The Central Aromatic Residue in Loop L2 of RecA Interacts with DNA

QUENCHING OF THE FLUORESCENCE OF A TRYPTOPHAN REPORTER INSERTED IN L2 UPON BINDING TO DNA*

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DNA repair is vital for both prokaryotic and eukaryotic cells. RecA is an ubiquitous and multi-functional enzyme involved in various steps of DNA repair: regulation of synthesis of DNA repair proteins (SOS induction), promotion of homologous recombination, and mutagenesis (For reviews, see Refs. 1–3). For these activities, RecA first binds to single-stranded DNA with a strong cooperativity and forms a filamentous complex in which RecA subunits are arranged in a helical manner around the DNA (4). This nucleofilament binds a second double-stranded DNA molecule for the strand exchange reaction (5) and interacts with repressors for the induction of the SOS system (6) and with UmuD and UmuD* proteins for mutagenesis (7, 8).

The molecular structure of the RecA filament in the absence of DNA has been determined by x-ray crystallography (9). A lower resolution structure of the nucleofilament (4) and of the DNA in the complex (5) has also been determined. These studies have shown that the DNA strands lie near the axis of the RecA-DNA filament. Determination of the DNA binding sites in RecA would allow us to build a higher resolution model of the RecA-DNA complex. Despite various studies, however, the DNA binding site of RecA has not yet been determined. From the comparison of sequence with the DNA binding domain of RecA protein, we have constructed a protein in which a modified protein is active both in vivo and in vitro. The binding of nucleotide cofactor (ATP or its analog adenosine 5’-O-3-thiotriphosphate) does not modify the fluorescence. By contrast, the binding of DNA, both in the absence and presence of cofactor, strongly decreases the fluorescence in intensity (40–65%) and shifts the emission peak from 344 to 337 nm. The change occurs both with single- and double-stranded DNA and also upon the binding of a second single-stranded DNA. The results indicate that the residue 203 is in fact close to the first and second DNA binding sites. However, the quenching is not total and depends only slightly on the nature of DNA bases, thus suggesting an indirect interaction with DNA bases.

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1 The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATP-γS, adenosine 5’-O-3-thiotriphosphate; wt, wild type; poly(dA), poly(1,N6-ethenodeoxyadenosine); DTT, dithiothreitol; PIPES, 1,4-piperazinedithanesulfonic acid.

2 O. N. Voloshin, L. Wang, and R. D. Camerini-Otero, submitted for publication.
DTT, 1 mM EDTA. The suspension was centrifuged in SS-34 Sorvall rotor for 30 min at 12,000 rpm, the supernatant was discarded, and the pellet was homogenized in 25 ml of 20 mM Tris-HCl (pH 7.5), 300 mM ammonium sulfate, 10% glycerol, 1 mM DTT, 1 mM EDTA. After centrifugation, the supernatant was loaded directly onto a MonoQ column (Pharmacia Biotech Inc.), and the column was developed with a linear gradient of NaCl (0–1 M) in 20 mM Tris-HCl (pH 7.5), 1 mM EDTA.

Finally, RecA<sub>203W</sub> fraction was run through a HiLoad Superdex 75 column (Pharmacia), and the protein was dialyzed against 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.4 mM DTT, 0.1 mM EDTA.

Materials—Poly(dA) (lot no. CJ 7836103) and poly(dT) (lot no. 2067834021) were from Pharmacia. Poly(dA) was prepared by modification of poly(dA) by chloroacetaldehyde (Merck), as described (20). ATP·γS was purchased from Boehringer Mannheim. Concentration of materials was estimated from UV absorption using the following absorption coefficients: ε<sub>280</sub> = 2.17 × 10<sup>4</sup> M<sup>−1</sup> cm<sup>−1</sup> for wt RecA, ε<sub>295</sub> = 2.73 × 10<sup>4</sup> M<sup>−1</sup> cm<sup>−1</sup> for RecA<sub>203W</sub>, ε<sub>280</sub> = 8.60 × 10<sup>4</sup> M<sup>−1</sup> cm<sup>−1</sup> (in bases) for poly(dA), ε<sub>295</sub> = 8.52 × 10<sup>4</sup> M<sup>−1</sup> cm<sup>−1</sup> (in bases) for poly(dT), ε<sub>295</sub> = 1.32 × 10<sup>4</sup> M<sup>−1</sup> cm<sup>−1</sup> (in base pairs) for dsDNA, and ε<sub>295</sub> = 3.7 × 10<sup>4</sup> M<sup>−1</sup> cm<sup>−1</sup> (in bases) for poly(dA). All DNA concentrations are expressed as phosphate concentrations.

