Allosteric Regulation of RecA Protein Function Is Mediated by Gln\textsuperscript{194}\# 

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Binding of ATP to the RecA protein induces a high affinity DNA binding required for activation of enzyme function. Screens for in vivo recombination and repressor cleavage activities show Gln\textsuperscript{194} to be intolerant of all substitutions. Analyses of three mutant proteins (Q194N, Q194E, and Q194A) show that although basal enzyme function is maintained, each protein no longer displays an ATP-induced increase in DNA binding affinity. High salt activation of RecA function is also disrupted by these mutations. In contrast, ATP-induced changes in the oligomeric structure of RecA are maintained in the mutant proteins. These results demonstrate that Gln\textsuperscript{194} is a critical “allosteric switch” for ATP-induced activation of RecA function but is not the exclusive mediator of ATP-induced changes in RecA.

The Escherichia coli RecA protein is a multifunctional enzyme that plays a central role in the processes of recombinational DNA repair, homologous genetic recombination, and the cellular SOS response to DNA damage (1–3). Each of these activities exhibits a common initial or activating step, formation of a RecA-ATP-ssDNA\textsuperscript{1} nucleoprotein filament (4–6). The binding of RecA to ssDNA is regulated in a classic allosteric fashion, whereby the binding of ATP induces a high affinity DNA binding state of the protein (7, 8). In the presence of ADP, or in the absence of cofactor, RecA exhibits a low affinity (\textgtrsim 20 \mu M) for ssDNA (7).

The crystal structure of the helical RecA protein filament has been solved in both the absence and presence of ADP (9, 10) and displays a helical pitch (82.7 Å) which is intermediate between the inactive nucleotide-free form (\textgtrsim 70 Å) and the active ATP-bound form (\textsim 95 Å) as determined by electron microscopy (reviewed in Ref. 11). Despite this, the ATP binding site shows a remarkable conservation of structure compared with several other nucleotide-binding proteins (e.g. p21\textsuperscript{160}, EF-Tu, and adenylate kinase), and Story and Steitz (10) were able to model specific determinants of both ATP binding and hydrolysis in the RecA structure. In addition, the structure provided valuable insight into a possible allosteric mechanism for ATP-induced high affinity binding to ssDNA. The carboxamidyl side chain of Gln\textsuperscript{194} extends into the ATP binding site and would be in very close proximity to the \(\gamma\)-phosphate of bound ATP (Fig. 1). Gln\textsuperscript{194} immediately precedes one of two disordered loops in the structure (L2, residues 195–209) that were proposed to be part of the DNA binding sites (9). Recent work has, in fact, provided strong evidence that L2 comprises all or a large part of the ssDNA binding site within RecA (12–14). Story and Steitz (10) proposed that upon ATP binding Gln\textsuperscript{194} interacts with the nucleotide \(\gamma\)-phosphate thereby causing L2 to assume a conformation with high affinity for ssDNA. Upon ATP hydrolysis this interaction would be lost, returning L2 to a low affinity DNA binding conformation.

In this study, we show that mutations at Gln\textsuperscript{194} prohibit the formation of a high affinity ssDNA binding state when ATP is bound. In addition, mutations at Gln\textsuperscript{194} block the high salt activation of RecA function. These results indicate that Gln\textsuperscript{194} is an important “on-off” switch required for the general activation of RecA function.

