Probing of DNA-Binding Sites of *Escherichia coli* RecA Protein Utilizing 1-Anilinonaphthalene-8-sulfonic Acid

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ABSTRACT: RecA protein of *Escherichia coli* plays an essential role in homologous recombination of DNA strands. To analyze the interaction of RecA with single-stranded DNA (ssDNA), we performed a fluorescence competition assay employing 1-anilinonaphthalene-8-sulfonic acid (ANS) as an extrinsic fluorescent probe. ANS bound to RecA at three sites, leading to enhancement of ANS fluorescence. Addition of synthetic polynucleotides to the RecA−ANS complex in the absence of a nucleotide quenched the ANS fluorescence, indicating displacement of ANS molecules by ssDNA. Less effective quenching by poly(dA) suggests that the nucleoprotein filament on poly(dA) may differ from those on poly(dT) and poly(dC). A titration experiment with poly(dT) and poly(dA) showed clear stoichiometric binding of 3.5 nucleotides per protein. The site size for poly(dC) was 7.0, which could be explained by the formation of a double helix of poly(dC). ATP and other nucleotides also displaced the ANS. To identify ANS-binding sites, ANS was incorporated into RecA by UV irradiation, and fluorescent peptides were isolated from the proteolytic digest. Sequence analysis suggested that ANS binds to or near the ATP-binding region. These results suggest that the fluorescence quenching and photoincorporation assay using ANS may be useful for the analysis of the interaction of a protein and its ligand.

The RecA protein of *Escherichia coli* (RecA) plays an essential role in homologous recombination (*1*, *2*). RecA polymerizes on the single-stranded DNA (ssDNA)[1] in the presence of ATP and produces a helical nucleoprotein filament, which is a functional species of RecA. This filament captures a double-stranded DNA (dsDNA) and searches for sequence homology in it. Recognition of sequence homology is followed by the strand exchange reaction, after which the displaced strand and heteroduplex are released.

It is unknown how the nucleoprotein filament recognizes homology and catalyzes the strand exchange reaction. One approach to this subject is the structural analysis of the interaction between RecA and DNA molecules. Although the structure of the RecA−DNA filament has been depicted with reconstituted electron microscopic images (*3*, *4*), its resolution was too low to identify the interface between the RecA and DNA molecules. The crystal structures of RecA and the RecA−ADP complex without DNA gave no definitive information about the sites responsible for binding of DNA molecules (*5*, *6*). Photochemical cross-linking experiments have been used to identify DNA-binding sites in RecA (*7*−*10*). However, these studies suggested that several distinct regions, including disordered loops L1 and L2, are involved in binding to DNA molecules. In addition, the different regions have also been proposed to be DNA-binding sites by alternative methods (*11*, *12*).

Spectroscopic techniques have often been used to investigate the interaction of a protein with DNA in solution. Among them, fluorescence spectroscopy is one of the most sensitive techniques, allowing one to estimate the binding constants for those interactions. Most researchers have used the intrinsic fluorescence of the aromatic amino acids of the protein as a probe. In some cases, the intrinsic fluorescence can indicate the location of particular aromatic residues within the DNA-binding site of the protein. However, the intrinsic fluorescence of RecA shows little change after the protein binds nucleic acids, which may result from the absence of a tryptophan residue near the DNA-binding sites (*13*). To overcome this difficulty, several approaches to the measurement of DNA binding have been examined, such as the incorporation of tryptophan residues into putative DNA-binding sites (*14*, *15*) or the modification of bases in the DNA molecule with fluorescent reagents (*16*−*18*).

As an alternative fluorescence method, one can use an extrinsic fluorescent probe, which interacts with or competes with a ligand for the binding site of the protein. In the latter case, the fluorescence of the probe bound to the protein is reduced as the ligand displaces the probe molecule. The method that utilizes this phenomenon is the called competitive fluorescence assay or dye-displacement assay (*19*). Several fluorescent dyes have been used to study protein−nucleotide (*20*, *21*) and protein−nucleic acid interactions (*22*−*25*). Furthermore, it was reported recently that a fluorescent probe can be used to obtain site-specific information by photoincorporation of the probe into the protein (*26*).

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[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; bis-ANS, 4,4′-bis(1-anilinonaphthalene-8-sulfonic acid); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; PAGE, polyacrylamide gel electrophoresis; UV, ultraviolet; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin.

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These observations prompted us to employ an extrinsic fluorescence method to study the interaction of RecA with DNA.

In this study, we analyzed the interaction of RecA with DNA by employing 1-anilinonaphthalene-8-sulfonic acid (ANS) as an extrinsic fluorescent probe. ANS is minimally fluorescent in polar environments, such as aqueous solutions, but its fluorescence is increased in less polar environments (*27*). ANS has long been used to study protein structure (*28*) by probing the conformational states of proteins (*29*, *30*) and the hydrophobic surfaces of proteins (*31*, *32*). We show that ANS can be used as an extrinsic fluorescent probe to analyze the RecA−ssDNA interaction. The results obtained suggest that the mode of interaction differs for respective polynucleotides. In addition, we also show that ANS can be incorporated into RecA by ultraviolet (UV) light irradiation, which enabled us to determine that the ANS-binding site is near the ATP-binding site. On the basis of these results, we discuss the binding site(s) for ssDNA in the RecA molecule.

EXPERIMENTAL PROCEDURES

Materials. 1-Anilinonaphthalene-8-sulfonic acid ("high purity") and 4,4′-bis(1-anilinonaphthalene-8-sulfonic acid) (bis-ANS) were purchased from Molecular Probes, Inc. (Eugene, OR). The ANS concentration was calculated from the molar extinction coefficient of 5.5×10^3 M^{-1} cm^{-1} at 370 nm in aqueous solution (*19*). *E. coli* RecA was purified as previously described (*33*). The RecA concentration was determined using an extinction coefficient of 2.15×10^4 M^{-1} cm^{-1} at 278 nm (*33*). Poly(dA), poly(dC), and poly(dT) were purchased from Pharmacia Biotech. The concentrations (nucleotide molar, M) of synthetic polynucleotides were determined by absorbance using the following extinction coefficients: 8.6×10^3 M^{-1} cm^{-1} at 257 nm for poly(dA) (*34*), 7.4×10^3 M^{-1} cm^{-1} at 268 nm for poly(dC) (*35*), and 8.52×10^3 M^{-1} cm^{-1} at 264 nm for poly(dT) (*35*). Adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) was purchased from Boehringer Mannheim, and other nucleotides were from Yamasa (Tokyo, Japan) and Life Technologies Oriental, Inc. (Tokyo, Japan). The nucleotide concentrations were determined using extinction coefficients of 1.54×10^4 M^{-1} cm^{-1} at 259 nm for ATP, ADP, and AMP and 1.50×10^4 M^{-1} cm^{-1} for ATPγS (*36*). Lysylendopeptidase was purchased from Takara Shuzo Co. (Kusatsu, Japan). All the other chemicals and reagents were purchased from commercial sources.