DNA Binding Assays—Oligonucleotide T-50 spanning positions 395–444 of the non-coding strand of the pUC19 plasmid and pGEM4 DNA linearized with EcoRI were used as single- and double-stranded substrates in DNA binding assays. 0.5 μM T-50 was incubated with 0–200 μM RecA in 40 μl of 25 mM Tris-HCl (pH 7.5), 20 mM NaCl, 10 mM MgCl<sub>2</sub>, 0.4 mM DTT, 10 μg/ml bovine serum albumin, 0.5 mM EDTA at 37°C. Because of the inability of RecA protein to bind dsDNA at pH 7.5 (21), the experiments on binding to dsDNA were done in buffer containing 25 mM PIPES (pH 6.2) instead of Tris-HCl (pH 7.5); dsDNA was 1.35 μM, and RecA was 0–500 μM. Complexes formed after a 30-min incubation period were filtered using a double-filter system (22) with BA85 nitrocellulose and NA-45 DEAE membranes (Schleicher & Schuell). Data were quantitated with a PhosphorImager (Molecular Dynamics).

Fluorescence Measurements—All experiments were performed at 20°C in a buffer containing 5 mM K<sub>2</sub>HPO<sub>4</sub>, 15 mM KH<sub>2</sub>PO<sub>4</sub>, and 1 mM MgCl<sub>2</sub>, pH 6.4. The concentration of RecA and ATP·γS was usually 3 μM and 10 μM, respectively. This concentration of ATP·γS should be sufficient to saturate 1 μM RecA (23, 24). DNA-RecA complexes were formed by addition of DNA to RecA solutions. For complete formation of ATP·γS-RecA-DNA complexes, the mixture was incubated at least for 30 min at 20°C.

Fluorescence was measured in a FP-777 spectrofluorometer (Jasco) at 20°C. The excitation wavelength was set at 295 nm with a bandwidth of 3 nm for selective excitation of tryptophan residues. The emission was usually measured at 345 nm with a bandwidth of 5 nm. For the measurement of excitation spectra of poly(dA) fluorescence, the fluorescence was observed at 450 nm to minimize the contribution of fluorescence from tryptophan residues. The bandwidths were 3 and 5 nm for excitation and emission, respectively. Fluorescence was measured in a 1.0 × 0.4-cm quartz cell to minimize the inner filter effect. The spectra were also corrected for background and Raman scattering by subtracting the buffer signal. Spectra were averaged over at least two scans. The absorption was measured in a J-710 spectrophotometer (Jasco) in UV absorption mode. The quartz cell was treated by silicon (Serva) to avoid the adsorption of protein to the wall.

Fluorescence polarization anisotropy was measured in a FP-777 spectrofluorometer with the aid of ADP-301 automatic anisotropy measurement apparatus (Jasco). The temperature was set at 20°C, and the excitation and emission wavelengths were set at 295 nm (bandwidth of 3 nm) and 345 nm (bandwidth of 5 nm), respectively. The signal was averaged over 25 measurements of 0.5 s.

RESULTS

Activity of RecA<sub>203W</sub>—Using site-directed mutagenesis, we introduced single amino acid substitution Phe<sub>→</sub>Trp in loop L2 of the RecA protein. Mutant RecA<sub>203W</sub> protein was tested in DNA binding assay, and its activity was compared with wt RecA (Fig. 1). One can see that mutant RecA<sub>203W</sub> protein is able to bind both ss- and dsDNA at the same, or even higher affinity, compared to wt protein. These data are in good agreement with our results on synthetic peptides spanning loop L2 region; Trp-substituted peptide possesses higher affinity to both ss- and dsDNA than the wild type Phe-containing peptide.