EXPERIMENTAL PROCEDURES

Materials—Labeled NTPs and dNTPs were from NE1 Life Science Products. PEI-cellulose chromatography plates were from J. T. Baker Inc. Single-stranded RV-1 DNA, an M13 derivative, was used for in vitro ATPase and repressor cleavage assays and was purified as described (15). An 86-base oligonucleotide used in the ssDNA binding assays and mutagenic oligonucleotides were made with an Applied Biosystems 392 DNA/RNA synthesizer. Double-stranded DNA (dsDNA) used in both the ATPase and dsDNA binding assays was Nool-linearized pTRecA332 (\textgtrsim 5, 500 base pairs (16)). LexA protein was a generous gift from Dr. John Little and Donald Shepley (Dept. of Biochemistry, University of Arizona). Restriction enzymes, T4 DNA ligase, T4 polynuclotide kinase, and Klenow DNA polymerase I large fragment were from New England Biolabs. Sequenase version 2.0 was from U.S. Biochemical Corp. Nitrocellulose filters were from Schleicher and Schuell. Isopropyl-1-thio-\(\beta\)-galactopyranoside and mitomycin C were from Sigma.

Mutagenesis—Mutations were introduced at position 194 using a modification of a previously described cassette mutagenesis procedure (17). Two 81-base oligonucleotides corresponding to the top and bottom strands encoding residues Thr\textsuperscript{196} to Aen\textsuperscript{113} were synthesized such that codon 194 read NN(G/C) and all other bases were the wild type recA sequence. Oligonucleotides were annealed, and the resulting cassette was ligated into A\textgtrsim III/Mlu\textgtrsim I-digested pTRecA332, a derivative of pTRecA332 (18) containing a unique A\textgtrsim III site at position 187 and a unique Mlu\textgtrsim I site at position 214. Plasmids were transformed into a \textgtrsim recA strain, DE1663* (18), and colonies were selected on LB-ampicillin plates. Amino acid substitutions were determined by DNA sequence analysis.

Recombinational DNA Repair Activity in Vivo—The recombinational DNA repair activity of each mutant protein was determined using two genetic screens as described previously (18), cell survival in the presence of mitomycin C and cell survival following exposure to different doses of UV light.

Determination of the Size of Mutant RecA Protein Oligomers—The oligomeric distribution of wild type and mutant RecA proteins was determined using gel filtration chromatography as described previously (19).

Purification of Wild Type and Mutant RecA Proteins—Wild type and mutant proteins (Q194A, Q194E, and Q194N) were purified using a previously described method (20) and were determined to be >95% pure.
as judged by Coomassie-stained gels. Proteins were quantitated using the Bio-Rad protein assay kit and by optical density, using an extinction coefficient of ε_{280} = 0.59 mg^{-1} cm^{-1} (21). No exonuclease activity was detected in any of these preparations, even at elevated concentrations of RecA (50 μM) in the nuclease assay. None of these mutations has any deleterious effect on the overall folded structure or thermal stability of the protein. Wild type RecA and each mutant protein showed identical circular dichroism profiles and measurements of θ_{222 nm} as a function of temperature (5–90 °C) gave a T_m = 53 ± 1 °C for all 4 proteins (data not shown).

RecA-mediated LexA Cleavage—RecA-mediated cleavage of the LexA repressor was measured in vitro using strain DE1663 as described previously (18). The ability of purified wild type and mutant RecA proteins to mediate the autocleavage of the LexA repressor was measured in vitro as described previously (18). RecA-mediated cleavage was assessed in the presence of ssDNA as described previously (18). Reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM DTT, 25 μM ssDNA (concentration of bases), 0.5 mM [γ-³²P]ATP (150 μCi/ml), and 2 μM purified RecA protein. These were incubated at 37 °C for the indicated times, and 1-μl aliquots were spotted onto PEI cellulose. High salt activation of ATP hydrolysis was measured essentially as described (24). Reaction mixtures contained 50 mM Tris-HCl (pH 7.1), 17.5 mM MgOAc_{2}, 5 mM ATP (150 μCi/ml), 1.5 mM NaCl, 2% (v/v) glycerol, 0.1 mM EDTA, and 5 μM purified RecA protein. These were incubated at 37 °C, and 1-μl aliquots were diluted 1:10 in water prior to spotting onto PEI-cellulose. PEI plates were chromatographed in 0.5 M LiCl, 1 mM formic acid, and percent ATP hydrolysis was quantitated using a Molecular Dynamics PhosphorImager and ImageQuant software (v5.6). Units of V/E are mol of ADP/min⁻¹ mol of RecA monomer⁻¹.