Fluorescence Measurements. A Hitachi model F-4500 spectrofluorometer was used for fluorescence measurements. Slit widths used for the excitation and emission were both 5 nm. Fluorescence emission intensity data were collected directly on a Hitachi computer connected to the spectrofluorometer and were analyzed using the software Igor (Wave Metrix). All the measurements were taken with a 0.5 cm × 0.5 cm quartz cuvette at 25 °C. The fluorescence intensity was corrected for the inner filter effect (*37*). All the titration experiments were performed by preparing the sample in a single aliquot for each point.

Dye Binding Studies. Fluorescence emission intensities (at 470 nm) obtained from titration with ANS and RecA were used for analysis. The apparent dissociation constant (K_d) and Hill constant (α_H) for ANS binding to RecA were obtained by fitting the data to the Hill equation (*38*). The number of molecules (n) of ANS bound to RecA was estimated from Scatchard plots (*38*). Scatchard plot fitting was carried out as follows. ANS was titrated with RecA, and the reciprocal of the fluorescence intensity was plotted against the reciprocal of the RecA concentration. The intercept at an infinite protein concentration gives the value of the fluorescence intensity at saturation (F_{sat}). Scatchard plots were then constructed using $n = F/F_{sat}$ = the number of ANS molecules bound per RecA molecule, where F is the fluorescence intensity for a given concentration of ANS.

Job plots (*38*) were constructed from the fluorescence intensities observed when the total concentration of ANS plus RecA was kept at 4 μM.

Competitive Fluorescence Assay. For the competitive fluorescence assay, 1 μM RecA and 100 μM ANS were incubated for at least 30 min at 25 °C. Binding of ANS to RecA was almost completed during manual mixing. After addition of the ligand, including DNA, nucleotides, or salts, the solution was further incubated for at least 30 min at 25 °C. In some cases, RecA was added after incubation of ANS with those ligands. The data from the titration experiment of ssDNA with RecA in the presence of ANS were analyzed according to an equation for bimolecular reaction (*36*) by assuming that the site sizes for the ssDNAs were the same as the values obtained in the titration experiments of RecA with ssDNAs. We assumed the following scheme for the interaction between RecA (E) and ssDNA (S),

$$E + S \overset{K}{\rightleftharpoons} ES \tag{1}$$

where K is the dissociation constant defined by

$$K = [E][S]/[ES] \tag{2}$$

The parentheses denote concentration. It should be noted that the concentration of ssDNA defined here is the value obtained by dividing the actual concentration by the respective site size.

The total concentrations of RecA ($[E]_0$) and ssDNA ($[S]_0$) are related to the respective free concentrations ([E] and [S]) as follows:

$$[E]_0 = [E] + [ES] \tag{3}$$

$$[S]_0 = [S] + [ES] \tag{4}$$

The concentration of free RecA is obtained from eqs 2−4,

$$[E] = [[E]_0 - [S]_0 - K + [([E]_0 - [S]_0 - K)^2 + 4K[E]_0]^{1/2}]/2 \tag{5}$$

Then, [ES] and [S] are calculated from eqs 3 and 4. The observed change of fluorescence intensity (ΔF_{470} in Figure 4) is expressed as

$$\Delta F_{470} = f[ES] \tag{6}$$

where f is the molar fluorescence intensity of the RecA−ssDNA complex. The two parameters K and f were determined by fitting eq 6 to the observed changes at various

concentrations $[E]_0$. Fluorescence measurements were carried out as described above.

Photoincorporation of ANS into RecA. For the labeling reaction, RecA (10 μM) was dissolved in 25 mM Tris-HCl (pH 7.5) in the presence of ANS at various concentrations. The samples (10 μL) were placed in the wells of a microtiter plate. The microtiter plate was placed upside down on the transilluminator, and ice was placed on the bottom of the plate. Irradiation at 312 nm was carried out with the transilluminator [CFL-40BX (Fluo-Link), Cosmobio, Japan] at a total power of 10 J/cm^2. The irradiated samples were then separated on 12.5% SDS−polyacrylamide gels (*39*). After electrophoresis, the gel was illuminated on a transilluminator (312 or 354 nm) and photographed using a camera (Printgraph, ATTO, Kyoto, Japan). The gel was then stained with Coomassie brilliant blue.

The irradiated sample (10 μM RecA and 2 mM ANS) was separated on a Sephacryl S-200 column (1.4 cm × 22 cm) in 50 mM Tris-HCl, 1 mM EDTA, and 100 mM KCl. One fraction from each peak was used for UV and fluorescence spectroscopy.

Separation of ANS-Bound Fragments. Photoincorporation of ANS (2 mM) into RecA (10 μM) and electrophoresis of the irradiated sample (total of 4 ng of RecA) were performed as described above. The fluorescent RecA bands were cut out from the gel on the transilluminator using a razor blade. The excised bands were cut into smaller pieces and transferred to microtubes. The pieces were washed twice with 50 mM Tris-HCl (pH 9.0) containing 50% (v/v) acetonitrile and dried in an evaporator. The lysylendopeptidase solution, 0.01 mg/mL in 100 mM Tris-HCl (pH 9.0), was added to the dried pieces, and digestion was performed at 37 °C for 20 h. The reaction was stopped by the addition of trifluoroacetate (TFA) at a final concentration of 1%. By addition of acetonitrile (final concentration of 60%), the resulting peptides were recovered in the supernatant fraction, which was concentrated in an evaporator. The peptide solution was injected onto a C_{18} column (4.6 mm × 250 mm, Waters) equilibrated with 0.05% TFA and separated with a linear gradient of acetonitrile from 0 to 50% using a Gilson HPLC system (model 303). Peptide elution was monitored using a UV detector and fluorescence detector (FP-920, Jasco, Japan) connected in tandem. The sequences of the peptides were determined using a gas-phase protein sequencer [Perkin-Elmer (Applied Biosystems), model 473A]. A control experiment was carried out using a solution of RecA and ANS without UV irradiation.

RESULTS

Binding of ANS to RecA. As seen in Figure 1A (broken line), ANS yielded little fluorescence with an emission maximum at 524 nm in aqueous buffer solutions upon excitation at 370 nm (*40*). In the presence of RecA, however, the fluorescence emission of ANS was considerably enhanced with a blue wavelength shift of the maximum fluorescence to about 470 nm (Figure 1A, solid lines). These changes suggest that ANS binds to RecA in a hydrophobic environment, which increases its quantum yield. The fluorescence titration of RecA with ANS is shown in Figure 1B. Binding of ANS at 1 μM RecA was slightly sigmoidal, suggesting cooperative interactions of multiple binding sites.