<sup>2</sup>E. Angov, O. Voloshin, and R. D. Camerini-Otero, unpublished observation.
fluorescence (not shown), the absence of a fluorescence change was not due to a reduced affinity of the modified protein for ATPγS. The addition of the physiological cofactor, ATP, up to 0.5 mM did not significantly modify the fluorescence of wt RecA or RecA F203W. The cofactor did not affect the fluorescence of Trp-203 and thus did not modify its environment. The anisotropy of Trp-203 fluorescence was also not modified upon binding to ATPγS. Thus the local motion of the residue was not affected by ATPγS. The anisotropy of RecA F203W was 0.150 and as large as that of wt RecA (0.145) both in the presence and absence of ATPγS. By subtraction of contribution from Trp-290 and Trp-308, which are also present in wt RecA, we got an anisotropy value r = 0.15 for Trp-203. The value was large and indicated that the local motion of Trp-203 was restricted. The local motion was slower than the lifetime of tryptophan, which is in the order of nanoseconds.

Quenching of Trp-203 fluorescence upon DNA Binding in the Absence of Nucleotide Cofactor—Fig. 3 shows the fluorescence change of RecA F203W upon the addition of poly(dA) or poly(dT) in absence of cofactor. There was a large decrease in the intensity of RecA F203W fluorescence in contrast to no significant change of wt RecA fluorescence. The signal was corrected for the increase of the inner filter effect upon the addition of polynucleotides. Since these fluorescence experiments were made with a selective excitation of tryptophan residues, the fluorescence change was certainly due to a quenching of Trp-203 upon the binding of ssDNA.

The fluorescence change was significantly smaller for poly(dA) (21% quenching) than poly(dT) (32% quenching). The former exhibits weaker binding affinity to RecA than poly(dT) (20). However, this difference was not due to an incomplete formation of RecA·poly(dA) complex because, as Fig. 4 shows, the fluorescence change was saturated about 5 bases of polynucleotide per RecA monomer for both poly(dA) and poly(dT).

**Fig. 2.** No significant fluorescence change of Trp-203 reporter in RecA upon the binding of nucleotide cofactor. In panel a, the fluorescence emission spectra of 1 μM RecA F203W with (dashed line) and without (heavy solid line) 20 μM ATPγS are shown and compared with the spectrum of wild type RecA with (dotted line) and without (thin solid line) ATPγS. The excitation wavelength was set at 295 nm for selective excitation of tryptophan residues. In panel b, the spectrum of wt RecA is subtracted from those of RecA F203W for an estimation of emission spectrum of Trp-203 reporter in the absence (thick solid line) and presence of ATPγS (dashed line). The two spectra are almost completely superimposable.

**Fig. 3.** Fluorescence change of Trp-203 reporter in RecA upon the binding of ssDNA in the absence of cofactor. Emission spectra of 1 μM RecA F203W are measured in the absence (thick solid line) and presence of 9 μM (in bases) of poly(dA) (dashed line) or poly(dT) (dotted line). The spectra of 1 μM wild type RecA with (broken line) and without (thin solid line) 9 μM poly(dT) are also shown with thinner lines.
similar binding stoichiometry was observed for wt RecA (29). Therefore, the slight difference between poly(dA) and poly(dT) may reflect a slight difference in the binding mode or the stability of the complexes. When we consider that the difference of fluorescence between RecA<sub>F203W</sub> and wt RecA simply corresponds to the fluorescence of Trp-203, the binding of poly(dA) and poly(dT) quench about 40 and 60% of Trp-203, respectively.