dsDNA Binding—Nitrocellulose filter binding assays were performed using a variation of a described procedure (25) and an apparatus similar to that described by Wong and Lohman (26). Filters were prepared by soaking in 0.4 M KOH for 10 min followed by several washes with double distilled H₂O until the pH approximated 7.0. Filters were equilibrated in binding buffer for at least 1 h prior to use. Reaction mixtures (50 μl) contained binding buffer (20 mM Tris-HCl, pH 7.5), 10 mM MgCl₂, 1 mM DTT, 0.5 mM EDTA, 20 mM NaCl, 40 μM 5'-end-labeled dsDNA (concentration of bases), and where indicated, 0.5 mM ATP·S. Reactions were started with the addition of the indicated amounts of protein and incubated at 37 °C for 15 min. Samples were applied to the filter under suction, and the filters were washed with 2.0 ml of binding buffer containing 150 mM NaCl. Filters were air-dried, and bound DNA was quantitated by analyzing air-dried filters with a Molecular Dynamics PhosphorImager and ImageQuant software (v5.6).

ssDNA Binding—Nitrocellulose filter binding assays were performed using a variation of a previously described procedure (25). Filters were pre-equilibrated in binding buffer (20 mM sodium maleate (pH 6.2), 10 mM MgCl₂, 1 mM DTT, 0.5 mM EDTA) for at least 1 h. Reaction mixtures contained 20 μM end-labeled dsDNA (concentration of bases), purified RecA protein, and where indicated, 0.5 mM ATP·S. Incubations were performed at 37 °C for 15 min, and samples were applied to the filter under suction and washed with 2.0 ml of binding buffer. Double-stranded DNA binding was quantitated by analyzing air-dried filters with a Molecular Dynamics PhosphorImager and ImageQuant software (v5.6).

RESULTS AND DISCUSSION

RecA Activity in Vivo—We obtained 16 of 19 possible substitutions at Gln<sup>194</sup>, including a conservative Asn, an isosteric Glu, and an Ala.<sup>2</sup> In vivo screens for recombinational DNA repair and LexA cleavage showed that these activities are completely dependent on the wild type Gln residue (data not shown). Each of the 16 mutants showed no survival greater than the ΔrecA control in the presence of mitomycin C or following exposure to UV, even under low dose conditions (0.3 μg/ml mitomycin C or 0.67 J/m²/s UV light). In addition, the LexA coprotease activity of each mutant was completely inhibited, a result which was confirmed by in vitro LexA cleavage assays (see below).

RecA Activity in Vitro—We obtained 16 of 19 possible substitutions at Gln<sup>194</sup>, including a conservative Asn, an isosteric Glu, and an Ala.<sup>2</sup> In vivo screens for recombinational DNA repair and LexA cleavage showed that these activities are completely dependent on the wild type Gln residue (data not shown). Each of the 16 mutants showed no survival greater than the ΔrecA control in the presence of mitomycin C or following exposure to UV, even under low dose conditions (0.3 μg/ml mitomycin C or 0.67 J/m²/s UV light). In addition, the LexA coprotease activity of each mutant was completely inhibited, a result which was confirmed by in vitro LexA cleavage assays (see below).

**FIG. 1. Position of Gln<sup>194</sup> in the RecA ATP binding site.** The structure of the RecA-ADP complex (10) shows the amide nitrogen of Gln<sup>194</sup> to be approximately 6.9 Å from the β-phosphate of ADP. Residues 195–209, not visible in the structure, correspond to region L2 which is likely to contain all or part of the primary DNA binding domain of RecA (see text). From the RecA-ADP structure Story and Steitz (10) modeled P-loop residues Lys<sup>72</sup> to interact with the γ-phosphate of ATP and Thr<sup>73</sup> with a Mg<sup>2+</sup> that bridges the β- and γ-phosphates of ATP. Atom colors are: yellow, carbon; red, oxygen; blue, nitrogen; orange, phosphorus.

