Differential Rates of NTP Hydrolysis by the Mutant [S69G]RecA Protein

EVIDENCE FOR A COUPLING OF NTP TURNOVER TO DNA STRAND EXCHANGE*

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The x-ray crystal structure of the Escherichia coli RecA protein indicates that the phosphate groups of the nucleotide cofactor are bound by a loop whose amino acid sequence (G^66PESSGKT^73) corresponds to a consensus phosphate binding loop sequence (GXXXGK(T/S)) found in many NTP-binding proteins. As part of an investigation of the role of the P-loop in ATP hydrolysis, we prepared a mutant RecA protein in which serine 69 was replaced by a glycine residue. We have found that the [S69G]RecA mutation has a differential effect on the hydrolysis of various nucleoside triphosphates. The [S69G]RecA protein catalyzes the single-stranded DNA-dependent hydrolysis of rATP, ddATP, and dATP with turnover numbers of 10, 20, and 36 min^(-1), respectively. The wild type RecA protein, in contrast, hydrolyzes each of these nucleoside triphosphates with similar turnover numbers of 20–24 min^(-1). Significantly, the [S69G]RecA protein promotes strand exchange with all three nucleoside triphosphates, and the rate of strand exchange is directly proportional to the rate of hydrolysis of each of the nucleotide cofactors. These findings with the [S69G]RecA protein provide support for the existence of a mechanistic coupling between NTP hydrolysis and DNA strand exchange.

The RecA protein of Escherichia coli (Mr, 37,842, 352 amino acids) is essential for homologous genetic recombination and for the postreplicative repair of damaged DNA. The purified RecA protein will promote a variety of DNA pairing reactions that presumably reflect in vivo recombination functions. The most extensively investigated DNA pairing activity is the ATP-dependent three-strand exchange reaction in which a circular ssDNA molecule and a homologous linear dsDNA molecule are recombined to yield a nicked circular dsDNA molecule and a linear ssDNA molecule. This reaction proceeds in three phases. In the first phase, the circular ssDNA substrate is coated with RecA protein to form a presynaptic complex; this complex will catalyze the hydrolysis of ATP to ADP and P	extsubscript{i}. In the second phase, the presynaptic complex interacts with a dsDNA molecule, the homologous sequences are brought into register, and pairing between the circular ssDNA and the complementary strand from the dsDNA is initiated. In the third phase, the complementary linear strand is completely transferred to the circular ssDNA by unidirectional branch migration to yield the nicked circular dsDNA and displaced linear ssDNA products.

The x-ray crystal structure of the RecA protein indicates that the phosphate groups of the nucleotide cofactor, ATP, are bound by a loop consisting of amino acids 66 through 73 (2). The sequence of this loop (G^66PESSGKT^73) corresponds to a variation of the well known phosphate binding loop (P-loop) consensus sequence (GXXXGK(T/S)) found in many NTP-binding proteins (3). The invariant lysine and threonine/serine residues in the P-loop motif are generally found to interact directly with the β and γ phosphates of ATP and have been shown for the RecA protein (as well as many other proteins) to be directly involved in the catalysis of phosphoryl transfer (4). Interestingly, although the four variable residues (XXXG) in this sequence can differ widely in different classes of proteins, the specific sequence, GPESSGKT, is highly conserved in over sixty different bacterial RecA proteins (1).

As part of an investigation of the role of the P-loop in ATP hydrolysis, we prepared a mutant RecA protein in which the serine residue at position 69 was replaced by a glycine. The biochemical properties of the [S69G]RecA protein provide new insight into the coupling of NTP hydrolysis and DNA strand exchange and are described in this report.

EXPERIMENTAL PROCEDURES

Materials—Wild type RecA protein was prepared as described previously (5). rATP was from Sigma; dATP, ddATP, and [α-32P]dATP, and [α-32P]ddATP were from Amersham Pharmacia Biotech. E. coli SSB was from Promega. Circular dX ssDNA (+−strand) and circular dX dsDNA were from New England Biolabs; linear dX dsDNA was prepared from circular dX dsDNA as described (6). Single- and double-stranded DNA concentrations were determined by absorbance at 260 nm using the conversion factors 36 and 50 µg/ml/abs260, respectively. All DNA concentrations are expressed as total nucleotides.

Preparation of the [S69G]RecA Protein—The mutant [S69G]RecA gene, in which the nucleotide sequence coding for serine 69 was replaced with a nucleotide sequence coding for glycine, was produced using the QuickChange™ protocol (Stratagene). The mutagenesis template consisted of pET21a (+) vector (Novagen) containing the wild type recA gene cloned into a NdeI/HindIII site. The mutagenesis primers were 5′−ATCTACGGACCGGAA and 5′−CTGGTTTACCGAGGCCTTGGCCGTCGTAGAT−3′ (the codon for serine 69 is underlined, and the nucleotide mismatch is in bold). The entire [S69G]RecA gene was sequenced to confirm that only the desired changes had been introduced during the mutagenesis procedure. The expression plasmid, pETRecA/S69G, was introduced into the E. coli recA deletion strain, BLR(DE3) (Novagen). The expression of the [S69G]RecA protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (1 mM final concentration) at A260 = 0.6 followed by a 3-h incubation at 37 °C. The [S69G]RecA protein was then purified to greater than 95% homogeneity by methods that have been described previously (5). The purified [S69G]RecA protein is shown in Fig. 1.