Quenching of Trp-203 Fluorescence upon DNA Binding in the Presence of Nucleotide Cofactor—Fig. 5 shows fluorescence change of RecA<sub>F203W</sub> upon the binding of poly(dA), poly(dT), or plasmid dsDNA in the presence of ATP·S. We can observe a large decrease of the fluorescence of RecA<sub>F203W</sub> upon complex formation in contrast to no significant fluorescence change for wt RecA upon interaction with any DNA. The absence of change in tryptophanyl fluorescence of wt RecA upon DNA binding has been reported previously (25). The addition of any DNA with a ratio of 3 bases (or base pairs for dsDNA) per RecA, which should be just enough to saturate first site (site I), decreases the fluorescence of the protein about 20%. There was no significant difference for different DNAs. Poly(dA), poly(dT), and linearized ds plasmid DNA quenched the fluorescence to a similar extent. Further addition up to 9 base pairs per RecA of dsDNA did not any more change the fluorescence, while the addition of single-stranded polynucleotide further decreased the fluorescence (Fig. 5). We note that RecA bound only one chain of dsDNA with a stoichiometry of 3 base pairs per subunit but can bind up to three chains of ssDNA with a stoichiometry of 3 bases per subunit for each chain (29, 30). The titration experiments indicate that RecA<sub>F203W</sub> binds to DNA with a similar binding stoichiometry as wt RecA (Fig. 4b). The fact that the addition of single-stranded polynucleotides exceeding the ratio of 3 bases per RecA further modifies the protein fluorescence is in contrast to the case with dsDNA and suggests that the binding of a second ssDNA (occupation of site II), as well as that of the first ssDNA (occupation of site I), decreases the fluorescence of Trp-203. The binding of the first DNA quenched about 40% of the Trp-203 fluorescence and that of the second DNA about 20%. The binding of a third DNA (occupation of site III) did not appear to affect the fluorescence (Fig. 4b). The same conclusion was obtained using heat-denatured calf thymus DNA as ssDNA. A significant blue shift in the emission spectra was observed upon the binding of two ssDNAs (not shown).

Absence of Efficient Energy Transfer between Trp-203 and DNA Bases—The results above indicate that Trp-203 could be close to the DNA. To gain more structural information, we have investigated the presence of singlet-singlet energy transfer from the tryptophanyl residue to the fluorescent nucleobase of poly(dA), an analog of poly(dA). The presence of energy transfer has been examined by a change in shape of excitation spectra of poly(dA) upon the binding of RecA as it was performed for a poly(dA)-GP32 protein complex by Toulme and Helene (31). The emission spectrum of tryptophan fluorescence partially overlaps with the absorption spectrum of poly(dA). Energy transfer, therefore, can occur and should be detected by enlargement of the excitation spectra of poly(dA) in the tryptophan absorption region (around 280 nm). In Fig. 6, the excitation spectrum of poly(dA) with RecA<sub>F203W</sub> is shown and compared with the spectrum of poly(dA) without RecA and that with wt RecA. The fluorescence signal from the protein is subtracted. The fluorescence of poly(dA) is strongly enhanced upon the binding of RecA<sub>F203W</sub>, although smaller than that by wt RecA (a 6.5-fold increase compared with a 9-fold increase by wt RecA).

To facilitate the comparison of spectral shape, we have computed the ratio of fluorescence of complex/fluorescence of free poly(dA) at various wavelengths. The ratio is almost constant and independent of wavelength. There is not a large change in spectral shape upon the binding of RecA<sub>F203W</sub>. No significant change is observed upon the binding of wt RecA as reported previously (25). Since the two chromophores (Trp-203 and DNA bases) in the complex should be oriented (judged from their large anisotropy value), the absence of a large energy transfer between them could be due to their unfavorable relative orientation. The increase of poly(dA) fluorescence by the modified RecA may occur by the mechanism described for wt RecA (20). Decrease in the mobility of the DNA bases upon RecA binding prevents the base-base collision and thus the dynamic quenching of poly(dA) fluorescence.