2 The following substitutions were obtained at position 194: Ala, Arg, Asn, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Phe Pro, Ser, Trp, Val.
Q194A, Q194E, and Q194N, exhibits a comparable apparent affinity, although the Q194N protein appears to have a slightly reduced binding capacity (Fig. 2A). In the presence of ATPγS, however, a dramatic increase is seen in the affinity of wild type RecA for ssDNA compared with the mutant proteins (Fig. 2B). Wild type RecA now binds the ssDNA substrate with an apparent $K_d \sim 1 \mu\text{M}$, whereas none of the 3 mutant proteins shows any change in affinity for ssDNA. These results indicate that the mutations at position 194 render the proteins unable to undergo the ATP-induced transition required for high affinity ssDNA binding.

ATP Binding—An essential control was to test the ability of the mutant proteins to bind ATP. Using a UV crosslinking procedure we show that ATP binding by each of the 3 mutant proteins (Q194A, Q194E, and Q194N) is very similar to that of wild type RecA (Fig. 3). Proteins that had been heat-denatured prior to UV exposure show no crosslinking. These results show that the inability of the mutant proteins to assume a high affinity ssDNA binding state is not due simply to a defect in ATP binding.

ATP-induced Effects on the Oligomeric Distribution of RecA—In solution RecA protein exists as a heterogeneous population of oligomers, ranging in size from monomers, dimers, and hexamer-sized rings to larger filaments and bundles of filaments (27–30). In the presence of ATP this distribution is shifted such that bundles of filaments are disrupted and the average filament length is shorter (27–29). We used a recently developed gel filtration assay (19) to assess any effect that the mutant proteins to bind ATP. Using a UV crosslinking procedure we show that ATP binding by each of the 3 mutant proteins (Q194A, Q194E, and Q194N) is very similar to that of wild type RecA (data not shown). Control experiments showed that wild type and mutant proteins bound equivalent low levels of ssDNA in the absence of ATPγS, yet only wild type RecA displayed a significant ATP-dependent increase in ssDNA binding (data not shown).

These results demonstrate that mutations at Gln194 block the DNA-dependent activation of ATPase activity but do not affect the basal level of DNA-independent ATP turnover.

LexA Coprotease Activity—In the presence of both nucleotide and ssDNA cofactors RecA mediates the autoproteolysis of the LexA repressor (5). While wild type RecA catalyzes a significant level of LexA autoproteolysis (95% cleaved/40 min) this activity is completely lacking in each of the 3 mutant proteins (data not shown).

High Salt Activation of RecA Activities—In the absence of any DNA cofactor, but in presence of high salt, wild type RecA protein is activated for both ATP hydrolysis (24) and LexA cleavage (22). Because mutations at Gln194 prohibit high affinity DNA binding required for both of these activities we determined whether the Q194A, Q194E, and Q194N mutations also affect high salt activation of RecA function. We found that although the ATPase activity of wild type RecA was greatly stimulated in the presence of 1.8 M NaCl ($V_{/E} = 23$) the 3 mutant proteins showed only very low activity under these conditions (Fig. 6). Similarly, in the presence of 0.6 M NaOAc wild type RecA catalyzed a significant level of LexA autoproteolysis (50% cleaved/40 min), yet this activity was lacking in each mutant protein (data not shown).

