The L2 loop is a DNA-binding site of RecA protein, a recombinase from *Escherichia coli*. Two DNA-binding sites have been functionally defined in this protein. To determine whether the L2 loop of RecA protein is part of the primary or secondary binding site, we have constructed proteins with site-specific mutations in the loop and investigated their biological, biochemical, and DNA binding properties. The mutation E207Q inhibits DNA repair and homologous recombination *in vivo* and prevents DNA strand exchange *in vitro* (Larminat, F., Cazaux, C., Germanier, M., and Defais, M. (1992) *J. Bacteriol.* 174, 6264–6269; Cazaux, C., Larminat, F., Villani, G., Johnson, N. P., Schnarr, M., and Defais, M. (1994) *J. Biol. Chem.* 269, 8246–8254). We have found that mutant protein RecA<sub>E207Q</sub> lacked one of the two single stranded DNA-binding sites of wild type RecA. The remaining site was functional, and biochemical activities of the mutant protein were the same as wild type RecA with ssDNA in the primary binding site. The second mutation, E207K, reduced but did not eliminate DNA repair, SOS induction, and homologous recombination *in vivo*. In the presence of ATP, mutant protein RecA<sub>E207K</sub> catalyzed DNA strand exchange *in vitro* at a slower rate than wild type protein, and ssDNA binding at site I was competitively inhibited. These results show that the L2 loop is or is part of the functional secondary DNA-binding site of RecA protein.

RecA protein is a recombinase from *Escherichia coli* that plays an important role in chromosomal rearrangements and DNA repair. RecA exerts its biological activity by formation of a nucleoprotein structure known as a presynaptic filament, which is highly conserved throughout evolution (1–4). In this structure RecA protein binds to 3–4 nucleotides per RecA monomer and polymerizes with a helical disposition on ssDNA. Several biochemical activities of the presynaptic filament have been characterized *in vitro* including ATP hydrolysis and DNA strand exchange, the central enzymatic activity of homologous recombination. Hydrolysis of ATP is associated with an ATP-dependent affinity cycle in which RecA-ATP binds most tightly to DNA and RecA-ADP binds the least tightly, whereas RecA with no cofactor has intermediate stability (5). The tightly bound RecA-ATP complex stretches DNA 1.5 times the length of B form duplex DNA. In this conformation the presynaptic filament can invade double stranded DNA and catalyze DNA strand exchange *in vitro*.

It has been proposed for some time that RecA protein has two DNA-binding sites (6). Subsequent experiments have shown that within the nucleoprotein filament, the RecA protein monomer binds two ssDNA molecules, each with a similar binding site size (4, 7), and these sites have been functionally defined. Occupation of the first site (site I) by ssDNA is sufficient for ATP hydrolysis (7) and stimulation of autocleavage of LexA repressor protein (8) by the nucleoprotein complex *in vitro*. Once assembled in a presynaptic filament, RecA is able to bind a second molecule of DNA (site II). dDNA and ssDNA competitively bind at this site. Fixation of a second ssDNA molecule at site II does not further stimulate LexA cleavage (8) or ATP hydrolysis (7). Binding ssDNA at site II does not require Watson-Crick base pair formation with ssDNA in the primary site and may play a role in the strand exchange reaction (9, 10). It is important to determine whether functionally defined sites I and II correspond to particular peptide sequences of the RecA protein.

On the basis of the crystal structure of RecA protein in the absence of DNA, it has been proposed that the L2 loop of RecA protein (185<sup>IRMKIGVMFGNPETT</sup>209) is a DNA-binding site (11). This sequence contains positively charged and aromatic residues that are typical constituents of DNA-binding sites. In addition it has a negatively charged residue Glu<sup>207</sup> that is highly conserved in bacterial recombinases (4). Various studies have attributed the L2 loop to the primary (11, 12) or secondary (13) DNA-binding site. A third possibility is that functional sites I and II do not correspond to distinct peptide sequences but require overlapping or identical surfaces of the RecA protein.

To address this question, we have investigated two mutant RecA proteins, RecA<sub>E207Q</sub> (14) and RecA<sub>E207K</sub> (this work), that eliminate the negative charge at position 207 and replace it with an uncharged or positively charge residue, respectively. We have compared the capacities of these proteins for DNA repair, SOS induction, and homologous recombination *in vivo* as well as the DNA strand exchange and DNA-dependent ATPase activities of purified proteins *in vitro*. Finally we have used fluorescence spectroscopy to compare the stoichiometry, structure, and stability of nucleoprotein filaments formed by mutant and wild type proteins.

