Biochemical and Biophysical Research Communications 421 (2012) 527–531

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Altered nucleotide cofactor-dependent properties of the mutant [S240K]RecA protein

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ARTICLE INFO

Article history:
Received 1 April 2012
Available online 10 April 2012

Keywords:
RecA protein
LexA protein
DNA strand exchange
Co-protease

ABSTRACT

Two mutant *Escherichia coli* RecA proteins were prepared in which the ATP active site residue, Ser240, was replaced with asparagine and lysine (these amino acids are found in the corresponding positions in other bacterial RecA proteins). The S240N mutation had no discernible effect on the ATP-dependent activities of the RecA protein, indicating that serine and asparagine are functionally interchangeable at position 240. The S240K mutation, in contrast, essentially eliminated the ability of the RecA protein to utilize ATP as a nucleotide cofactor. The [S240K]RecA protein was able to catalyze the hydrolysis of dATP, however, suggesting that the absence of the 2′-hydroxyl group reduced an inhibitory interaction with the Lys240 side chain. Interestingly, the [S240K]RecA protein was able to promote an efficient LexA cleavage reaction but exhibited no strand exchange activity when dATP was provided as the nucleotide cofactor. This apparent separation of function may be attributable to the elevated $S_{0.5}$ value for dATP for the [S240K]RecA protein (490 μM, compared to 20–30 μM for the wild type and [S240N]RecA proteins), and may reflect a differential dependence of the LexA co-protease and DNA strand exchange activities on the nucleotide cofactor-mediated stabilization of the functionally-active state of the RecA–ssDNA complex.

1. Introduction

The RecA protein (37.8 kDa, 352 amino acids) plays a central role in DNA repair in *Escherichia coli*, acting both as a DNA recombinase that participates directly in the repair of damaged DNA, and as a co-protease that facilitates the auto-cleavage of the LexA repressor protein during the SOS response to DNA damage [1]. Both processes are carried out by a complex formed by the binding of RecA protein to regions of cellular single-stranded DNA (ssDNA). During the recombinase reaction, the RecA–ssDNA complex interacts with a homologous region of double-stranded DNA (dsDNA) and then transfers the complementary strand of the dsDNA to the ssDNA within the RecA–ssDNA complex [1]. During the co-protease reaction, the RecA–ssDNA complex binds to the LexA protein and promotes the transition of the LexA protein from the non-cleavable to the auto-cleavable conformation [2]. Both reactions are dependent on the ATP hydrolysis activity of the RecA–ssDNA complex [1,2].

The RecA protein binds cooperatively to ssDNA, forming a helical, filament-like structure with one RecA monomer per three nucleotides of ssDNA, and six RecA monomers per helical turn [1]. The functionally-active conformational states of the RecA–ssDNA complex are induced by the binding and hydrolysis of ATP [1]. Recent structural studies have shown that MgATP binds at the interface between adjacent monomers in the RecA–ssDNA complex [3]. In one RecA monomer, Lys72, Thr73, Thr74, Glu96 and Asp144 interact with the Mg^{2+} ion or phosphate groups; Asp100 and Tyr103 interact with the adenine group; and Ser240 is positioned close to the 2′- and 3′-hydroxyl groups of the ribose ring of MgATP. The primary contacts from the adjacent monomer are made by Lys248 and Lys250, which interact with the terminal phosphate of MgATP (Fig. 1A).

We have been carrying out a comparative analysis of the biochemical properties of the *E. coli* RecA protein with those of the RecA proteins from *Bacillus subtilis* and *Streptococcus pneumoniae* [4,5]. A comparison of the amino acid sequences reveals that all of the amino acids in the ATP binding site of the *E. coli* RecA protein noted above are also found in the *B. subtilis* and *S. pneumoniae* RecA proteins, except for the residue at position 240. Whereas this residue is a serine in the *E. coli* RecA protein, the corresponding residue in the *B. subtilis* RecA protein is an asparagine and in the *S. pneumoniae* RecA protein is a lysine (see Ref. [6] for sequence alignments). To explore the potential functional significance of the variability at this position, we have prepared two new mutant *E. coli* RecA proteins in which Ser240 has been replaced with asparagine and lysine. The biochemical properties of the [S240N]RecA and [S240K]RecA proteins are described in this report.