These results demonstrate that the wild type Gln194 side chain is necessary for the general activation of RecA function and supports the suggestion by Pugh and Cox (24) that salt activation of RecA is “functionally mimicking the ionic interaction of the protein with DNA.” In the presence of ATP and elevated salt concentrations, if 3 to 4 anions bind to the same sites within RecA as do phosphate groups on the DNA backbone (24, 31), our data show that Gln194 mediates the ATP-induced occupancy of these sites giving rise to an activated RecA-ATP-DNA (or RecA-ATP-salt) complex. In future studies it would be interesting to determine the ion occupancy of the Gln194 mutant proteins.
NTP-induced conformational change is a common theme for activation of enzyme function. Studies of a number of NTP-binding proteins, including G-protein complexes (32–34), the myosin motor domain (35), the kinesin and Ncd microtubule motor proteins (36, 37), and the ADP$^\alpha$AlF$_4$$^\delta$-stabilized nitrogenase complex (38) have identified specific protein-nucleotide interactions which mediate conformational changes leading to activation of protein function. Additionally, the position of residues in the ATP binding site of the PcrA helicase from Bacillus stearothermophilus shows a striking similarity to those in RecA (39). For example, in addition to residues proposed to be involved in Mg$^{2+}$ binding (Asp223 in PcrA, Asp144 in RecA) and the catalysis of ATP hydrolysis (Glu224 in PcrA, Glu96 in RecA), Gln254 occupies a position in PcrA very similar to Gln194 in RecA. Subramanya et al. (39) propose that ATP-induced effects on the DNA binding affinity by neighboring regions are mediated by Gln254.

Our data identify Gln194 as a NTP-binding site “g-phosphate sensor” in that mutations at this residue disrupt only the ATP-induced increase in RecA activities and not the basal level functions or properties of the protein. For example, the low level DNA-independent ATP hydrolysis seen with wild type RecA is maintained for each of the Gln194 mutant proteins indicating that Gln194 is not an important component of the intrinsic RecA ATPase catalytic mechanism. In addition, Gln194 appears not to be an important determinant of ATP binding because Asn, Glu, and Ala mutations show binding profiles similar to wild type RecA. We also show that each of the 3 mutant proteins maintains a low affinity binding to ssDNA in the absence of ATP similar to wild type RecA, and therefore, Gln194 is not a determinant of this DNA binding property. We cannot necessarily exclude the possibility that Gln194 interacts with DNA in the activated RecA-ATP-DNA complex. Using synthetic peptides corresponding to residues 193–212, which include the entire L2 region, Voloshin et al. (14) identified position 203 as an important determinant of DNA binding. Studies using peptides with substitutions at Gln194 could assist in defining a potential role for this residue in DNA binding.

Although our data demonstrate the importance of Gln194 in transmitting allosteric information within the RecA protein structure it certainly does not exclude other residues, perhaps within the L2 DNA binding region, as also participating in ATP-induced allosteric effects. We note that specific ATP-induced changes in the oligomeric structure of RecA are not
blocked by mutations at Gln^{194} (Fig. 4). Therefore, other residues must play important roles in mediating nucleotide-induced changes in RecA structure and/or function as the protein progresses through the catalytic cycle.

