Observation of RecA protein monomer by small angle X-ray scattering with synchrotron radiation

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Received 18 July 2000; revised 5 September 2000; accepted 6 September 2000

Edited by Hans Eklund

Abstract RecA protein is capable of forming homo-oligomers in solution. The oligomeric and monomeric states of *Thermus thermophilus* RecA protein were studied by small angle X-ray scattering, a direct method used to measure the overall dimensions of a macromolecule. In the presence of 3 M urea or 0.2 M lithium perchlorate, RecA dissociates from higher oligomeric states to form a hexamer with a radius of gyration (R_g) of 52 Å. The value of R_g decreased to 36 Å at a higher lithium perchlorate concentration (1.0 M). The zero angle intensity, $I(0)$, was consistent with the identification of the former state as a hexamer and the latter as a monomer.

Key words: RecA; Oligomer; Monomer; Dissociation; Small angle X-ray scattering; *Thermus thermophilus* HB8

1. Introduction

RecA and its many homologues play an essential role in homologous DNA recombination [1,2]. The properties of *Escherichia coli* RecA protein have been well characterized, and it has been shown to be involved in the following processes: recombination between homologous DNA strands, DNA-dependent ATPase activity and stimulation of autocleavage of repressor proteins [2–4]. RecA protein is active only when it forms a nucleoprotein filament, which consists of many RecA monomers arranged around single-stranded DNA. Even in the absence of DNA, RecA protein can self-assemble into a variety of multimeric forms.

In the presence of 1 M urea, dissociation of *E. coli* RecA protein from its oligomeric state and unfolding of an N-terminal α-helix have been observed [5,6]. This suggests that folding of the N-terminal domain is dependent upon the protein–protein interaction. The remainder of *E. coli* RecA gradually denatured as the urea concentration was increased above 1 M. Therefore, the study of oligomerization of *E. coli* RecA protein is impossible at high urea concentration due to its instability. RecA protein from *Thermus thermophilus* HB8, an extremely thermophilic bacterium that can grow at temperatures over 75°C, was stable under a variety of conditions [7,8].

Stability and self-association of *T. thermophilus* RecA protein have been studied by circular dichroism (CD) spectroscopy and size exclusion chromatography. The results suggest that oligomerization of the protein is strongly concentration- and salt-dependent [7]. Using CD measurements, it was demonstrated that *T. thermophilus* RecA protein was stable up to 3.5 M of urea. The results of the size exclusion chromatography demonstrated that it is highly oligomerized at low salt or urea concentrations, but dissociates and forms a hexamer in the presence of 3 M urea. In the presence of a chaotropic salt, such as sodium perchlorate ($NaClO_4$) or lithium perchlorate ($LiClO_4$), highly oligomeric states of the protein are similarly dissociated. Below 0.1 M of $NaClO_4$, the molar ellipticity at 222 nm was not changed, suggesting a hexameric form, whereas above 0.5 M, unfolding of a N-terminal α-helix was observed, suggesting further dissociation to monomeric form. In the presence of a chaotropic salt, the results of the size exclusion chromatography suggested that the protein exists as a hexamer at a low salt concentrations and as a monomer at higher salt concentrations [7].

Although the oligomeric states of *T. thermophilus* RecA protein could be regulated by addition of a chaotrope, there is no direct evidence for the existence of the hexameric and monomeric states of the protein. CD measurements have given information regarding the folding state of an N-terminal α-helix, which is required for the interaction between RecA monomers, but gave no direct information about molecular size. Size exclusion chromatography measures apparent molecular mass but is equivocal, since the protein is diluted over the column. Small angle X-ray scattering (SAXS) is a powerful technique for measurement of the size and shape of the molecule at equilibrium. It is well known that the SAXS pattern is sensitive to the size and shape of a scattering molecule [9]. The size of a molecule can be estimated using the radius of gyration (R_g), obtained by analysis of the SAXS patterns using Guinier plots. R_g is more sensitive to subunit configuration than Stokes' radius (obtained by size exclusion chromatography), because R_g is the second momentum of excess

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Abbreviations: SAXS, small angle X-ray scattering; CD, circular dichroism; BSA, bovine serum albumin

0014-5793/00/$20.00
PII: S0014-5793(00)02053-6

electron distribution whereas Stokes' radius is a function of the shape of the molecule only.