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http://dx.doi.org/10.1016/j.bbrc.2012.04.038

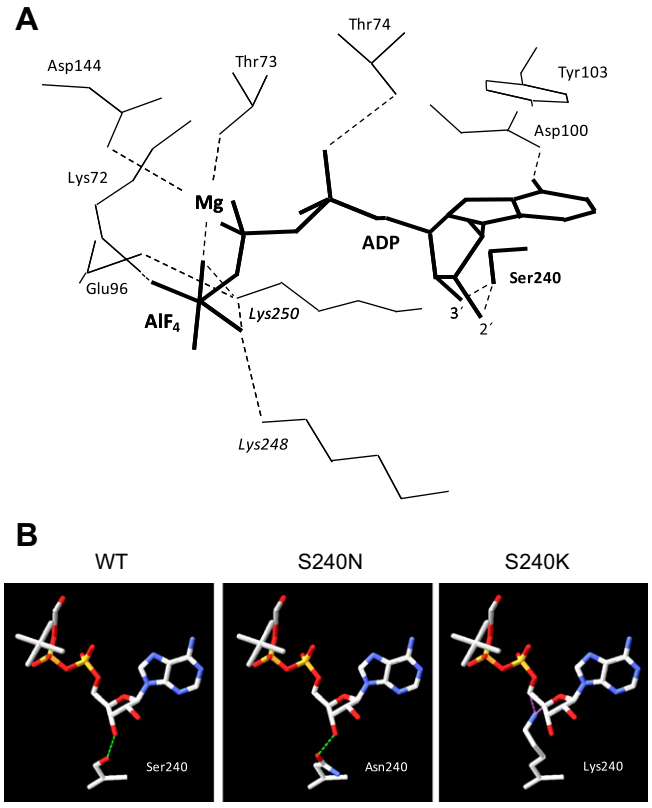

Fig. 1. ATP binding site of the *E. coli* RecA protein. (A) Schematic diagram of the ATP binding site. Residues labeled with normal font are from the same RecA monomer and those labeled in italics are from the adjacent monomer. The ATP analog, ADP–AlF_4–Mg, and Ser240 are highlighted in bold. (B) Diagram showing the bound ADP–AlF_4 and the side chains of the wild type RecA, [S240N]RecA, and [S240K]RecA proteins. The green lines represent a potential hydrogen bonding interaction and the purple lines indicate a potential steric clash. The diagrams in panels A and B were based on the structure of the $RecA_5$–$(dT)_{15}$–$(ADP–AlF_4–Mg)_5$ complex (PDB code 3CMW; Ref. [3]), and were generated using the DeepView/Swiss-Pdb Viewer version 4.0.1 (http://www.expasy.org/spdbv/; Ref. [15]).

2. Materials and methods

2.1. Materials

E. coli RecA protein was prepared as described previously [7]. *E. coli* LexA protein was prepared as described [8], using the pET construct pJWL228 (generously provided by Dr. John Little, University of Arizona). *E. coli* SSB protein was from Promega. ATP, dATP, [α-^{32}P]ATP, and [α-^{32}P]dATP were from Amersham Biosciences. Circular φX ssDNA (+ strand) and circular φX dsDNA were from New England Biolabs. Linear φX dsDNA was prepared from circular φX dsDNA by *Pst*I digestion as described [9]. Single- and double-stranded φX DNA concentrations were determined by absorbance at 260 nm using the conversion factors 36 and 50 μg ml^{-1} A_{260}^{-1}, respectively. All DNA concentrations are expressed as total nucleotides.