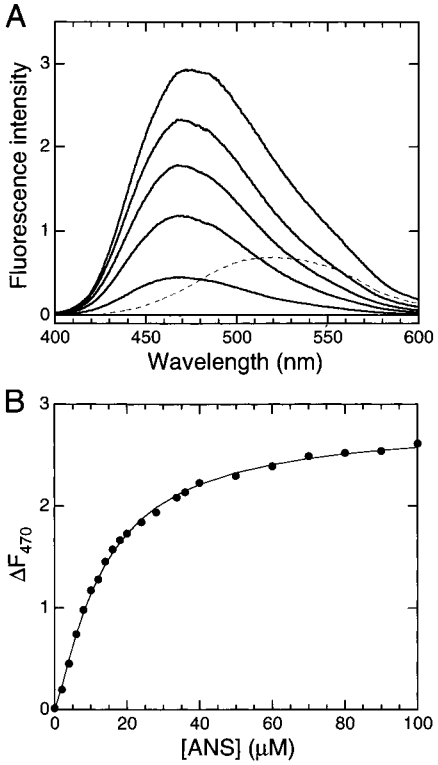

FIGURE 1: Enhancement of ANS fluorescence emission upon binding to RecA. (A) Typical fluorescence emission spectra of 1 μM RecA in the presence of ANS at 0, 4, 10, 20, 40, and 100 μM (solid lines, from bottom to top). These spectra were derived by subtracting the spectrum of ANS alone at each concentration (broken line) from each observed spectrum. Measurements were taken at 25 °C with an excitation wavelength of 370 nm in 50 mM Tris-HCl (pH 7.5) in a 5 mm × 5 mm cuvette. (B) Fluorescence titration curve for the interaction of RecA (1 μM) with ANS. The solid line represents the theoretical curve ΔF_{470} (the ordinate) = $f[\mathrm{ANS}]^{\alpha_H}/(K_d\alpha_H + [\mathrm{ANS}]^{\alpha_H})$, where f, K_d, and α_H are the fluorescence intensity at saturation, the dissociation constant, and the Hill constant, respectively.

When the data in Figure 1B were fit to the Hill equation, the apparent dissociation constant (K_d) and Hill constant (α_H) for ANS binding to RecA were calculated to be 13 μM and 1.2, respectively.

The number of molecules (n) of ANS bound to RecA was obtained from a Scatchard plot (Figure 2A). The plot was obtained from the data in Figure 1B, using the fluorescence intensity at saturation (described in Experimental Procedures). The convex upward curve in the Scatchard plot suggests positive cooperative binding of ANS to RecA. The intercept on the abscissa gave a value of 2.8 per protein for the number of binding sites. When the cooperativity is ignored, the number of binding sites was determined to be 3.0.

The stoichiometry of the ANS−RecA complex was also investigated by the method of continuous variation (*38*). Fluorescence data were collected as a function of the molar fraction of the RecA, at a constant total molar concentration of the two components of 4 μM. The results are shown in a Job plot (Figure 2B). The maximum fluorescence intensity occurred between a mole fraction of 0.25 and 0.35. The extrapolated maximum occurred at 0.26, corresponding to a ratio of RecA to ANS of 1:3. Therefore, these results indicate that ANS binds to three sites on RecA. Table 1

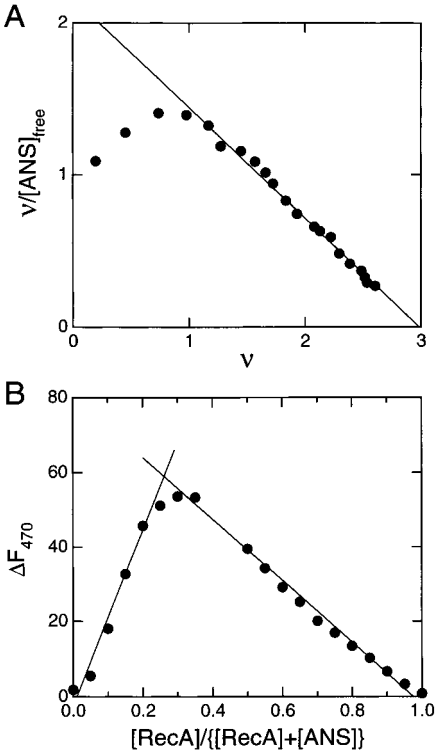

FIGURE 2: Stoichiometry of ANS bound to RecA. (A) Scatchard plot. The abscissa (ν) indicates the molar ratio of bound ANS to RecA. The ordinate indicates the ratio of ν to the concentration of unbound ANS. The solid line represents the theoretical curve assuming that there are three ANS-binding sites and no cooperative binding (see Experimental Procedures for details). (B) Job plot. Fluorescence emission spectra were measured keeping the total concentration of ANS and RecA at 4 μM. The intensities at 470 nm were plotted as a function of the molar fraction of RecA. The solid lines were obtained by fitting the data points to a linear equation in the range of 0−0.2 and 0.35−1.0 in the abscissa by the least-squares method.

Table 1: Binding Parameters for the RecA−ANS Interaction[a]

[RecA] (μM)	K_d (μM)	α_H	n
0.4	34.0	1.1	3.5
1.0	13.5	1.2	2.8
5.0	13.3	1.8	2.6

[a] K_d, α_H, and n represent the dissociation constant, Hill coefficient, and the average number of ANS molecules bound to each RecA monomer, respectively (see Results for details).

shows the dependence of K_d, α_H, and n on RecA concentration.

Similar analyses were carried out for bis-ANS, a dimeric form of ANS, and the results indicated that there were more than 40 bis-ANS binding sites on RecA (data not shown). Such multiplicity of binding sites might hinder further analysis of the interaction of RecA with its ligands by dye fluorescence techniques. Therefore, we chose ANS as the extrinsic fluorescent probe for the following experiments.

Quenching of ANS Fluorescence by ssDNA. Figure 3 shows the effect of the addition of poly(dT) on the fluorescence emission spectra of ANS bound to RecA. As the concentration of poly(dT) was increased, the fluorescence emission spectra were considerably quenched with a red wavelength shift (Figure 3A) and the intensity decreased to the level of free ANS alone at concentrations above 4 μM poly(dT) (Figure 3B, squares). In the absence of RecA,