In this study, we carried out SAXS measurements with and without chaotropic salt and urea. The results demonstrate that *T. thermophilus* RecA protein indeed exists as a hexamer or monomer in different conditions. We calculate the R_g and discuss the value in comparison with the X-ray crystal structure [10] and with the model derived from electron microscopy [11,12].

2. Materials and methods

2.1. Materials

T. thermophilus RecA protein [13] was purified as previously described [8]. Bovine serum albumin (BSA) was purchased from Nacalai Tesque (Japan), ultrapure urea and $LiClO_4$ were from Wako Pure Chemical (Japan).

2.2. Synchrotron SAXS measurement

Solution X-ray scattering experiments were carried out at the solution scattering station (SAXES camera) installed at BL-10C, the Photon Factory, Tsukuba, Japan [14,15]. The sample cell was 50 μl in volume, with a 1-mm path length. The measurements were carried out at protein concentrations ranging from 1 to 5 mg/ml at 25°C. X-ray scattering intensities in the small angle region are given as $I(Q) = I(0)\exp(-R_g^2Q^2/3)$, where Q and $I(0)$ are the momentum transfer and intensity at zero scattering angle, respectively [9]. Q is defined by $Q = (4\pi \sin\theta)/\lambda$, where 2θ and λ are the scattering angle and the wavelength (1.488 Å) of the X-rays, respectively. The R_g value is obtained from the slope of the Guinier plot, $\ln I(Q)$ vs. Q^2. It has been demonstrated that the values of R_g and $I(0)$ are sensitive to the intermolecular association and dissociation of protein complexes [16–18]. The globularity of the protein molecule was examined with a Kratky plot, $I(Q)\ Q^2$ vs. Q.

2.3. Calculation of R_g from model structure

The value of R_g for the RecA monomer was calculated from the X-ray crystal structure [10] and that for the hexamer from the model structure of a hexameric ring [12]. The calculation was carried out using the program CRYSOL [19]. The hydration shell width was set at 3 Å, and the maximum harmonic number was 12.

3. Results

3.1. SAXS measurement for hexameric state of RecA protein in urea

In order to elucidate size and shape of the particle of RecA protein in solution, SAXS measurements were carried out. As our previous study showed, *T. thermophilus* RecA protein is highly oligomerized in the absence of urea or salt [7,8]. Under the same conditions, a Guinier plot of the scattering curve at low angles (below $Q^2 < \sim 0.001$ Å^{-2}) indicated a highly oligomerized state (Fig. 1A). Three peaks were observed at a higher angle region (Fig. 1A, arrows), suggesting that RecA monomers are assembled in an ordered sequence, ultimately generating a helical filament. These results are consistent with previous observations [20,21]. In the presence of 3 M urea, under which condition *T. thermophilus* RecA protein dissociates into hexamers, the non-linear slope in the low angle region and the peaks in a higher angle region disappeared and an approximately linear Guinier region was observed (Fig. 1A). R_g was calculated from the slope of the linear region at each protein concentration. In order to investigate the effect of protein concentration, the experiment was repeated in the range of 1–4 mg/ml of RecA. As shown in Fig. 2, protein concentration did not significantly affect R_g and $I(0)$. The values of R_g and $I(0)$ at infinite dilution were calculated to be 52.0 ± 0.3 Å and 359 ± 22, respectively, by data extrapolation (Table 1).