**Experimental Procedures**

Chemicals—Buffers, salts, and enzymes were purchased from commercial sources. ADP and ATP<sub>S</sub> were purchased from Sigma. eDNA was synthesized from single stranded M13 phage DNA (15) and freshly distilled chloroacetaldehyde (Aldrich) as described (16–18). eDNA in-
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tegrity was controlled by agarose gel electrophoresis, which revealed a single high molecular weight band. UV spectral ratios, $A_{305}/A_{280} = 1.1$ and $A_{260}/A_{280} = 1.7$, indicated a low level of etheno modification (7, 17, 18). eDNA concentration (moles nucleotide) was determined in 0.1 M Tris, pH 7.4, at 25 °C using an extinction coefficient of $e_{260} = 7000$ M$^{-1}$ cm$^{-1}$.

Mutations—recA was polymerase chain reaction amplified using primers GAGGATCCCTGTGGCAACATTTCC and CTGGAGTCCAAAGCAGTTAAATATCTTCG from a M13 phage replicative form DNA carrying the recA gene (19, 20). Upon restriction enzyme digestion a BamHI fragment containing only the recA sequence was obtained and cloned into pBR322 DNA to create pCCrecA. Mutants recA207K and recA207Q were constructed by using a 5′ polymerase chain reaction primer (CTCTACTCCGGTCTGCCTTTCATCGGA) harboring the AgeI site situated about 500 base pairs upstream the L2 site and a 3′ primer (TACACCGTATGTTGTTTTCGATACCGA) matching the L2 loop up to a second AgeI site downstream. The DNA template used was the pC8605 plasmid carrying the recA207Q, mutant (20). The &′ oligonucleotide not only brings the E207K mutation but also eliminates the HpaI site related to the G204V substitution through the reversion of this mutation toward the wild type 204 residue. This strategy allowed us to easily screen recombinant clones by restriction analysis. Upon AgeI digestion, the amplified DNA fragment was cloned into the large fragment of AgeI-digested pCCrecA to give pCCE207K recombinant plasmid. recA207Q (14), was cloned in the same vector.

Cell Survival, SOS Induction, and in Vivo Recombination Assays—UV irradiation and cell survival were measured as described previously (21). SOS induction was measured using a bacterial strain carrying a cea::lacZ fusion and measuring β-galactosidase expression as described elsewhere (14, 15). The induction factor is the ratio of β-galactosidase activities after and before irradiation. Intrachromosomal and interchromosomal recombination assays were measured using published protocols (14).

Protein Purification—RecA207Q was purified as described previously (20, 22). Wild type RecA and RecA207K were isolated from SOS-deregulated strain PC1421 (21) freshly transformed by the plasmids pCCrecA and pCCE207K, respectively, and proteins were purified as above. Protein concentrations (moles protein) were determined using $e_{280} = 2.17 \times 10^4$ M$^{-1}$ cm$^{-1}$. The purity of the final material was estimated by gel electrophoresis to be greater than 95%.

DNA Strand Exchange Assay—Strand exchange promoted by wild type and mutant proteins was followed as described (22). 9 μM M13 ssDNA, 0.5 μM E. coli single stranded binding protein, 5 μM protein, and 1 mM ATP-S were incubated at 37 °C in 25 mM Tris-HCl, 4 mM MgCl$_2$, 0.05 mM dithiothreitol, pH 7.5. The reaction was initiated by addition of 9 μM M13 dsDNA. In some experiments ATP-S was replaced by an ATP regenerating system consisting of 5 units/ml pyruvate kinase, 5 mM ADP, 1 mM ATP, 0.8 mM MgCl$_2$, 1 mM dithiothreitol, pH 7.5. The reaction was monitored at 260 nm at a constant temperature of 37 °C using a DNA strand exchange reaction between single and double stranded M13 phage DNA in the presence of E. coli single stranded binding protein and the stable ATP analog ATP-S. wtRecA protein carries out strand exchange in these reaction conditions, and RecA207Q is inactive (22); RecA207K catalyzed strand exchange as efficiently as wild type protein.

\[ \text{RecA}_{207Q} \]

\[ \text{RecA}_{207K} \]

FIG. 1. Survival of bacteria exposed to UV radiation. ΔrecA bacteria were transformed by vectors carrying wtRecA (●), RecA207K (▲), or RecA207Q (■). X, survival of ΔrecA bacteria (14).

the 4-mm path oriented in the direction of the exciting light to minimize inner filter corrections. Protein-DNA complexes formed during titration were subsequently dissociated by aliquots of a 2 M NaCl solution in TMD buffer, pH 7.4.