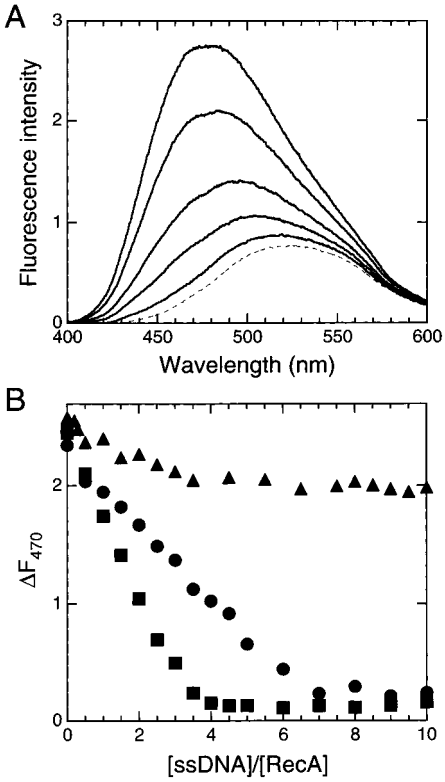

FIGURE 3: Fluorescence quenching of ANS bound to RecA by poly(dT). (A) Emission spectra of 1 μM RecA and 100 μM ANS in the presence of poly(dT) at 0, 1, 2, 3, and 4 μM (solid lines, from top to bottom). RecA and ANS were incubated at 25 °C for 30 min in 50 mM Tris-HCl (pH 7.5); poly(dT) was added, and the solution was further incubated at 25 °C for 30 min. Each point represents a separate sample. Other measurement conditions were the same as those described in the legend of Figure 1A. The broken line represents the spectrum of 100 μM ANS alone. (B) Titration of 1 μM RecA with each polynucleotide in the presence of 100 μM ANS. The measurement conditions were the same as those described for panel A. The difference between the emission intensities at 470 nm in the presence and absence of RecA was plotted against the molar ratio of each polynucleotide to RecA: (■) poly(dT), (●) poly(dC), and (▲) poly(dA).

addition of poly(dT) had no effect on ANS fluorescence (data not shown). The fluorescence quenching could be due to decreased binding of ANS to RecA or a decreased quantum yield of bound ANS. However, the latter possibility can be excluded since the maximum of the fluorescence emission was red shifted to about 520 nm (Figure 3A), which indicates that ANS was dissociated from the protein. Therefore, we concluded that poly(dT) dissociates ANS, which is bound to RecA, from its binding sites. This means that the quenching of ANS fluorescence promoted by ssDNA binding to the RecA−ANS complex can be used to measure the binding isotherm for ssDNA.

Next, we plotted the difference of the fluorescence intensities of samples with and without RecA against the molar ratio of poly(dT) to RecA (Figure 3B, squares). The fluorescence intensity decreased linearly in proportion to the poly(dT) concentration. At ratios above 3.5, all the fluorescence derived from the RecA−ANS complex was essentially quenched. The fluorescence decrease showed a sharp break point at a poly(dT):RecA ratio of 3.5. The observation that the break point in this binding curve was sharp indicates a small dissociation constant compared with the protein concentration used. Therefore, this result indi-

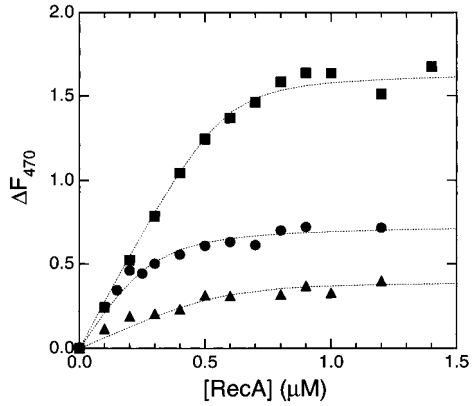

FIGURE 4: Fluorescence titration of ssDNA with RecA in the presence of ANS. Each ssDNA (2 μM) was incubated with 100 μM ANS in 50 mM Tris-HCl at pH 7.5 and 25 °C for 30 min, and then the solution was titrated with RecA. The differences between fluorescence intensity at 470 nm (excited at 370 nm) in the absence and presence of ssDNA were plotted against the RecA concentration. Squares, circles, and triangles represent the data for poly(dT), poly(dC), and poly(dA), respectively. The dotted lines represent the theoretical curves (see Experimental Procedures for details).

cates that 3.5 nucleotides were bound per protein subunit.

The effects of other polynucleotides, poly(dC) and poly(dA), on a preformed RecA−ANS complex were also examined in a similar manner (Figure 3B, circles and triangles), and the results differed from those of poly(dT). Poly(dC) quenched virtually all the ANS fluorescence as did poly(dT); however, the stoichiometric amount required for complete quenching was about 7.0, twice that required for poly(dT). By contrast, the binding curve for poly(dA) appeared to show a break point at a poly(dA):RecA ratio of 3.5; however, it quenched only about 20% of the ANS fluorescence.

The titration of ANS with RecA was also carried out in the absence or presence of ssDNA. Figure 4 shows the plots of the difference between the fluorescence intensities at each titration point for samples, with and without ssDNA, against protein concentration. As these differences represent the amount of bound DNA, these plots are considered to be binding curves for the RecA−ssDNA interaction. When the site size for each ssDNA polynucleotide is assumed, the dissociation constants were calculated from these binding curves. The data for poly(dT), poly(dC), and poly(dA) closely fitted the equation when the site sizes were assumed to be 3.5, 7.0, and 3.5, respectively. The dissociation constants for poly(dT), poly(dC), and poly(dA) were determined to be 19, 43, and 33 nM, respectively. However, the curves for the increase in the fluorescence with increasing polynucleotide concentrations fell close to the lines expected for fully stoichiometric binding. These limited the precision to which the dissociation constants could be determined.

As the competitive fluorescence assay was found to be useful for analyzing the interaction of RecA with ssDNA, we tested whether this method could be applied to the analysis of the interaction of RecA with ssDNA in the presence of ATP (or ATPγS). However, such analysis was unsuccessful because ATP and ATPγS quenched the fluorescence of ANS bound to RecA, as described in the following section. The quenching of ANS fluorescence by ATP also prevented the analysis of the interaction of RecA with dsDNA, because the binding of RecA to dsDNA requires ATP or ATPγS.

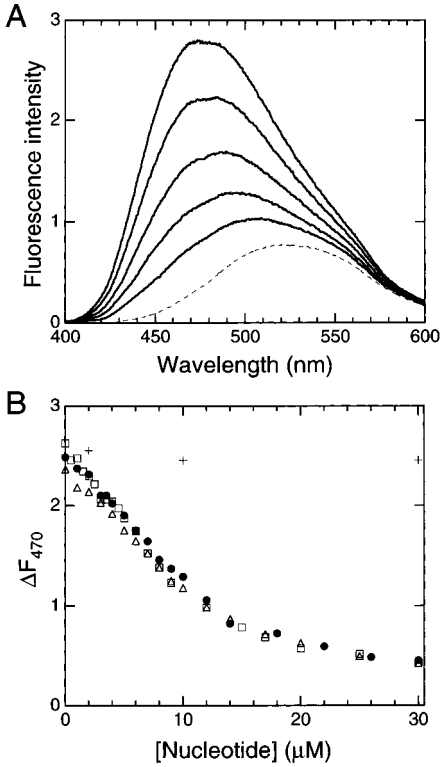

FIGURE 5: Fluorescence quenching of ANS bound to RecA with adenine nucleotides. (A) Emission spectra of 1 μM RecA and 100 μM ANS in the presence of ATP at 0, 5, 10, 14, and 30 μM (solid lines, from top to bottom). The measurement conditions were the same as those described in the legend of Figure 3A except for the presence of 5 mM $MgCl_2$. The broken line represents the spectrum of 100 μM ANS alone. (B) Titration of 1 μM RecA with adenine nucleotides in the presence of 100 μM ANS. The measurement conditions were the same as those described for panel A. The difference between the emission intensities at 470 nm in the presence and absence of RecA was plotted against the concentration of each nucleotide: (●) ATP, (□) ATPγS, (▲) ADP, and (+) AMP.