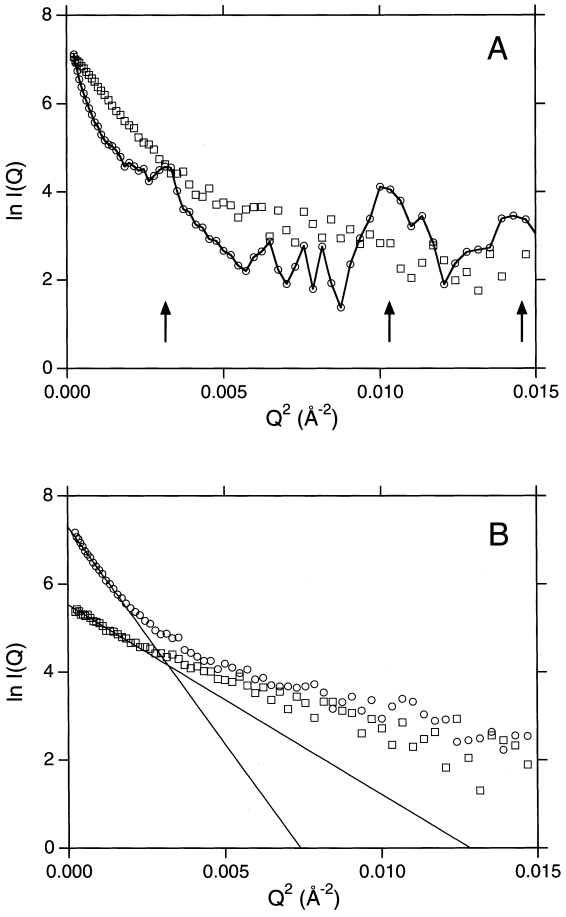

Fig. 1. Guinier plots of scattering curves for *T. thermophilus* RecA protein under various conditions. RecA was dissolved in 50 mM Tris–HCl buffer (pH 7.5) to a final concentration of 4 mg/ml. A: Plots in the presence (squares) and absence (circles) of 3 M urea. The arrows indicate the peaks of a curve generated without urea. B: Plots in the presence of 0.2 M (circles) or 1.0 M (squares) $LiClO_4$.

Table 1
Structural parameters obtained by SAXS

Solution (M)		R_g (Å)	$I(0)$	No. of subunits
Urea	3.0	52.0 ± 0.3	359 ± 22	6
$LiClO_4$	0.2	51.3 ± 0.4	359 ± 12	6
$LiClO_4$	0.5	44.4 ± 0.7	172 ± 2	3
$LiClO_4$	1.0	36.1 ± 0.9	59 ± 9	1

3.2. Changes in the R_g and $I(0)$ values with different chaotrope concentrations

As previously shown, chaotropes stimulate the dissociation of *T. thermophilus* RecA oligomer, with a stronger effect observed with $LiClO_4$ than with $NaClO_4$ [7]. In this work, we used $LiClO_4$ as a chaotrope because a high protein concentration, at which protein association is stimulated, is required for SAXS measurement. SAXS experiments in the presence of 0.1, 0.2, 0.5 and 1.0 M $LiClO_4$ were carried out. At 0.1 M $LiClO_4$, the Guinier plot of the scattering curve was the same as that obtained without urea or chaotrope (data not shown),

suggesting a highly oligomeric state. Above 0.2 M $LiClO_4$, the non-linearity of the low angle region disappeared and an approximately linear Guinier region was observed (Fig. 1B). Experiments repeated at 2–5 mg/ml of RecA showed little effect of protein concentration (Fig. 2). As before, $I(0)$ and R_g at infinite dilution were calculated by data extrapolation (Table 1).

The values of R_g and $I(0)$ at 3 M urea and 0.2 M $LiClO_4$ were very similar (Table 1). This suggests that *T. thermophilus* RecA forms hexamers in the presence of 0.2 M $LiClO_4$. As the concentration of $LiClO_4$ increased, R_g and $I(0)$ decreased (Table 1). These decreases indicate dissociation of molecules from the oligomeric state to monomers. Because the value of $I(0)$ depends on the total number of electrons in the protein, the six-fold lower $I(0)$ observed in 1.0 M $LiClO_4$, as compared to 0.2 M $LiClO_4$, suggests that dissociation of hexamers to monomers takes place. The R_g value at 1.0 M $LiClO_4$ was calculated to be 36.1 ± 0.9 Å. At a moderate concentration of $LiClO_4$ (0.5 M), the values of R_g and $I(0)$ were intermediate between those at 0.2 M and 1.0 M $LiClO_4$. This suggests that the intermediate state of RecA dissociation from hexamer to monomer was observed at 0.5 M $LiClO_4$.