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**REFERENCES**

1. West, S. C. (1992) *Annu. Rev. Biochem.* **61**, 603–640
2. Kowalczykowski, S. C., Dixon, D. A., Eggelston, A. K., Lauder, S. D., and Rehrauer, W. M. (1994) *Microbiol. Rev.* **58**, 401–465
3. Roca, A. I., and Cox, M. M. (1997) *Prog. Nucleic Acid Res. Mol. Biol.* **56**, 129–222
4. Craig, N. L., and Roberts, J. W. (1980) *Nature* **283**, 26–30
5. Little, J. W., Edmiston, S. H., Pacelli, L. Z., and Mount, D. W. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 3225–3229
6. Radding, C. M. (1989) *Biochim. Biophys. Acta* **1008**, 131–145
7. Silver, M. S., and Fersht, A. R. (1982) *Biochemistry* **21**, 6066–6072
8. Menetski, J. P., and Kowalczykowski, S. C. (1985) *J. Mol. Biol.* **181**, 281–295
9. Story, R. M., Weber, I. T., and Steitz, T. A. (1992) *Nature* **355**, 318–325
10. Story, R. M., and Steitz, T. A. (1992) *Nature* **355**, 374–376
11. Egelman, E. H. (1993) *Curr. Opin. Struct. Biol.* **3**, 189–197
12. Gardner, R. V., Voloshin, O. N., and Camerini-Otero, R. D. (1995) *Eur. J. Biochem.* **233**, 419–425
13. Malkov, V. A., and Camerini-Otero, R. D. (1995) *J. Biol. Chem.* **270**, 30230–30233
14. Voloshin, O. N., Wang, L., and Camerini-Otero, R. D. (1996) *Science* **272**, 868–872
15. Zagursky, R. J., and Berman, M. L. (1984) *Gene (Amst.)* **27**, 183–191
16. Skiba, M. C., and Knight, K. L. (1994) *J. Biol. Chem.* **269**, 3823–3828
17. Konola, J. T., Logan, K. M., and Knight, K. L. (1994) *J. Mol. Biol.* **237**, 20–34
18. Nastri, H. G., and Knight, K. L. (1994) *J. Biol. Chem.* **269**, 26311–26322
19. Logan, K. M., Skiba, M. C., Eldin, S., and Knight, K. L. (1997) *J. Mol. Biol.* **266**, 306–316
20. Konola, J. T., Nastri, H. G., Logan, K. M., and Knight, K. L. (1995) *J. Biol. Chem.* **270**, 8411–8419
21. Craig, N. L., and Roberts, J. W. (1981) *J. Biol. Chem.* **256**, 8039–8044
22. DiCapua, E., Ruigrok, R. W. H., and Timmins, P. A. (1990) *J. Struct. Biol.* **104**, 91–96
23. Weinstock, G. M., McIntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256**, 8829–8834
24. Pugh, B. P., and Cox, M. M. (1988) *J. Biol. Chem.* **263**, 76–83
25. McIntee, K., Weinstock, G. M., and Lehman, I. R. (1981) *J. Biol. Chem.* **256**, 8835–8844
26. Wong, I., and Lehman, T. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5428–5432
27. Brenner, S. L., Zlotnik, A., and Griffith, J. D. (1988) *J. Mol. Biol.* **204**, 959–972
28. Heuser, J., and Griffith, J. D. (1989) *J. Mol. Biol.* **210**, 473–484
29. Wilson, D. H., and Benight, A. S. (1990) *J. Biol. Chem.* **265**, 7351–7359
30. Ruigrok, R. W. H., and DiCapua, E. (1991) *Biochimica* **191**, 121–137
31. Leahy, M. C., and Radding, C. M. (1986) *J. Biol. Chem.* **261**, 6954–6960
32. Milburn, M. V., Tong, L., deVos, A. M., Brunger, A., Yamazumi, Z., Nishimura, S., and Kim, S. H. (1996) *Science* **274**, 939–945
33. Noel, J. P., Hamm, H. E., and Sigler, P. B. (1993) *Nature* **366**, 654–663
34. Lambright, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) *Nature* **369**, 621–628
35. Fischer, A. J., Smith, C. A., Thoden, J. B., Smith, R., Sutoh, K., Holden, H. M., and Reymont, I. (1995) *Biochemistry* **34**, 8960–8972
36. Kull, P. J., Sabin, E. P., Lau, R., Fletterick, R. J., and Vale, R. D. (1996) *Nature* **380**, 550–555
37. Sabin, E. P., Kull, P. J., Cooke, R., Vale, R. D., and Fletterick, R. J. (1996) *Nature* **380**, 555–559
38. Schindelin, H., Kisker, C., Schlesman, J. L., Howard, J. B., and Rees, D. C. (1997) *Nature* **387**, 370–376
39. Subramanya, H. S., Bird, L. E., Brannigan, J. A., and Wigley, D. B. (1996) *Nature* **384**, 379–383