Quenching of ANS Fluorescence by Nucleotides. When ATP was added to a solution of RecA equilibrated with ANS in the presence of $MgCl_2$, the ANS fluorescence emission intensity was decreased and a red shift in the ANS fluorescence emission maximum was observed in a way similar to that of the effect of ssDNA (Figure 5A). As the ATP concentration was increased, the intensity of the fluorescence decreased in a slightly sigmoidal way (Figure 5B). The lack of $MgCl_2$ led to no quenching of the fluorescence emission (data not shown), which indicated no binding of ATP to RecA. The effects of other adenine nucleotides or an analogue are shown in Figure 5B. ATPγS and ADP, both of which can bind to RecA, were shown to have similar effects on the fluorescence emission of ANS bound to RecA. When the data were fit to the Hill equation, the apparent dissociation constants for these nucleotides were calculated to be approximately 9 μM. No effect of ATP, ADP, or ATPγS on the fluorescence spectrum of free ANS in aqueous solution was observed. No significant effect on ANS fluorescence was observed upon addition of AMP, which cannot bind to RecA (*36*). These results suggest that nucleotides capable of binding to RecA can dissociate ANS from RecA.

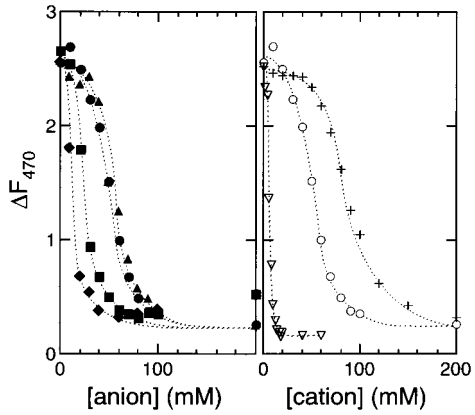

FIGURE 6: Fluorescence quenching of ANS bound to RecA with salts. Emission spectra of 1 μM RecA and 100 μM ANS in the presence of various concentrations of salts. The measurement conditions were the same as those described in the legend of Figure 3A. The difference between the emission intensities at 470 nm in the presence and absence of RecA was plotted against concentration of each salt: (left panel) (●) potassium chloride, (▲) sodium chloride, (■) magnesium chloride, and (◆) calcium chloride and (right panel) (○) potassium chloride, (▽) potassium sulfate, and (+) potassium acetate.

Quenching of ANS Fluorescence by Salts. To evaluate the importance of electrostatic affinity in ANS binding to RecA, the effect of salts on the binding of ANS to RecA was examined. Potassium chloride was the first salt tested in the competitive fluorescence assay. The KCl was able to quench the fluorescence of the RecA−ANS complex. The decrease of the fluorescence intensity at 470 nm was sigmoidal, and emission was quenched completely at concentrations of KCl above 100 mM, as shown in Figure 6 (circles).

To determine whether the quenching observed with KCl was due to an anion- or cation-specific interaction with RecA, the test was repeated for the chloride salts of Ca^{2+}, Mg^{2+}, and Na^{+}. Each of the chloride salts examined quenched the fluorescence of the RecA−ANS complex (Figure 6, left panel). The salts composed of divalent ions, Ca^{2+} and Mg^{2+}, quenched the fluorescence more efficiently than the monovalent ions. Next, the relative ability of anions such as the potassium salt to dissociate ANS from RecA was examined for SO_4^{2-}, CH_3COO^{-}, and Cl^{-} (Figure 6, right panel). Again, the efficiency of quenching by the salt composed of divalent ions was greater than that of monovalent ones. Potassium sulfate, in particular, proved to be a most effective quencher of the fluorescence of the RecA−ANS complex. Nevertheless, all the salts examined were able to dissociate ANS from RecA, regardless of their type of charge. These results suggest that a hydrophobic interaction is not necessarily the predominant force in ANS binding to RecA.

Quenching of ANS Fluorescence by Urea. ANS has often been used to study the conformational states of a protein through a folding pathway. Folding intermediates with compact but loosely folded tertiary structures promote a dramatic increase in the fluorescence intensity of ANS, suggesting a molten globule state in proteins. To characterize the relationship between ANS binding and the conformational state of RecA, the effect of urea on the RecA−ANS complex was investigated.

As shown in Figure 7, the fluorescence emission of ANS decreased as the urea concentration increased. These results have been corrected for ANS fluorescence quenching due to urea alone. The fluorescence emission was abolished completely at concentrations of urea above 6 M. The titration curve seems to be biphasic: 0−2 and 2−6 M urea. This behavior may be associated with the denaturing of RecA by urea, which we have investigated intensively (*41*) (see Discussion).

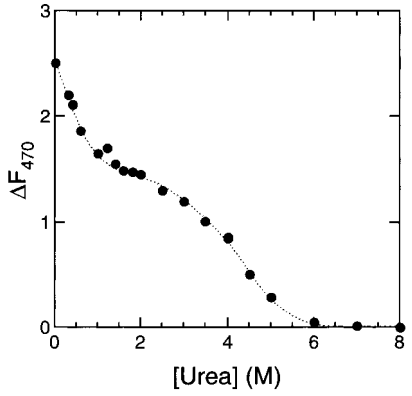

FIGURE 7: Fluorescence quenching of ANS bound to RecA with urea. Emission spectra of 1 μM RecA and 100 μM ANS in the presence of various concentrations of urea. The measurement conditions were the same as those described in the legend of Figure 3A. The difference between the emission intensities at 470 nm in the presence and absence of RecA was plotted against the concentration of urea.

Photoincorporation of ANS into RecA. It has been thought that the use of hydrophobic probes is not adequate to gain specific information about the location of binding sites on a protein. Recently, Seale and co-workers reported that bis-ANS can be incorporated into several proteins, including GroEL, by UV irradiation (*26*). Their report prompted us to investigate whether ANS, a monomeric form of bis-ANS, can be photoincorporated into RecA.