2.2. Preparation of the [S240N]RecA and [S240K]RecA proteins

The genes for the [S240N]RecA and [S240K]RecA proteins, in which the nucleotide sequence coding for Ser240 was replaced with a sequence coding for asparagine and lysine, respectively, were produced by PCR site-directed mutagenesis using Pfu polymerase (Stratagene). The mutagenesis template consisted of a pUC19 vector which contained an *E. coli* DNA fragment carrying the wild-type RecA gene. The mutagenesis primers were 5′-CGTGGTGGGT**AAC**GAAACCCGCGTG-3′ and 5′-CACGCGGGTTTC**GTT** ACCCACCACG-3′ for the S240N mutation, and 5′-CGTGGTGGGT**AAA** GAAACCCGCGTG-3′ and 5′-ACGCGGGTTTC**TTT**ACCCACCACG-3′ for the S240K mutation (the codons for asparagine 240 (AAC) and lysine 240 (AAA) are shown in bold).

The primers 5′-AAGCTTCTGTCATGGCATATCCT-3′ and 5′-GCGC CATATGGCTATCGACGAAAACAAACAGAAAGCG-3′ were then used in a second PCR reaction to engineer *HindIII* and *NdeI* restriction sites into the mutant genes (the restriction sites are underlined). The blunt ended PCR products were ligated into pCR-Blunt™ (Invitrogen). Insert-containing clones were identified by *NdeI* and *HindIII* restriction analysis and subcloned into the *NdeI*/*HindIII* site of a pET-21a expression vector to yield the constructs pETrecA(S240N) and pETrecA(S240K). Both constructs were sequenced to confirm that only the desired mutations had been introduced into the RecA gene.

The pETrecA(S240N) and pETrecA(S240K) expression constructs were introduced into the *E. coli recA*-deletion strain BLR(DE3)pLysS (Novagen), and the [S240N]RecA and [S240K]RecA proteins were purified from the resulting cells using procedures analogous to those that have been described previously for the wild type RecA protein [7]. The final fractions of the [S240N]RecA and [S240K]RecA proteins were greater than 95% pure as judged by SDS polyacrylamide gel electrophoresis.

2.3. ATP and dATP hydrolysis assay

The reaction solutions contained 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 30 μM φX ssDNA, 1 μM [S240N]RecA protein or [S240K]RecA protein, and the concentrations of [α^{32}P]ATP/ATP or [α-^{32}P]dATP/dATP indicated in Fig. 2. The reactions were initiated by the addition of the RecA proteins and were carried out at 37 °C. The ATP and dATP hydrolysis reactions were monitored using a thin layer chromatography method as described previously [10]. For each reaction, a time course of hydrolysis was generated and the initial rate of hydrolysis was derived from the initial linear phase of the time course (each of the data points in Fig. 2 represents the average of three independent measurements; the variation between measurements was less than ±10% in all cases). In this assay the φX ssDNA (30 μM total nucleotide) is provided in excess relative to the RecA protein (1 μM monomer; binding stoichiometry = 3 nucleotides of ssDNA per monomer) to ensure that there is sufficient φX ssDNA to bind all of the RecA protein in the reaction solution.

2.4. Three-strand exchange assay

The reaction solutions contained 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 5 μM circular φX ssDNA, 15 μM linear φX dsDNA, 0.3 μM *E. coli* SSB, 6 μM [S240N]RecA protein or [S240K]RecA protein, and 5 mM ATP or dATP. All reaction components except for the RecA proteins were incubated for 5 min at 37 °C. The RecA proteins were then added and the incubation was continued for 1 min. The reactions were initiated by the addition of *E. coli* SSB protein and the incubation was continued for 60 min at 37 °C. Aliquots (20 μL) were then removed from the reaction solutions and quenched with SDS (1% final concentration)/EDTA (15 mM final concentration). The quenched aliquots were analyzed by electrophoresis on an agarose gel (0.8%) using a Tris acetate–EDTA buffer system. The substrates and products of the reactions were visualized by ethidium bromide staining [9]. In this assay the RecA protein (6 μM monomer; binding stoichiometry = 3 nucleotides of ssDNA per monomer) is provided in excess relative to the circular φX ssDNA (5 μM total nucleotide) to ensure that there is sufficient RecA protein to cover all of the circular φX ssDNA in the reaction solution. The reaction solutions also contained *E. coli* SSB protein which is routinely