RESULTS

Biological Effects—To investigate the effect of charge at position 207 of the RecA protein on overall DNA repair, we measured the colony forming ability after UV irradiation of ΔrecA bacteria transformed by vectors carrying wild type and mutant recA genes (Fig. 1). Transformation of ΔrecA bacteria by a vector carrying utrecA restored survival to the level of wild type bacteria; RecA207K increased survival less efficiently than wt protein but more efficiently than RecA207Q (14).

RecA protein can modulate DNA repair by participating in the SOS response, which induces the expression of an ensemble of gene products under control of the LexA repressor. To evaluate the capacity of the mutant proteins to induce the SOS response, we measured β-galactosidase production in bacteria carrying a cea::lacZ fusion (Table I), where cea possesses a promoter regulated by the LexA protein. β-Galactosidase induction in ΔrecA bacteria transformed with pCCrecA reached a plateau of 4.5 times basal level 30 min after irradiation. Bacteria expressing RecA207K induced β-galactosidase by a factor of 2 after 120 min, and RecA207Q protein did not induce the SOS response as previously reported (14).

RecA protein also catalyzes homologous recombination. Bacteria expressing RecA207K protein carried out intrachromosomal recombination with 12% efficiency of wild type (Table I); no activity was found with RecA207Q as previously reported (14). The mutation E207K also reduced interchromosomal recombination by 2-fold (Table I), whereas bacteria expressing RecA207Q were inactive (14).

Biochemical Activities—To understand the molecular bases of these phenotypic differences, we purified wild type and mutant proteins and compared their biochemical activities in vitro. DNA bases of homologous recombination are exhibited in vitro by a DNA strand exchange reaction between single and double stranded M13 phage DNA in the presence of E. coli single stranded binding protein and the stable ATP analog ATP-S. wtRecA protein carries out strand exchange in these reaction conditions, and RecA207Q is inactive (22); RecA207K catalyzed strand exchange as efficiently as wild type protein.

\[ \text{RecA}_{207Q} \]

\[ \text{RecA}_{207K} \]

\[ \text{RecA}_{207Q} \]
TABLE I

Phenotype of bacteria expressing wild type and mutant RecA proteins

| Phenotype                        | SOS induction factor | Intrachromosomal recombination | Interchromosomal recombination |
|----------------------------------|----------------------|-------------------------------|-------------------------------|
| wtRecA                           | 2.5                  | 100                           | 100                           |
| RecA_E207K                       | 2                    | 12                            | 50                            |
| RecA_E207Q                       | 1                    | 0                             | 0.0004                        |

* Maximum level of β-galactosidase after exposure to 10 J/m² UV radiation relative to uninduced cells.
* Number of Papillae relative to wild type.
* Number of genetic crosses relative to wild type.
* Ref. 14.

(2.3). We also compared strand exchange activity of these proteins in the presence of an ATP regenerating system. Under these conditions the extent of the reaction increased; both wtRecA and RecA_E207K converted 80% ssDNA into dsDNA products. However, kinetics were significantly slower for the RecA_E207K protein; half reaction of mutant protein was observed after 30 min as compared with 10 min with wtRecA. RecA_E207Q was inactive.

RecA protein also exhibits DNA-dependent ATPase activity. ATP hydrolysis was examined in nucleoprotein filaments formed by poly(dT) (25). In one set of experiments, initial rates of ATP hydrolysis were measured using 3.0 μM poly(dT) and varying the concentration of wtRecA or mutant RecA proteins. As shown in Fig. 3a hydrolysis rates were the same for wtRecA and RecA_E207K and reached a maximum value of 0.2 μM/min. The maximum hydrolysis rate of RecA_E207Q was 50% of wild type protein. Stoichiometries of the saturated nucleoprotein filament were calculated by extrapolating the low concentration region of this titration curve to the plateau. In our experiments RecA protein, RecA_E207K and RecA_E207Q all bound poly(dT) with a stoichiometry of 3 nucleotides/protein monomer (Table II). DNA binding at site I is both necessary and sufficient for ATPase activity of RecA protein, and the stoichiometry for binding at this site is 3 nucleotides/protein monomer (7). In a second set of experiments ATP hydrolysis rates were determined using 1 μM protein as a function of poly(dT) concentration. A double reciprocal plot of these data, 1/V versus 1/(poly(dT)), gave values for V-max and K_d, apparent (Fig. 3b). Poly(dT), which lacks secondary structure, gives the maximum rate of DNA-dependent ATP hydrolysis; our kinetic parameters for wtRecA protein, k_cat = 29 min⁻¹ and K_d = 1.5 μM (Table II), are in agreement with previously reported values (25). The turnover number for ATP hydrolysis by mutant proteins RecA_E207K and RecA_E207Q was the same as that of the wild type protein. However, the apparent dissociation constant of the mutant nucleoprotein filaments were 1.4 and 4 times larger, respectively, than wild type.