RecA was irradiated with UV light in the presence of various concentrations of ANS, and the amount of labeled protein was assessed on polyacrylamide gels after electrophoresis. Figure 8 shows the dependence on the ANS concentration of the photoincorporation of ANS into RecA. The labeled protein on a polyacrylamide gel was visualized with UV light. The amount of labeled RecA increased with increasing ANS concentration. Photoincorporation appeared to reach saturation around 2 mM ANS for 10 μM RecA. No fluorescence was observed for the sample without UV irradiation even at a high concentration of ANS (Figure 8, −UV). These results indicate that UV irradiation can cause incorporation of ANS into RecA. This reaction is also dependent on the intensity of the UV light, but no significant increase in the degree of photolabeling was found above 10 J/cm^2 (data not shown). However, as described later, the efficiency of photoincorporation was very low. Thus, the proportion of RecA molecules that were fluorescence labeled was low, although labeled and unlabeled molecules could not be distinguished from each other on the gel.

Covalent binding of ANS to RecA was also examined by gel filtration analysis of the UV-irradiated solution of RecA and ANS. Three peaks were found on the elution profile, as shown in Figure 9A. Peak 1 was eluted in the void volume of the column and contained RecA protein with fluorescence, which was identified by SDS−PAGE (data not shown). This RecA band contained both labeled and

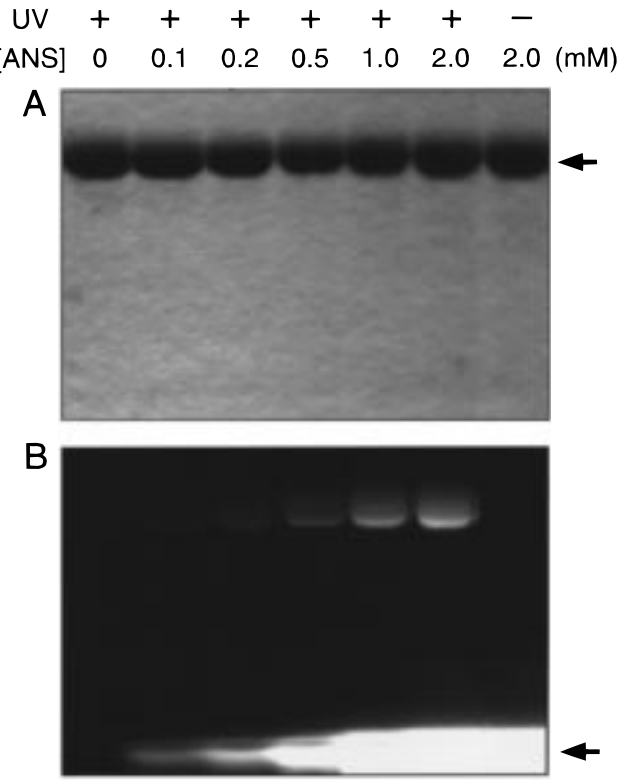

FIGURE 8: Photoincorporation of ANS into RecA. The solutions of 10 μM RecA and ANS at the indicated concentrations were irradiated by UV light (312 nm) in 25 mM Tris-HCl (pH 7.5). The irradiated samples were subjected to SDS−PAGE. (A) RecA was stained with Coomassie brilliant blue. The arrow indicates the position of free RecA. (B) The fluorescence from ANS was visualized on a transilluminator (312 nm). The arrow indicates the position of free ANS.

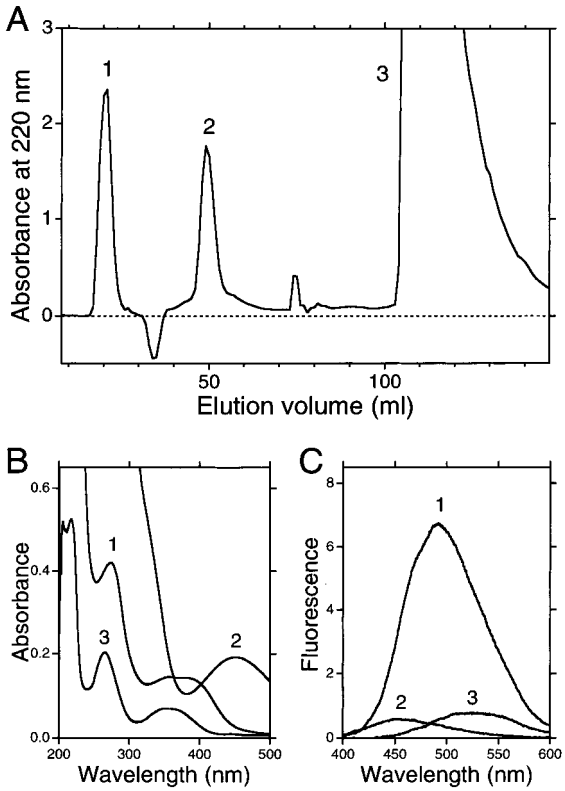

FIGURE 9: Properties of ANS-incorporated RecA. (A) Elution profile of an UV-irradiated RecA−ANS solution on a Sephacryl S-200 column. The sample was prepared by irradiation of 10 μM RecA in the presence of 2 mM ANS in 25 mM Tris-HCl (pH 7.5). (B) UV spectra of fractions from the peaks indicated in panel A. The spectrum of one fraction from each peak was measured at 25 °C with a 1 cm light path cell. (C) Fluorescence emission spectra of fractions from the peaks indicated in panel A. The spectrum of one fraction from each peak was measured at 25 °C with an excitation wavelength of 370 nm in a 5 mm × 5 mm cell.

unlabeled RecA molecules because unlabeled RecA alone also eluted in the void volume of this column. This result suggests that the RecA−ANS complex retains the ability to interact with other RecA molecules. In addition, peak 1 had an absorption maximum at around 360 nm in addition to that at 280 nm and an emission maximum at 480 nm by excitation at 370 nm (Figure 9B,C). These results indicate that ANS was bound to RecA in peak 1. Peaks 2 and 3 were eluted in elution volumes, approximately 50 and 110 mL, respectively, that were larger than the bed volume of the column (approximately 34 mL), which suggested nonspecific adsorption of the materials in these peaks to the resin. Peak 3 showed UV and fluorescence spectra that were typical of free ANS, whereas peak 2 showed spectra that were distinct from those of ANS. Peak 2 was likely to be a derivative of ANS formed by UV irradiation since this peak was found in the chromatogram of UV-irradiated ANS in the absence of RecA (data not shown). Nonspecific adsorption to chromatographic resin has been reported for several dyes (*42*, *43*).

To determine the ANS-binding site on the protein, proteolytic fragmentation of photolabeled RecA was performed, followed by isolation of ANS-bound peptides. The fragmentation was carried out by in-gel digestion of labeled RecA (see Experimental Procedures). The digest recovered from the gel pieces was separated on a reverse-phase HPLC column at acidic pH. The elution was monitored by both absorbance and fluorescence. The elution profiles are shown in Figure 10A. The absorbance profiles of the irradiated and nonirradiated samples showed no significant difference (data not shown). However, several peaks with fluorescence emission were detected only in the UV-irradiated sample (Figure 10B). This indicates that the ANS-labeled peptides were identified by their fluorescence derived from bound ANS.