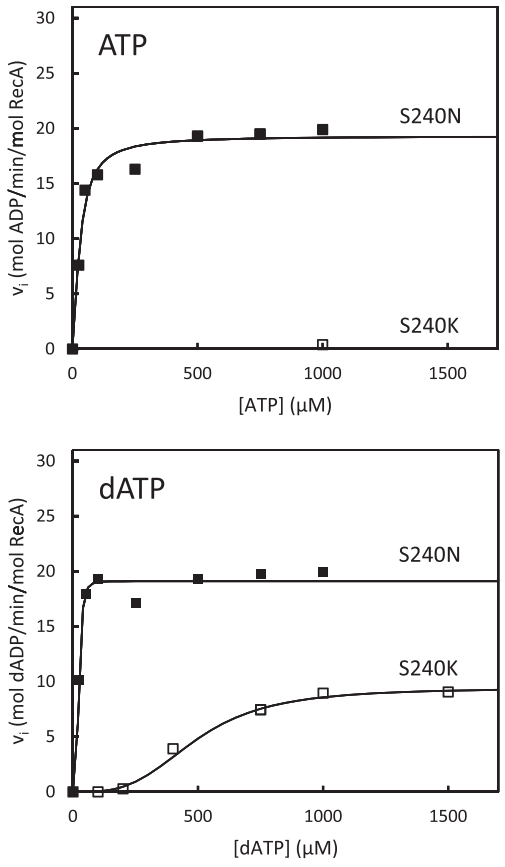

Fig. 2. Single-stranded DNA-dependent ATP and dATP hydrolysis reactions. The reaction solutions contained 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 30 μM φX ssDNA, 1 μM [S240N]RecA protein (■) or [S240K]RecA protein (□), and the indicated concentrations of ATP or dATP. The reactions were carried out at 37 °C. The data points represent the initial rates of ATP or dATP hydrolysis. The solid lines were generated using the Hill equation and the following parameters: V_{max} = 19.3 min^{-1}, $S_{0.5}$ = 30 μM, n = 1.4 ([S240N]RecA/ATP); V_{max} = 19.1 min^{-1}, $S_{0.5}$ = 24 μM, n = 3.78 ([S240N]RecA/dATP); V_{max} = 9.6 min^{-1}, $S_{0.5}$ = 490 μM, n = 3.3 ([S240K]RecA/dATP); and no reaction ([S240K]RecA/ATP). There was no detectable ATP or dATP hydrolysis by either the [S240N]RecA or [S240K]RecA protein when the circular φX ssDNA was omitted from the reaction solution (data not shown).

included as an accessory factor in RecA-promoted strand exchange assays (see Ref. [1]).

2.5. LexA cleavage assay

The reaction solutions contained 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 30 μM circular φX ssDNA, 4 μM *E. coli* LexA protein, 1 μM [S240N]RecA protein or [S240K]RecA protein, and either 5 mM ATP or dATP. All reaction components except for the RecA proteins were incubated for 10 min at 37 °C. The reactions were initiated by the addition of the RecA proteins and the incubation was continued for 60 min at 37 °C. Aliquots (20 μL) were then removed, added to 10 μL of quench solution (250 mM Tris–HCl (pH 6.8)/4% SDS/10% glycerol/10% β-mercaptoethanol/0.5% bromophenol blue), and heated to 100 °C for 3 min. The quenched aliquots were analyzed by electrophoresis on an SDS–polyacrylamide gel (5% stacking gel/13% resolving gel) using a Tris glycine buffer system. The substrates and products of the reactions were visualized by Coomassie Brilliant Blue staining. In this assay the φX ssDNA (30 μM total nucleotide) is provided in excess relative to the RecA protein (1 μM monomer; binding stoichiometery = 3 nucleotides of ssDNA per monomer) to ensure that there is sufficient φX ssDNA to bind all of the RecA protein in the reaction solution.

3. Results

3.1. ATP hydrolysis

The [S240N]RecA and [S240K]RecA proteins were first analyzed for ssDNA-dependent ATP hydrolysis activity. The reactions were carried out with circular φX ssDNA (5386 nucleotides) as the ssDNA cofactor. The initial rates of ATP hydrolysis were determined as a function of ATP concentration (Fig. 2).