**DISCUSSION**

By monitoring the fluorescence change of a tryptophan reporter residue inserted in loop L2 of RecA, we have investigated the role of this residue in the loop in its interactions with cofactor and DNA. Loop L2 in RecA crystal does not provide x-ray diffraction and is considered to be disordered or in several different conformations (9). This loop was proposed as a ssDNA binding site by Story et al. (9). Subsequent work has confirmed this proposal (15) and extended it by showing that this loop also binds to dsDNA.²

The tryptophan reporter was inserted in the place of phenyl-
alanine, another aromatic amino acid, and does not appear to significantly alter the activities in vitro or in vivo of RecA. The modified RecA enhances the fluorescence of the DNA analog poly(dεA) as does wt RecA. The binding stoichiometries are not affected by this substitution. These results also suggest that the structure of the DNA-RecA complex is not so much altered by the insertion of the tryptophan. Therefore, the conclusions obtained from this fluorescence study of this tryptophan reporter should reflect the role of Phe-203 in wt RecA. The fluorescence changes of the modified RecA can be used in future, for kinetic and thermodynamic analysis of RecA-DNA interaction.

Restricted Motion of Trp-203—The distance between the two ends (residue 194 and residue 210) of loop L2 in the crystal is only 0.7 nm, whereas loop L2 is 14 amino acid residues long. The loop could exist in various conformations, and residue 203, which is at the center of the loop, could be far from the ends of the loop. The fluorescence of Trp-203 estimated from the difference between the fluorescence of modified and wt RecA is centered around 345 nm and thus indicates that the residue is exposed to solvent. A large anisotropy value indicates that the local motion of Trp-203 is restricted. Thus, the loop should not be in fast motion, and the absence of diffraction from the loop in the x-ray analysis is probably due to the presence of various conformations.

No Interaction with Nucleotide Cofactor—Absence of a fluorescence change upon the binding of nucleotide cofactor indicates that the residue is far from the cofactor. Story and Stetitz (32) proposed that Gln-194 in the loop may interact with γ phosphate of ATP, but we did not observe a significant fluorescence change of Trp-203. There may be a rather large distance between Phe-203 and the γ phosphate of ATP. The absence of fluorescence change upon cofactor binding also suggests that the residue may not be in the subunit-subunit interface because the cofactor is known to modify the helical organization of the protein (5, 33) probably by changing subunit-subunit interactions. In fact, the fluorescence intensity of RecA<sub>203W</sub> as that of wt RecA, is simply proportional to the protein concentration from 10 nM to 2 μM (not shown). The fluorescence is thus independent of association and dissociation of RecA subunits.

The Aromatic Residue in Loop L2 Is Probably Involved in DNA Binding—The large fluorescence change of Trp-203 upon DNA binding both in the presence and absence of cofactor suggests that this region could be a DNA binding domain, although the fluorescence change can also occur indirectly upon a conformation change induced by DNA binding. Site-directed mutagenesis made in L2 loop by Cazaux et al. (13) show that loop L2 is important for various activities of RecA. These results indicate that this residue is involved in DNA binding. However, the quenching of fluorescence is not total. There may not be strong stacking interaction of Trp-203 with DNA bases, although intercalation of aromatic residues between DNA bases has been proposed (34). The degree of quenching of Trp-203 fluorescence depends little upon the nature of the DNA bases. This, together with the absence of efficient energy transfer from Trp-203 to DNA bases, also suggests absence of a direct interaction between Trp-203 and the DNA bases.

Binding of Various DNA Occurs in the Same Domain—The fluorescence change occurs both in the presence and absence of cofactor. This indicates that DNA interacts with the same region of RecA in the presence and in the absence of cofactor, although the cofactor modifies the DNA binding mode of RecA. Our results also suggest that the binding of the second ssDNA occurs in the same part of RecA as that of the first ssDNA, although the fluorescence change upon the binding of the second DNA is smaller and not exactly the same. This suggests that site I and site II are very close to each other. Another interesting observation is that the fluorescence change promoted by dsDNA is similar to that by one ssDNA but smaller to that by two ssDNAs. One of the strands of dsDNA may interact with RecA in a similar way as the first ssDNA. By contrast, the second strand of dsDNA may not be interacting with this part of RecA. Such a model has been proposed previously (5). The binding of a third DNA may occur in a region different from loop L2. This is rather expected because the binding mode of a third ssDNA is clearly different from the binding mode of the first and second ssDNA (18, 30).
Interaction of Loop L2 of RecA with DNA

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