Sequence analyses of the peak fractions detected by fluorescence revealed that they contained at least two distinct peptides. Then, the fluorescence fractions were further purified by repeated chromatography on the same column but with an improved elution gradient. The obtained fractions were subjected to sequence analysis, but only two of them could be matched to amino acid sequences in RecA. One of these sequences was TXAXXDAE, which was matched to residues 89−96 (TCAFIDAE). On the basis of the specificity of the protease, this peptide was likely to correspond to residues 89−106, since residues 88 and 106 are lysine. The other sequence, SGAVDVIVVDXVAALTPK, corresponded to residues 135−152 (X is serine). It should be noted here that residue 134 is arginine. Although this peptide appears to be inconsistent with the substrate specificity of lysylendopeptidase, nonspecific cleavage of peptide bonds after an arginine residue has been reported in several cases (*44*−*46*). The former peptide was only partly sequenced, but for the latter peptide, significant amounts of PTH−amino acids were recovered for all residues (a low yield of PTH−Ser is expected under normal conditions).

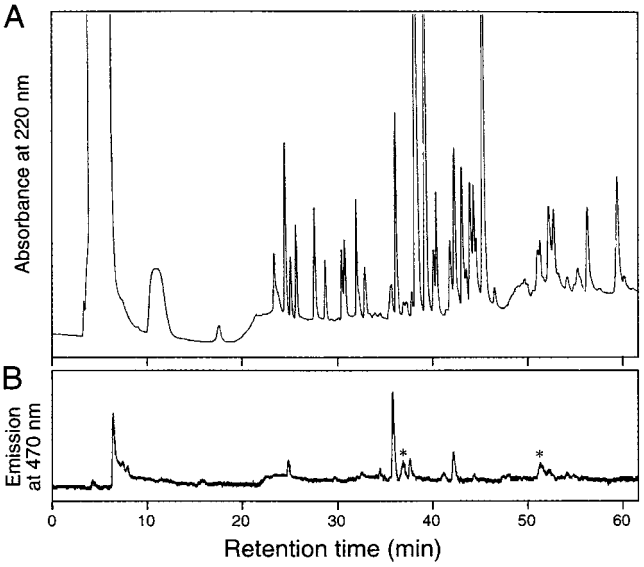

FIGURE 10: Isolation of the ANS-labeled peptides of RecA. The UV-irradiated solution of RecA (10 μM) and ANS (2 mM) was subjected to SDS−PAGE, and fluorescent RecA bands were excised and digested in the gel with lysylendopeptidase. The recovered digest was separated with a reverse-phase HPLC column. (A) Elution profile monitored by absorbance at 220 nm. (B) Elution profile monitored by fluorescence emission by excitation at 370 nm. The peaks with asterisks contained peptides which could be matched to the amino acid sequences in RecA.

Thus, cross-linked residues were not identified in either peptide. The other fluorescent fractions showed no significant amount of amino acids in sequencing, implying that they contained very small amounts of peptides or unknown derivatives of ANS.

DISCUSSION

In this study, we have demonstrated that ANS can be used as an extrinsic fluorescent probe for analyzing the interactions of RecA with several ligands, especially ssDNA. RecA was shown to have approximately three binding sites for ANS. Multiple binding sites for ANS have been reported for other proteins in their native conformation (*47*). Although ANS and its derivatives have been called hydrophobic probes, the salts affected the binding of ANS to RecA (Figure 6), suggesting that charged groups contribute to the interaction. Several lines of evidence (*48*, *49*) suggest that these fluorescent probes bind to hydrophobic regions situated near water; thus, the planar aromatic rings are in contact with hydrophobic regions, whereas the charged groups are in contact with water.

One candidate for an ANS-binding site in RecA is the nucleotide-binding site, which is more hydrophobic than the rest of the surface area (*5*). It has been reported that hydrophobic probes, including ANS, have a particular affinity for proteins with nucleotide-binding sites (*21*, *23*, *47*). This possibility is supported by the observation that the cross-linked regions, identified as residues 89−106 and 135−152, include residues which form part of the nucleotide-binding pocket of RecA (*5*). In addition, the apparent affinity of RecA for adenine nucleotides observed in this study was close to that obtained in the circular dichroism spectral change study (*36*). Therefore, some of the bound ANS molecules seem to be located at or near the nucleotide-binding site of RecA. It has been suggested that the aromatic rings and sulfonate group of ANS may mimic the base and phosphate group of the nucleotides (*19*). Involvement of the sulfonate group may be supported by the effective quenching of ANS fluorescence by potassium sulfonate (Figure 6).

Another candidate is the subunit interface of RecA in oligomeric states. RecA at 1 μM exists predominantly as a monomer and a hexamer (*50*, *51*), and in crystalline forms, the subunit interfaces of RecA show relatively high hydrophobicity (*6*). This possibility is supported by significant quenching of ANS fluorescence by about 1 M urea, which causes RecA oligomers to dissociate to monomers with partial unfolding of the N-terminal domain (*41*). The observation that binding of ANS to RecA became more cooperative at higher protein concentrations (α_H in Table 1) also supports this possibility because RecA forms larger oligomers as the protein concentration increases (*51*).

The release of ANS can be explained in two ways. One is the displacement of ANS directly by the ligand; in this model, ANS competes with the ligand for the binding site of the protein, which means there is overlap of the ANS-binding site and the ligand-binding site. If the nucleotide-binding site is one of the ANS-binding sites, the displacement of ANS by adenine nucleotides would be explained by this model. On the other hand, it is uncertain whether this notion can explain the displacement by ssDNA because the ssDNA-binding sites of RecA have not been determined. Several regions are proposed to be ssDNA-binding sites (*7*−*10*, *15*, *52*). Of these regions, residues 89−106 and residues 61−72 are of special interest because they include an element of the nucleotide-binding site (*6*). If these regions are involved in ssDNA binding, ANS molecules binding at or near the nucleotide-binding site would be displaced by binding of ssDNA as well as by nucleotides.

The other explanation is that ANS is released indirectly as the result of a conformational change of its binding site on the protein. The contacts between RecA subunits in the hexameric ring in solution are thought to differ from those in the spiral filaments that form on ssDNA (*53*). Within the subunit itself, a conformational transition from loop L2 to a β structure may occur upon binding to ssDNA (*54*). It has also been reported that binding of adenine nucleotides affects the oligomeric state of RecA (*55*−*57*). Such structural changes could result in the release of ANS by affecting the ANS-binding site.

Few instances have been reported of the application of a competitive fluorescence assay to a protein−DNA interaction (*19*). In this study, the binding of each of the three polynucleotides examined decreased the ANS fluorescence of the RecA−ANS complex. Although the affinities of the polynucleotides for the RecA−ANS complex were not significantly different from each other, the order of the calculated dissociation constants for RecA was as follows: poly(dT) > poly(dA) > poly(dC). This result is consistent with a previous study in which the affinities of polynucleotides were assessed by competition experiments using fluorescent DNA (*17*). However, the site size for the polynucleotides depended on their base compositions; it was 3.5 nucleotides per RecA monomer for poly(dT) and poly(dA), whereas it was 7.0 nucleotides for poly(dC).