The [S240N]RecA protein catalyzed ATP hydrolysis with a turnover number (V_{max}/E_t) of 19.3 min^{-1} and an $S_{0.5}$ for ATP of 30 μM (Fig. 2). These results are similar to those that have been obtained for the wild type RecA protein under these conditions [11], and indicate that the S240N mutation has no apparent effect on the ssDNA-dependent ATP hydrolysis activity of the RecA protein. The [S240K]RecA protein, in contrast, exhibited no detectable ATP hydrolysis activity, even at the highest ATP concentrations that were tested (Fig. 2). These results indicate that the S240K mutation eliminates the ssDNA-dependent ATP hydrolysis activity of the RecA protein.

3.2. dATP hydrolysis

The absence of ATP hydrolysis activity for the [S240K]RecA protein suggested that the lysine residue at position 240 may interact unfavorably with ATP, possibly due to interference with the 2′- and 3′-hydroxyl groups of the ribose ring (Fig. 1A). To explore this possibility, the [S240N]RecA and [S240K]RecA proteins were also examined for their ability to catalyze the ssDNA-dependent hydrolysis of dATP, which lacks a hydroxyl group at the 2′-position (the wild type RecA protein catalyzes ATP and dATP hydrolysis with similar $S_{0.5}$ values and turnover numbers [11]). The reactions were carried out under the same conditions as those used for the ATP hydrolysis reactions (Fig. 2).

The [S240N]RecA protein catalyzed dATP hydrolysis with a turnover number of 19.1 min^{-1} and an $S_{0.5}$ for dATP of 24 μM. These values are similar to those that were obtained with ATP, and demonstrate that the [S240N]RecA protein, like the wild type RecA protein, is able to catalyze the hydrolysis of ATP and dATP with similar efficiencies. These results indicate that the S240N mutation has no apparent effect on the dATP hydrolysis activity of the RecA protein.

In contrast to the results that were obtained with ATP, the [S240K]RecA protein was able to catalyze the hydrolysis of dATP, with a turnover number of 9.6 min^{-1} and an $S_{0.5}$ for dATP of 490 μM. Although the $S_{0.5}$ value for dATP was 20-fold higher, the rate of dATP hydrolysis by the [S240K]RecA protein was only 2-fold lower than that by the [S240N]RecA protein at dATP concentrations of 1 mM or higher. These results indicate that the inhibitory effect of the S240K mutation that is apparent in the ATP hydrolysis reaction is at least partially relieved when dATP is presented as the nucleotide cofactor.

3.3. Three-strand exchange

The [S240N]RecA and [S240K]RecA proteins were next analyzed for DNA recombinase activity using the φX DNA three-strand exchange assay. In this assay, stoichiometric amounts of RecA protein bind to the circular φX ssDNA substrate (5386 nucleotides) to form a presynaptic complex which then interacts with the linear φX dsDNA substrate (5386 base pairs). The complementary strand of the linear dsDNA is then transferred to the circular ssDNA to form a nicked circular dsDNA and a displaced linear ssDNA as the reaction products [1,9]. The reactions were carried out with either ATP or dATP as the nucleotide cofactor. The substrates and products of

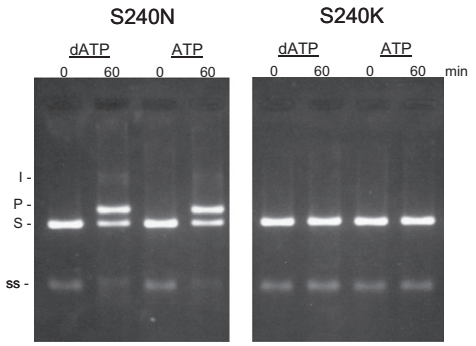

Fig. 3. Three-strand exchange reactions. The reaction solutions contained 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 0.3 μM *E. coli* SSB, 5 μM circular φX ssDNA, 15 μM linear φX dsDNA, 6 μM [S240N]RecA protein or [S240K]RecA protein, and 5 mM ATP or dATP, as indicated. The reactions were carried out at 37 °C for the indicated times and then analyzed by agarose gel electrophoresis. Labels: *S*, linear φX dsDNA substrate; *I*, partially exchanged reaction intermediates; *P*, nicked circular φX dsDNA product; *ss*, φX ssDNA substrate and product. The circular φX ssDNA substrate (5 μM total nucleotide) was limiting relative to the linear φX dsDNA (15 μM total nucleotide = 7.5 μM base pairs) and therefore the maximum amount of linear dsDNA that can be converted to nicked circular dsDNA product is 67%.