DNA Binding—Binding and hydrolysis of ATP modulates nucleoprotein filament structure and stability (5). This phenomenon can be investigated using eDNA, a fluorescent derivative of phage M13 ssDNA (16–18). eDNA conformation can be followed by the fluorescence increase, which is a result of lengthening eDNA in the nucleoprotein filament. Stoichiometry of the protein-DNA complexes (nucleotides/protein monomer) can be determined from the concentration of protein necessary to saturate the fluorescence increase. Finally, stability of the protein−eDNA complex can be characterized by the salt titration midpoint, the salt concentration that produces loss of 50% fluorescence increase.

Titrations of eDNA with RecA_E207Q protein resembled in part those with wild type protein (7, 16–18, 26) (Fig. 4 and Table III). Without cofactor or in the presence of 100 μM ADP, eDNA fluorescence increased by a factor of 2 reflecting the formation of extended single stranded structures in both protein-DNA complexes. Titration with RecA or RecA_E207Q in the presence of 100 μM ATPγS increased fluorescence 2.7 times, and the additional fluorescence signal is associated with further stretching of DNA. Although the complexes with mutant protein were dissociated by slightly higher NaCl concentrations, relative stability depended on nucleotide cofactor in the same way for

FIG. 2. Kinetics of the strand exchange reaction catalyzed by wtRecA (circles) or RecA_E207Q (triangles). Closed symbols, ATPγS; open symbols, ATP regenerating system. RecA_E207Q was inactive with both nucleotide cofactors.

FIG. 3. a, ATP hydrolysis as a function of protein concentration; reaction mixtures contained 3.0 μM poly(dT) 1.0 mM ATP and the indicated concentrations of protein. b, ATP hydrolysis rate as a function of poly(dT) concentration. 1 μM protein was titrated with 0–25 μM poly(dT). In the double reciprocal plot, the y intercept gives the value of 1/V_max and the x intercept is 1/K_d (apparent). Initial hydrolysis rates were measured at 37°C in buffer A using a coupled-enzyme assay containing an ATP regenerating system (see “Experimental Procedures”). ●, RecA; □, RecA_E207K; ○, RecA_E207Q.

TABLE II

Kinetic parameters for ssDNA dependent ATP hydrolysis by wild type and mutant RecA proteins

| Protein       | Stoichiometry | k_cat | K_d apparent |
|---------------|---------------|-------|--------------|
|               | nucleotides/protein | min⁻¹ | μM           |
| wtRecA        | 3.2 ± 0.2     | 29 ± 2 | 1.8          |
| RecA_E207K    | 2.9 ± 0.4     | 28 ± 2 | 2.5          |
| RecA_E207Q    | 3.0 ± 0.2     | 28 ± 3 | 7.0          |

a Titration of 3.0 μM poly(dT) with protein (Fig. 3a).

b Titration of 1 μM protein with poly(dT) (Fig. 3b); k_cat = V_max/total protein concentration.
RecA and RecAE207Q, the fluorescence of III). In contrast complexes of DNA with RecAE207Q had the same stoichiometry and stability as with wtRecA protein. In some conditions nucleoprotein filaments equilibrate slowly, and the nature of the RecA-DNA complex may vary with the order of addition (7, 17). To investigate a possible effect of kinetic trapping on the stoichiometry of the RecAE207Q filament, we carried out reverse titrations where protein was titrated with DNA (Fl and Table III). For both wild type RecA and RecAE207Q, the fluorescence of DNA increased to the same extent in filaments with protein alone and in the presence of ADP, whereas fluorescence increased to a greater extent with ATP·S. However, saturation of RecA protein by DNA gave a complex with twice the fluorescence intensity of the corresponding complex with RecAE207Q; as observed for titration with protein, the stoichiometry of the complexes showed that each wtRecA molecule bound twice as much DNA (Table III).