Several lines of enquiry have established that RecA filaments bind to ssDNA at a site with a size of 3 nucleotides in the presence of ATPγS or ATP analogues (*2*). By contrast, the binding stoichiometries of RecA−ssDNA filaments in the absence of a nucleotide cofactor are not well-established, and different methods have produced different results. A flow linear dichroism and fluorescence spectroscopy study (*58*) indicated that 3.5−4.0 nucleotides bind to each RecA monomer, which is very close to the values obtained in this study for poly(dT) and poly(dA). However, electron microscopic analysis suggested the stoichiometry of 5 nucleotides per RecA monomer (*59*). It is uncertain at present whether these differences are significant or not. In either case, it is almost certain that the addition of ATPγS reduces the stoichiometry to 3 nucleotides per RecA monomer. A recent NMR study revealed the extended structure of the ssDNA bound to RecA in the presence of ATPγS (*60*). Similar studies may also determine the structure of ssDNA bound to RecA in the absence of ATPγS.

The site size for poly(dC) obtained in this study was approximately twice as large as that for poly(dT). A site size of 6−8 nucleotides has also been observed when other methods, such as fluorescence quenching using etheno-DNA, were employed (*61*). This apparent inconsistency has been explained by a model in which the RecA filament has two distinct ssDNA-binding sites (*62*). According to this model, monitoring of the RecA−ssDNA interaction by a DNA-based signal reflects the occupancy of both ssDNA-binding sites, which may lead to an overestimation of the site size. In addition, RecA may be kinetically trapped in a complex, in which both ssDNA-binding sites are occupied, when RecA is added in small amounts to ssDNA. In this study, however, interaction between RecA and ssDNA was monitored by a protein-based signal, that is, the fluorescence from the protein modified with an extrinsic fluorescent probe, and titration experiments were performed by directly mixing RecA with ssDNA at various concentrations.

In this regard, it is of interest that poly(dC) can form a double helix, in which the bases are hemiprotonated and the strands are parallel (*63*, *64*). This self-assembly is stable at neutral pH (*64*). To confirm the presence of a double-helix form of poly(dC) under the conditions employed in this study, we measured the CD spectrum in the near-UV region (Figure 11). At pH 7.5, poly(dC) alone showed a positive peak at 286 nm and a negative peak at 265 nm. These features are characteristic of protonated C·C$^+$ base pairs in a double helix (*64*). When RecA was added to poly(dC), the CD spectrum still had a significant CD band above 290 nm, suggesting the presence of a double helix. Therefore, the observation that the binding site of poly(dC) was twice as large as the size of the binding sites of the other ssDNAs examined can be explained by the notion that poly(dC) strands bind to RecA as a double helix. This notion also explains the results of our reverse titration experiment (Figure 4). In this experiment, poly(dC) was saturated by half the amount of RecA required to saturate the other two polynucleotides, which resulted in a small fluorescence change for poly(dC), which was half of that observed for the other polynucleotides. It is unclear at present whether two parallel strands of poly(dC) double helix bind to one ssDNA-binding site or whether each strand binds to a separate ssDNA-binding sites.

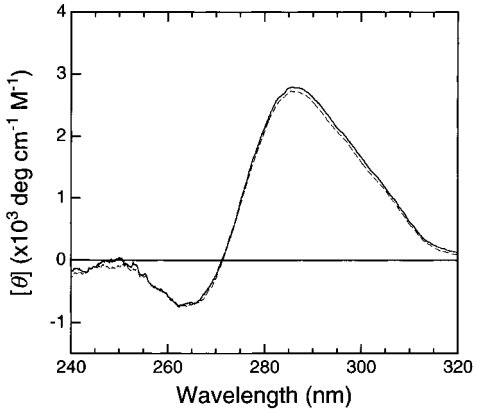

FIGURE 11: CD spectra of poly(dC) in the near-UV region. The spectra of 7 μM poly(dC) were measured in the absence (solid line) and presence (dashed line) of 1 μM RecA. The latter spectrum was derived by subtracting the spectrum of RecA alone from the observed spectrum. Measurements were performed at 25 °C in 50 mM Tris-HCl (pH 7.5) in a 5 mm cell.

A significant difference was also observed in the extent of quenching by the respective polynucleotides; poly(dT) and poly(dC) completely quenched the ANS fluorescence, whereas quenching by poly(dA) was less effective. This result suggests that only a small fraction of the bound ANS was dissociated from RecA by poly(dA). This phenomenon may be explained by the differences in the binding site on RecA and/or by conformational change upon formation of the complex. Although it seems unlikely that the binding site of poly(dA) on RecA is distinct from those of the other polynucleotides, it is probable that the conformational change (and structure of the resulting nucleoprotein filament) caused by poly(dA) binding is different from those caused by the other polynucleotides. The conformational changes that occur upon binding of RecA to ssDNA in the absence of nucleotide cofactors have not been reported in detail. In terms of ssDNA-dependent ATPase activity, poly(dA) had a lower turnover than poly(dT) and poly(dC) (*65*, *66*). This observation suggests that the structure of the nucleoprotein filament formed by each polynucleotide differs in the presence of ATP. Similar differences may exist between the filaments formed in the absence of ATP. The intrinsic properties of each polynucleotide may be responsible for these differences.

To obtain further specific structural information, we examined the photoincorporation of ANS into RecA, inspired by a recent experiment which showed that bis-ANS can be photoincorporated into proteins (*26*), and which indicated that ANS could be incorporated into RecA using UV irradiation. To our knowledge, this is the first report of covalent binding of ANS to a protein. Neither the addition of UV-irradiated ANS to nonirradiated RecA nor the addition of UV-irradiated RecA to nonirradiated ANS resulted in incorporation of ANS into the protein (data not shown). These results may support a mechanism of photoincorporation similar to photosensitized oxidation, as proposed by Brandt and co-workers (*67*).

Fluorescent peptides were obtained by protease digestion; however, the fluorescence intensity of the peptides was very weak, and they could not be detected by gel electrophoresis. This may be explained by the disappearance of the hydrophobic surfaces, which enhanced the fluorescence emission

of ANS bound to the intact protein. In addition, the yield of the fluorescent peptides was very low, which was evident from the absence of differences between the absorption profiles of UV-irradiated and nonirradiated samples. This outcome was not unexpected because ANS is not a strong photochemical cross-linker.

In summary, the fluorescence quenching assay using ANS as an extrinsic probe was useful for analyzing the interaction between RecA and ssDNA. Furthermore, photoincorporation of ANS into the protein was utilized to gain site-specific information about the interaction. The combination of these two methods will also aid the analysis of interactions of other proteins with their ligands.

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