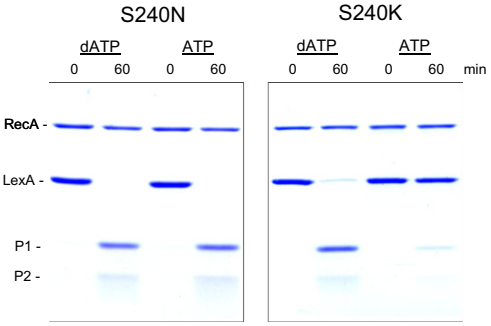

Fig. 4. LexA protein cleavage reactions. The reaction solutions contained 25 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 5% glycerol, 1 mM dithiothreitol, 30 μM circular φX ssDNA, 4 μM *E. coli* LexA protein, 1 μM [S240N]RecA protein or [S240K]RecA protein, and 5 mM ATP or dATP, as indicated. The reactions were carried out at 37 °C for the indicated times and then analyzed by SDS–polyacrylamide gel electrophoresis. Labels: *RecA* (37.8 kDa); *LexA* (22 kDa), *P1*, LexA cleavage product 1 (13 kDa); *P2*, LexA cleavage product 2 (9 kDa). There was no detectable LexA protein cleavage by the either the [S240N]RecA or the [S240K]RecA protein when either the circular φX ssDNA or the nucleotide cofactor was omitted from the reaction solution (data not shown).

the reactions were monitored by agarose gel electrophoresis (Fig. 3).

The [S240N]RecA protein was able to promote the strand exchange reaction when either ATP or dATP was provided as the nucleotide cofactor (Fig. 3). In both cases, virtually all of the circular ssDNA substrate was converted into the nicked circular dsDNA reaction product within the 60 min reaction period. These reactions are comparable to the reactions that are promoted by the wild type RecA protein under these conditions [11], and are consistent with the results in Fig. 2 which showed that the [S240N]RecA protein was able to catalyze the hydrolysis of both ATP and dATP. These results indicate that the S240N mutation has no apparent effect on either the ATP or dATP-dependent strand exchange activities of the RecA protein.

The [S240K]RecA protein exhibited no strand exchange activity with ATP as the nucleotide cofactor (Fig. 3). This result is consistent with the results in Fig. 2 which showed that the [S240K]RecA protein had no detectable ATP hydrolysis activity. However, the [S240K]RecA protein also exhibited no strand exchange activity when dATP was provided as the nucleotide cofactor, even though the results in Fig. 2 showed that it was able to catalyze the hydrolysis of dATP (Fig. 3). These results indicate that the S240K mutation eliminates both the ATP and dATP-dependent strand exchange activities of the RecA protein.

3.4. LexA cleavage

The [S240N]RecA and [S240K]RecA proteins were also analyzed for co-protease activity using a LexA cleavage assay. The reactions were carried out with circular φX ssDNA as the ssDNA cofactor, and either ATP or dATP as the nucleotide cofactor. The substrates and products of the reactions were monitored by SDS–polyacrylamide gel electrophoresis (Fig. 4).

The [S240N]RecA protein was able to promote the cleavage of LexA protein (22 kDa, 202 amino acids) when either ATP or dATP was provided as the nucleotide cofactor (Fig. 4). The cleavage products were identical in size to the 13 and 9 kDa peptides that are produced under these conditions by the wild type RecA protein [12], and correspond to the auto-cleavage reaction between Ala84–Gly85 in the hinge region that connects the amino and carboxy domains of the LexA protein [8]. These results are consistent with the results in Fig. 2 which showed that the [S240N]RecA protein was able to catalyze the hydrolysis of both ATP and dATP, and indicate that the S240N mutation has no apparent effect on either the ATP or dATP-dependent LexA cleavage activities of the RecA protein.