RESULTS

ssDNA-dependent NTP Hydrolysis Activity of the [S69G]RecA Protein—The ssDNA-dependent hydrolysis of structurally related nucleoside triphosphates, ATP, dATP, and ddATP, by the
Properties of the [S69G]RecA Protein

wild type and [S69G]RecA proteins was analyzed at pH 7.5 and 37 °C. The reaction solutions contained 30 μM dX ssDNA and 1 μM wild type or [S69G]RecA protein; these conditions ensured that there was sufficient ssDNA to bind all of the RecA protein present. The dependence of the rate of NTP hydrolysis on NTP concentration is shown in Fig. 2, and the steady-state kinetic parameters for the hydrolysis of each nucleoside triphosphate are presented in Table I.

The wild type RecA protein catalyzed the hydrolysis of the three nucleoside triphosphates at similar rates, with turnover numbers (V_max/[E]) of 20, 24, and 24 min⁻¹ for ATP, dATP, and ddATP, respectively. The S_0.5 values for each of these nucleoside triphosphates were also similar (12–40 μM) under these reaction conditions.

The [S69G]RecA protein also catalyzed the hydrolysis of each of the three nucleoside triphosphates. In contrast to the wild type protein, however, the rate of the [S69G]RecA protein-catalyzed hydrolysis reaction depended on the identity of the nucleoside triphosphate, with turnover numbers of 10, 20, and 36 min⁻¹ for ATP, ddATP, and dATP, respectively. Although the turnover numbers differed, the S_0.5 values for the three nucleoside triphosphates were similar (9–20 μM) to those determined for the wild type protein.

Three-strand Exchange Activity of the [S69G]RecA Protein—The three-strand exchange activities of the wild type and [S69G]RecA protein were evaluated in the presence of either ATP, dATP, or ddATP. In the three-strand exchange assay, a circular dX ssDNA molecule and a linear dX dsDNA molecule are recombined to form a nicked circular dsDNA molecule and a linear ssDNA molecule; the substrates and products of this reaction are readily monitored by agarose gel electrophoresis.

As shown in Fig. 3A, the wild type RecA protein was able to promote strand exchange in the presence of either ATP, dATP, or ddATP. At early times in the reaction, partially exchanged intermediates are visible. In later time points, these intermediates are replaced by the fully exchanged circular dsDNA product. The rates for the formation of the intermediate structures and the final circular dsDNA products that were obtained with ATP, dATP, and ddATP were similar, indicating that the wild type RecA protein uses these nucleoside triphosphates equally efficiently as cofactors for the strand exchange reaction.

The [S69G]RecA protein was also active in the three-strand exchange reaction (Fig. 3A). In contrast to the wild type protein, however, the rate of the [S69G]RecA protein-promoted strand exchange reaction varied depending on the nucleoside triphosphate that was supplied as the cofactor. The strand exchange reaction was fastest with dATP and slowest with ATP, with ddATP giving an intermediate rate. Thus, the rates of the [S69G]RecA protein-promoted strand exchange reaction appeared to parallel the turnover numbers that were measured for the hydrolysis of each of these nucleoside triphosphates by the [S69G]RecA protein (Table I).

In order to quantify the rates of the wild type and [S69G]RecA protein-promoted strand exchange reactions, the intensity of the agarose gel bands corresponding to the linear dsDNA substrate, the strand exchange intermediates, and the circular dsDNA strand exchange product at each time point in the reactions shown in Fig. 3A was measured by scanning densitometry. The percentage of the linear dsDNA substrate that had been converted to the circular dsDNA product was then plotted as a function of time in order to generate time courses for the strand exchange reactions (Fig. 3B). The rates of strand exchange were then determined from these time course both by 1) determining the time at which the circular dsDNA product first appears (this represents the minimal time required to

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2 S_0.5 is the substrate concentration required for half-maximal velocity.

**Fig. 1.** SDS-polyacrylamide gel electrophoresis of purified [S69G]RecA protein. Lane 1, [S69G]RecA protein; lane 2, wild type RecA protein; lane 3, molecular mass standards (Life Technologies, Inc.) comprised of myosin H-chain (220,950 Da), phosphorylase b (96,730 Da), bovine serum albumin (71,775 Da), ovalbumin (45,475 Da), and carbonic anhydrase (28,865 Da). The acrylamide concentration was 5% in the stacking gel and 10% in the separating gel. The gel was stained in 0.1% Coomassie Brilliant Blue R-250.