**DISCUSSION**

The L2 loop (196IRMKGVFGNPETT209) is a likely candidate for one of the ssDNA-binding sites of RecA protein. These amino acids are disordered in the RecA-ADP crystal and disposed toward the central cavity where they can interact with DNA (11). Mutations in this region inactivate RecA protein (14, 20, 22, 27) and perturb DNA binding (28). Photocross-linking experiments (12, 13) show that, in the nucleoprotein filament, DNA is near the L2 loop. Finally a peptide with the sequence of RecA protein. The allosteric conformation and stability changes characteristic of wild type RecA protein (5) are also observed with RecAE207Q (Figs. 4 and 5 and Table III), i.e., upon binding ssDNA to site I without binding at the L2 loop, site II. Hence, the formation of the presynaptic filament may be both necessary and sufficient for the cofactor-dependent affinity cycle of wild type RecA protein.

No major differences in stoichiometry, structure, or stability were observed for nucleoprotein filaments of wtRecA and RecAE207Q in the presence of ADP, ATP·S, or without cofactor (Table III). It is therefore not surprising that RecAE207Q catalyzed DNA strand exchange in vitro in the presence of ATP·S as efficiently as wild type protein (Fig. 2). However, when strand exchange was carried out with an ATP regenerating system, the reaction rate of RecAE207Q was slower than wt type protein, although both proteins eventually gave 80% dsDNA products. $k_{cat}$ for the ATP hydrolysis reaction was the same for RecAE207Q as for wild type protein, whereas the apparent dissociation constant for the DNA binding was larger for mutant protein (Fig. 3b and Table II). Hence, inefficient ssDNA substrate binding at site I of RecAE207Q might explain the kinetic inhibition of strand exchange in the presence of ATP.

Model peptide studies suggested that the negative charge at position 207 might regulate affinity of the L2 loop for ssDNA (30). However, we observe no correlation of biochemical or biological activities of RecA with increasing positive charge at this position. An uncharged side chain (E207Q) inhibited biological functions, whereas a positive charge (E207K) reduced but did not eliminate biological activity (Fig. 1 and Table I). Therefore, a negative charge at position 207 is not necessary for RecA function. We speculate that Glu207 contributes to the water solubility of the L2 loop and that the point mutation E207Q may reduce the solubility and collapse this peptide sequence onto the protein surface where it is inaccessible to DNA. In this hypothesis, a positive or a negative charge at position 207 would equally solubilize the L2 loop and re-
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Stoichiometry, fluorescence increase, and stability of complexes formed between protein and eDNA and subsequently denatured by NaCl

The values are the means ± range of three or more experiments.

| Stoichiometry | Stoichiometry | Fluorescence increase | Salt titration midpoint |
|---------------|---------------|-----------------------|-------------------------|
| RecA          | No cofactor   | 7.2 ± 1.2             | 2.0 ± 0.1               | 275 ± 10 |
|               | ADP           | 7.4 ± 0.7             | 2.1 ± 0.2               | 170 ± 15 |
|               | ATPγS         | 10.2 ± 0.9            | 2.7 ± 0.2               | >500     |
| RecAE207K     | No cofactor   | 6.9 ± 0.2             | 2.2 ± 0.1               | 280 ± 10 |
|               | ADP           | 6.8 ± 0.7             | 1.9 ± 0.1               | 180 ± 10 |
|               | ATPγS         | 7.1 ± 0.1             | 2.8 ± 0.1               | >500     |
| RecAE207Q     | No cofactor   | 4.0 ± 0.5             | 2.2 ± 0.1               | 390 ± 10 |
|               | ADP           | 4.3 ± 0.5             | 2.2 ± 0.3               | 320 ± 15 |
|               | ATPγS         | 5.1 ± 0.5             | 2.7 ± 0.2               | >500     |

a 5.7 μM eDNA titrated by protein.
b 1 μM protein titrated by eDNA.

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store activity. The biochemical and DNA binding properties of RecAE207K protein are consistent with this proposition, even though Lys207 may also perturb DNA binding and reduce the efficiency of this protein compared with wild type RecA. If this argument is correct, the role of the negative charge Gly207 would be to increase the solubility of the L2 loop without engendering parasitic electrostatic protein-DNA bonds. Negatively charged amino acids that are found in the L2 region of recombinases from phage to man (4) may also have this function.

ATP hydrolysis requires DNA binding at site I of RecA protein. The Lineweaver-Burke plot for ATP hydrolysis as a function of poly(dT) (Fig. 3b) shows that the effect of our point mutations in the L2 loop resembles competitive inhibition (31) for ssDNA binding at site I, i.e. point mutations E207Q and E207K increased the apparent $K_D$ for ATP hydrolysis without changing $V_{max}$; the E207Q mutation was a more potent "inhib-
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