The [S240K]RecA protein exhibited only a trace level of LexA cleavage activity in the presence of ATP (Fig. 4). The [S240K]RecA protein was able to promote an efficient LexA cleavage reaction, however, when dATP was provided as the nucleotide cofactor (Fig. 4). These results are consistent with the results in Fig. 2 which showed that the [S240K]RecA protein was able to catalyze the hydrolysis of dATP, but had no detectable ATP hydrolysis activity. These results indicate that the S240K mutation, in effect, converts the RecA protein into a dATP-specific LexA co-protease.

4. Discussion

The S240N mutation has no discernible effect on the biochemical activities of the RecA protein, thus indicating that serine and asparagine are functionally interchangeable at position 240. The S240K mutation, in contrast, essentially eliminates the ability of the RecA protein to utilize ATP as a nucleotide cofactor. The [S240K]RecA protein does retain a significant dATP hydrolysis activity, however, indicating that the absence of a 2′-hydroxyl group in dATP at least partially reduces an inhibitory interaction with the Lys240 side chain. The finding that the [S240K]RecA protein is able to promote an efficient LexA cleavage reaction but exhibits no strand exchange activity when dATP is provided as a nucleotide cofactor is mechanistically significant because it indicates that these activities differ in their dependence on the nucleotide cofactor. This difference (which is not apparent from the properties of the wild type or [S240N]RecA proteins) may have been revealed with the [S240K]RecA protein because of its elevated $S_{0.5}$ value for dATP.

We have shown previously that the stability of the RecA–ssDNA–NTP complex is dependent on the $S_{0.5}$ value of the nucleoside triphosphate being hydrolyzed, with those nucleoside triphosphates with higher $S_{0.5}$ values leading to decreased filament stability [13,14]. Therefore, although the [S240K]RecA protein is able to catalyze the ssDNA-dependent hydrolysis of dATP, the [S240K]RecA–ssDNA–dATP filaments are likely to be less stable than those that are formed by the wild type and [S240N]RecA proteins, due to the elevated $S_{0.5}$ value for dATP (490 μM, compared to 20–30 μM for the wild type and [S240N]RecA proteins). The

efficient dATP-dependent LexA cleavage reaction shows that the [S240K]RecA protein is able to form tracts of functionally-active filaments on ssDNA in the presence of dATP that are at least stable enough to capture and facilitate the auto-cleavage of individual LexA protein molecules (the binding of LexA protein may conceivably even serve to stabilize the filaments). The absence of a dATP-dependent strand exchange activity, however, suggests that the [S240K]RecA protein may not be able to form the more extensive and stable presynaptic filaments on ssDNA that are required to engage and promote a strand exchange reaction with a linear dsDNA molecule [1,13,14]. By comparison, the [S240N]RecA protein, which would be expected to form more stable filaments on ssDNA owing to its lower $S_{0.5}$ value for dATP (24 μM), is able to promote LexA cleavage and DNA strand exchange with dATP (or ATP) as the nucleotide cofactor. The differential dependence of the LexA cleavage and DNA strand exchange reactions on nucleotide cofactor-mediated RecA filament stability may be relevant to the physiological functions of the wild type RecA protein and merits further investigation.

The effects of the S240K mutation on the ATP and dATP hydrolysis activities of the RecA protein suggest that the long and flexible Lys240 side chain may interfere with the positioning of the nucleotide cofactor or perturb the overall structure of the nucleotide cofactor binding site (Fig. 1B). The smaller serine and asparagine side chains of the wild type and [S240N]RecA proteins, in contrast, may be more sterically accommodating and may even form favorable hydrogen bonds with the nucleotide cofactor or with neighboring amino acid residues (Fig. 1B). Although the *S. pneumoniae* RecA protein has a lysine residue at the sequence position that corresponds to Ser240 in the *E. coli* RecA protein [6], we have shown previously that it is able to catalyze the hydrolysis of both ATP and dATP, and is able to promote the LexA cleavage and DNA strand exchange reactions with either ATP or dATP as the nucleotide cofactor [12]. The possible functional role of this lysine residue in the active site mechanism of the *S. pneumoniae* RecA protein remains to be determined.

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