those of ecRecA; in the case of ttRecA some regions other than the N-terminal region may make a greater contribution to the protein−protein interaction and enable the molecules to self-associate even with the N-terminal region unfolded. The nonessential role of the N-terminal region in protein−protein interactions in the filament may be consistent with the observation that the eukaryotic Rad51 proteins, whose N-terminal regions are not homologous to that of bacterial RecA, can form nucleoprotein filaments similar those formed by bacterial RecA (*20*).

Besides the CD spectral change described above, a significant decrease in the ellipticity intensity was observed for wavelengths below 215 nm. A similar spectral change upon binding of ecRecA to DNA was previously reported by Wittung et al. (*21*). They suggested that a change in the protein's conformation, in addition to an electronic perturbation of the peptide chromophore by the DNA, is responsible for this spectral change. As this spectral change was also observed for monomeric ttRecA, it is unlikely that the putative conformational change is related to the N-terminal region.

There were some differences in the ssDNA-dependent ATPase activities of filaments formed from ttRecA in different oligomeric states. Whereas the steady state was quickly achieved in the presence of KCl, it took a much longer time in the presence of $NaClO_4$ or $LiClO_4$. Filament assembly of RecA monomers on ssDNA is divided into at least two phases: nucleation and extension (*3*). The nucleation phase contains a slow transition, possibly a conformational change. The nucleation and extension phases of ecRecA filament assembly on ssDNA are so rapid that the kinetics are difficult to analyze. However, the measurement in this study of ttRecA ATPase activity at 25 °C, which is much lower than the optimal temperature for ATP hydrolysis by ttRecA, may have enabled us to observe these phases as a time lag in the ssDNA-dependent ATPase activity. Alternatively, the time lag may be the period needed for ttRecA to dissolve the secondary structure of ssDNA. A similar time lag has been observed for ecRecA when natural ssDNA or poly(dA) was used (*15*). If this is the case, it is possible that the oligomeric state, which is affected by chaotropic salts, influences the ability of RecA to dissolve the secondary structure of DNA.

The k_{cat} values determined in the presence of various salts differed significantly. The rate of ATP hydrolysis can be considered to reflect the rate of DNA binding, so these results suggest that the chaotropic ions affect the interaction between ttRecA and ssDNA in the filament. It is uncertain at present whether the effect of the chaotropic ions was direct or indirect. In the latter case, chaotropic ions may influence ATPase activity through their effect on the protein−protein interactions in the filament. Note that the dissociation of RecA molecules from the filament on linear ssDNA is coupled to ATP hydrolysis. If the presence of various ions also affects the subunit interactions in the filament, it is possible that the ease of dissociation of the filament is associated with the rate of ATP hydrolysis. However, disassembly is limited to the 5′-end region of the linear DNA, which may account for only a small portion of the total hydrolysis reaction, because ATP hydrolysis occurs throughout the nucleoprotein filament.

In our previous study, we proposed that the N-terminal region of RecA is involved in the kinetic regulation of the self-association process (*14*). This regulation may be associated with the local folding of the N-terminal region upon self-association. In this study, we investigated some properties of monomeric ttRecA, at the equilibrium state or steady state, which has an unfolded N-terminal region. As the N-terminal region of ttRecA was unfolded even in the filament form under some conditions, the kinetics of such filament formation may be different from that under normal conditions. Further kinetic studies about the activity of monomeric ttRecA are important for understanding the dynamic processes involved in the reactions catalyzed by RecA.

REFERENCES

1. Roca, A. I., and Cox, M. M. (1990) *Crit. Rev. Biochem. Mol. Biol. 25*, 415−456.
2. Kowalczykowski, S. C., and Eggleston, A. K. (1994) *Annu. Rev. Biochem. 63*, 991−1043.

3. Roca, A. I., and Cox, M. M. (1997) *Prog. Nucleic Acid Res. Mol. Biol. 56*, 129−223.
4. Williams, R. C., and Spengler, S. J. (1986) *J. Mol. Biol. 187*, 109−118.
5. Takahashi, M., Strazielle, J., Pouyet, J., and Daune, M. (1986) *J. Mol. Biol. 189*, 711−714.
6. Brenner, S. L., Zlotnick, A., and Griffith, J. D. (1988) *J. Mol. Biol. 204*, 959−972.
7. Brenner, S. L., Zlotnick, A., and Stafford, I. W. F. (1990) *J. Mol. Biol. 216*, 949−964.
8. Morrical, S. W., and Cox, M. M. (1985) *Biochemistry 24*, 760−762.
9. Ruigrok, R. W. H., and Dicapua, E. (1991) *Biochimie 73*, 191−197.
10. Kato, R., and Kuramitsu, S. (1993) *J. Biochem. (Tokyo) 114*, 926−929.
11. Oshima, T., and Imahori, K. (1974) *Int. J. Syst. Bacteriol. 24*, 102−112.
12. Angov, E., and Camerini-Otero, R. D. (1994) *J. Bacteriol. 176*, 1405−1412.
13. Masui, R., Mikawa, T., and Kuramitsu, S. (1997) *J. Biol. Chem. 272*, 27707−27715.
14. Mikawa, T., Masui, R., Ogawa, T., Ogawa, H., and Kuramitsu, S. (1995) *J. Mol. Biol. 250*, 471−483.
15. Mikawa, T., Masui, R., and Kuramitsu, S. (1998) *J. Biochem. (Tokyo) 123*, 450−457.
16. Timasheff, S. N. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 67−97.
17. Yu, X., Angov, E., Camerini-Otero, R. D., and Egelman, E. H. (1995) *Biophys. J. 69*, 2728−2738.
18. Yu, X., and Egelman, E. H. (1997) *Nat. Struct. Biol. 4*, 101−104.
19. Yu, X., and Egelman, E. H. (1992) *J. Mol. Biol. 227*, 334−346.
20. Ogawa, T., Yu, X., Shinohara, A., and Egelman, E. H. (1993) *Science 259*, 1896−1899.
21. Wittung, P., Nordén, B., and Takahashi, M. (1995) *Eur. J. Biochem. 228*, 149−154.

BI981296C