**Fig. 2.** Single-stranded DNA-dependent NTP hydrolysis by the wild type and [S69G]RecA proteins. The reaction solutions contained 25 mM Tris acetate (pH 7.5), 10 mM Mg(acetate)_2, 30 μM dX ssDNA, 1.0 μM wild type or [S69G]RecA protein, and the indicated concentrations of [α-32P]ATP, [α-32P]dATP, or [α-32P]ddATP. The reactions were initiated by the addition of protein and were carried out at 37 °C. ATP, dATP, and ddATP hydrolysis reactions were measured using a thin-layer chromatography method as described previously (12). The points represent the initial rates of ATP (circles), dATP (squares), or ddATP (triangles) hydrolysis that were measured at the indicated concentrations of NTP. The solid lines represent fits of the data by the Hill equation.
generate the strand exchange product and provides an estimate of the intrinsic rate of strand exchange), and by 2) determining the time required for each of the strand exchange reactions to reach near-completion (defined operationally as when the level of the circular dsDNA product reaches 80% of the maximal value). The rate of strand exchange by the wild type RecA protein in the presence of ATP, as determined by either of these methods, was assigned a relative rate of 1, and the rate of strand exchange by the wild type protein in the presence of the other nucleoside triphosphates (dATP, ddATP), and the rate of strand exchange by the [S69G]RecA protein in the presence of all three nucleoside triphosphates, were expressed relative to the rate of the wild type RecA/ATP reaction (Table II).

As shown in Fig. 3B and Table II, the strand exchange reaction of the wild type RecA protein proceeded at a similar rate (relative rates; 1) in the presence of either ATP, dATP, or ddATP. In contrast, the strand exchange reaction of the [S69G]RecA protein proceeded at approximately the same rate as the wild type protein reaction in the presence of ddATP (relative rate = 0.9–1.0), slower than the wild type reaction in the presence of ATP (relative rate = 0.6–0.8) and faster than the wild type reaction in the presence of dATP (relative rate = 1.6–1.8). The relative rates of strand exchange were essentially independent of whether the rates were based on the minimal time required for the fully exchanged reaction products to

| NTP     | V_max/| E | S0.5 |
|---------|-------|---|------|
|         | min⁻¹ | μM |
| Wild type |       |   |
| ATP     | 20    | 40 |
| dATP    | 24    | 20 |
| ddATP   | 24    | 12 |
| [S69G] |       |   |
| ATP     | 10    | 20 |
| dATP    | 36    | 17 |
| ddATP   | 20    | 9  |

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**TABLE II**

Properties of the [S69G]RecA Protein

| NTP        | Time  | Relative rate | Time  | Relative rate |
|------------|-------|---------------|-------|---------------|
|            | min   |               | min   |               |
| Wild type  |       |               |       |               |
| ATP        | 9     | 1             | 16    | 1             |
| dATP       | 9     | 1             | 16    | 1             |
| ddATP      | 7     | 1.3           | 14    | 1.1           |
| ddGTP      | 11    | 0.8           | 27    | 0.6           |
| [S69G]     |       |               |       |               |
| ATP        | 5     | 1.8           | 10    | 1.6           |
| dATP       | 9     | 1             | 18    | 0.9           |

* Time required for the first appearance of the circular dsDNA product (in min).
* Time required for the first appearance of circular dsDNA product, expressed relative to that for the wild type protein reaction in the presence of ATP.
* Time required for the level of the circular dsDNA product to reach 80% of the maximal value (in min).

![Fig. 4. Correlation between rates of NTP hydrolysis and DNA strand exchange.](image)

DISCUSSION

Although it is well established that the RecA protein-promoted strand exchange reaction requires ATP, a coupling of ATP hydrolysis to strand exchange has yet to be demonstrated. In a recent study, Cox and co-workers (11), on the other hand, have argued that ATP hydrolysis is required only for a dissociation and redistribution of RecA monomers during the exchange of longer DNA substrates and during the bypass of structural barriers in the DNA substrates. Both of these models predict that the rate of strand exchange will be related to the rate of NTP hydrolysis and, therefore, are consistent with the results presented in this paper.

REFERENCES

1. Roca, A. I., and Cox, M. M. (1997) *Prog. Nucleic Acid Res.* 56, 129–223
2. Story, R. M., Weber, I. T., and Steitz, T. A. (1992) *Nature* 355, 318–325
3. Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990) *Trends Biochem. Sci.* 15, 430–434
4. Rehbrauer, W. M., and Kowalczykowski, S. C. (1993) *J. Biol. Chem.* 268, 1292–1297
5. Cotterill, S. M., Satterthwait, A. C., and Fersht, A. R. (1982) *Biochemistry* 21, 4332–4337
6. Cox, M. M., and Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U. S. A.* 78, 3433–3437
7. Bedale, W. A., and Cox, M. M. (1996) *J. Biol. Chem.* 271, 5725–5732
8. Menetski, J. P., Bear, D. G., and Kowalczykowski, S. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 87, 21–25
9. Cox, M. M. (1994) *Trends Biochem. Sci.* 19, 217–222
10. Shan, Q., and Cox, M. M. (1996) *J. Biol. Chem.* 271, 5712–5724
11. Bianco, P. R., Tracy, R. B., and Kowalczykowski, S. C. (1998) *Front. Biosci.* 3, 570–603
12. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U. S. A.* 76, 129–130