Upon addition of urea or chaotrope, the packing of protein molecules was found to be more pronounced in the Kratky plot. Fig. 3 shows the Kratky plot of the scattering curves at different concentrations of $LiClO_4$ compared to 3 M urea. *T. thermophilus* RecA protein in 3 M urea or 0.2 M $LiClO_4$ shows a clear peak at a Q value of about 0.03 $Å^{-1}$, indicating the formation of a large globular particle. The peak position shifted to larger values of Q, from about 0.03 to 0.06 $Å^{-1}$. The position of the peak is inversely related to molecular size; therefore, the shift of the peak to a larger Q corresponds to a decrease in the size of the particle. This suggests that intermolecular dissociation occurred as the chaotrope concentration was increased. In the Kratky plot, the peak demonstrates the presence of globular particles at 1.0 M $LiClO_4$.

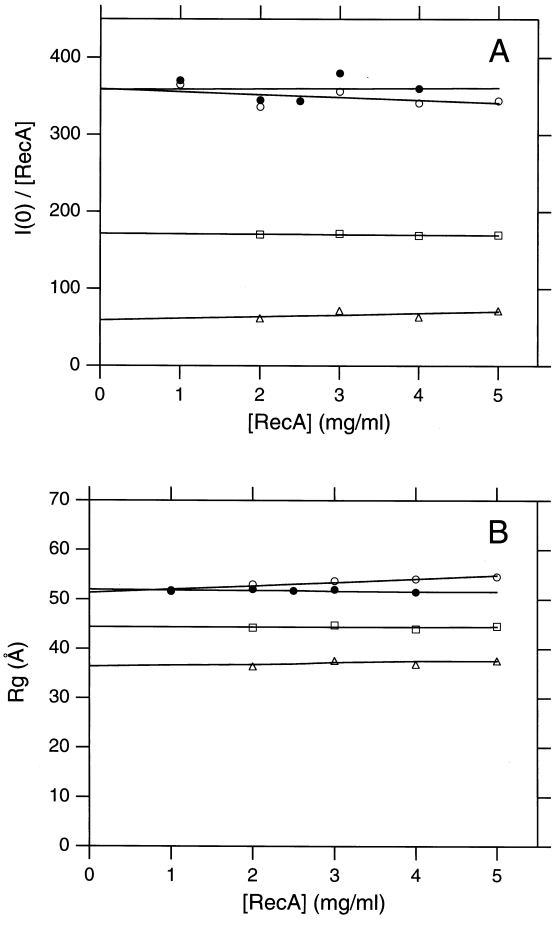

Fig. 2. Concentration dependence of $I(0)$ (A) and R_g (B) of *T. thermophilus* RecA protein under various conditions. Sample solutions contained 3 M urea (closed circles), or 0.2 M (open circles), 0.5 M (squares) or 1.0 M (triangles) $LiClO_4$.

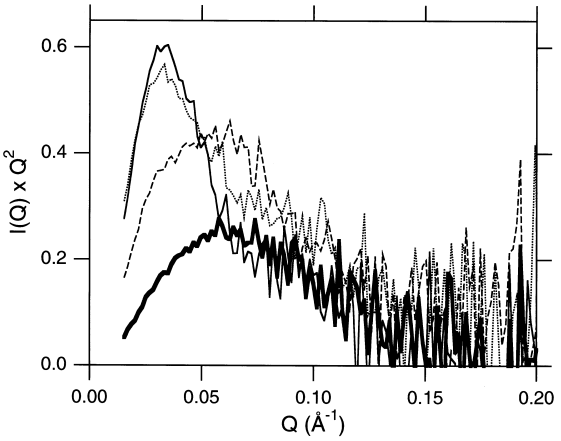

Fig. 3. Kratky plots of scattering curves of *T. thermophilus* RecA protein under various conditions. RecA was dissolved in 50 mM Tris–HCl buffer (pH 7.5) to a final concentration of 4 mg/ml. Sample solutions contained 3 M urea (continuous line), or 0.2 M (dotted line), 0.5 M (broken line) or 1.0 M (thick line) $LiClO_4$.

4. Discussion

We previously reported, using size exclusion chromatography, that highly oligomeric aggregates of *T. thermophilus* RecA protein dissociate to form hexamers in 3 M urea or 0.1 M $NaClO_4$, and monomers above 0.5 M $NaClO_4$ [7]. The initial protein concentrations used in that experiment were 0.072–0.36 mg/ml, which would be diluted over 10 times as the protein flowed through the column. Since the oligomeric state of RecA protein is dependent upon its concentration, it is necessary to evaluate the oligomeric state at equilibrium. In this paper, it was shown that *T. thermophilus* RecA protein exists as a hexamer in 3 M urea or 0.2 M $LiClO_4$ at protein concentrations between 1 and 5 mg/ml. This is the first demonstration of hexamer formation at equilibrium, with such high protein concentrations. Electron microscopy revealed that the *T. thermophilus* RecA hexamers form a ring-like structure (data not shown). The same structure of *Thermus aquaticus* RecA protein, with a ring diameter of about 100 Å, has been observed by electron microscopy [11,12]. Based on these observations from electron microscopy and the X-ray crystal structure of *E. coli* RecA protein, a 110 Å wide donut-shaped hexameric ring model was proposed [12]. The R_g value of the hexameric model structure is predicted to be 50.3 Å, which is consistent with the value of 52 Å obtained with our SAXS measurements. The two distance distribution functions, $P(r)$, were calculated from the hexameric model structure and from our scattering data, respectively. These ob-

served $P(r)$ curves are similar (data not shown), suggesting the solution structure resembles the structure of the model.

Self-oligomerization is intrinsic to RecA protein and is related to its highly cooperative enzymatic activity [22]. In *E. coli*, it has been shown that the interaction between RecA proteins is regulated by an N-terminal α-helix domain [5,6]. A truncated *T. thermophilus* RecA mutant, with deletions in the domain, lost oligomerization activity (T. Mikawa, R. Kato and S. Kuramitsu, unpublished results). These observations suggest that the mechanism of oligomerization is similar between *E. coli* and *T. thermophilus* RecA proteins. *E. coli* RecA oligomers dissociate at low protein concentrations or by addition of a low concentration of urea, however, a hexameric state is not observed [6]. This suggests that the hexameric state is more stable for *Thermus* RecA than for *E. coli* RecA. The stronger interaction between *Thermus* RecA monomers may be related to their thermostability.

The dissociation of *T. thermophilus* RecA hexamers induced by increasing the chaotrope concentration was monitored directly with the SAXS technique. The decrease in the values of the R_g and $I(0)$ shows clearly that oligomer dissociation occurs at $LiClO_4$ concentrations of 0.2–1.0 M. At 1.0 M $LiClO_4$, the molecular mass of *T. thermophilus* RecA was calculated to be 25 kDa by comparing the measured $I(0)$ value to that of BSA. This value is slightly smaller than 34 kDa, which is calculated from the RecA amino acid sequence [13] or observed using SDS–PAGE [8]. This difference may be explained by different measurement conditions of RecA and BSA; the former was measured under high salt, whereas the latter was acquired in the absence of salt.

The X-ray crystal structure of *E. coli* RecA has been reported [10]. Using this structure, the R_g value of the RecA monomer was calculated to be 27.0 Å. This value is considerably smaller than the 36 Å obtained in our experiment. The difference may be due to the following: (1) the reported crystal structure does not contain polypeptide loops, L1 and L2 and a part of C-terminal domain; (2) high chaotrope concentrations may result in a larger hydration shell. We previously showed that an N-terminal α-helix is unfolded at high chaotrope concentrations [7], which may be another reason for the large observed R_g value. We have already shown that, in the presence of chaotrope, the *T. thermophilus* RecA monomer possesses properties of the native state: ATPase, DNA binding and filament formation activities [7]. Moreover, size exclusion chromatography demonstrates that *T. thermophilus* RecA eluted at a monomeric position in the presence of chaotrope [7]. We confirmed the folded state of the RecA monomer at equilibrium by the existence of a peak at 1.0 M $LiClO_4$ in the Kratky plot.

Acknowledgements: This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. The experiment at BL-10C of the Photon Factory was performed under the approval of the Photon Factory Advisory Committee (Proposal No. 98G208).

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