Physiological Relevance of Salt Environment for *in vitro* recA System

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RecA protein can promote strand assimilation, homologous pairing, and strand exchange. All these reactions require DNA-dependent ATP hydrolysis by recA protein, and the activities of recA protein are affected by the ionic environment. In this experiment, DNA-dependent ATPase activity showed different sensitivity to anionic species. ATP hydrolysis and strand exchange were relatively sensitive to salt in the reactions with NaCl, strongly inhibited at 100 mM NaCl. However, the inhibition by sodium acetate or sodium glutamate was not observed at 50~100 mM concentration. Addition of sodium glutamate to the standard reaction condition increased the apparent efficiency of ATP hydrolysis during strand exchange. The condition including 50~100 mM sodium-glutamate might be similar to the physiological condition.

Key words: recA protein, DNA strand exchange, ATP hydrolysis, heteroduplex formation, *in vivo* salt environment

In *E. coli*, recA protein has been shown to play an important role in the process of genetic recombination and repair of UV-damaged DNA (4, 14). The enzymatic activities of this protein include strand assimilation, homologous pairing, and strand exchange. All these reactions require DNA-dependent ATP hydrolysis by recA protein. *In vitro* DNA strand exchange promoted by recA protein is analogous to homologous recombination *in vivo* (3). The process of strand exchange reaction involves presynaptic complex formation, homologous pairing. and strand exchange (Fig. 1). RecA protein binds cooperatively to single-stranded DNA under buffer conditions optimal for *in vitro* strand exchange, forming a nucleoprotein filament (7). In electron microscope, the complexes of recA protein with DNA have been shown to be highly ordered filamentous structures that can cover the entire length of the DNA molecule (6, 7). Protein-protein interactions appear to play an important role in the formation of filaments, as indicated by recA protein cooperatively binding to DNA. These structures are active species and have central importance in recA mediated strand exchange reaction. The rate and efficiency of strand exchange reaction is a function of both binding density of recA protein in the complex and stability of the complex (3, 5). In the

homologous pairing step, joint molecule is formed at the end of homologous duplex DNA. This heteroduplex joint is extended in the phase of branch migration. The branch migration steps involve ATP hydrolysis by recA protein. A number of standard assays, including agarose gel electrophoresis, S1 nuclease protection, and electron microscopy, are available to follow the fate of DNA species during the reaction *in vitro* (3, 19).

On DNA molecule, recA protein binds stoichiometrically to form extended nucleoprotein filaments. When bound to DNA, recA protein can mediate the hydrolytic reaction of ATP. ATP is hydrolyzed throughout these filaments (2). Monomers within complexes hydrolyze ATP with turnover number of 25 30 min^{-1}. In the absence of DNA, the level of ATP hydrolysis by recA protein decreases by 2,000 fold (15). DNA-independent ATPase activity can be stimulated by high salt concentration (2 M).

RecA protein is bound to the DNA along the phosphate backbone (9). The affinity of recA protein to DNA is shown to be dependent on pH, salt, and the presence of nucleotide cofactors (18). Salts are known to have dramatic effect on DNA binding properties and enzymatic activities of recA protein. In this work, the effects of salt on the activity of recA protein were investigated. Traditional standard reaction conditions which are not physiological are optimized for strand exchange. These experiments help establish the physiological relevance

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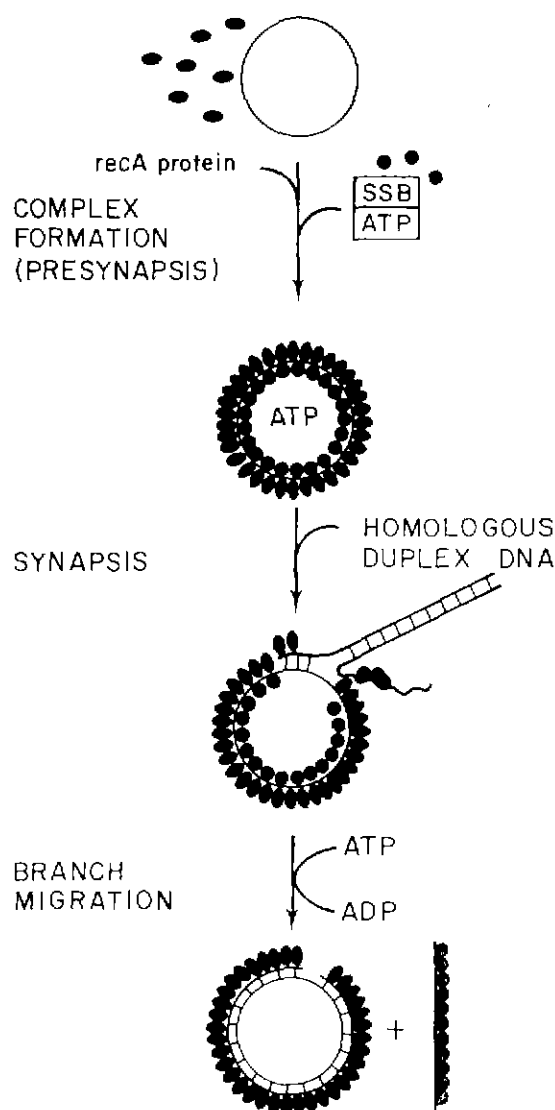(E-mail) jikim@swift2.swu.ac.kr

Fig. 1. A scheme of recA protein-mediated DNA strand exchange reaction (4).

of the fundamental properties of the recA system *in vitro*.

Materials and Methods

Proteins and Biochemicals

RecA protein was purified by a procedure previously described (3). The concentration of recA protein was estimated by measuring the absorbance at 280 nm using an extinction coefficient ε_{280} =0.59 A_{280} mg^{-1}ml. SSB protein was isolated by a modification of published procedure (11). Its concentration was calculated from the extinction coefficient, ε_{280}=2.83 × 10^4 M^{-1}cm^{-1}. Substrate DNAs (ssDNA and duplex DNA of M13mp18) were prepared according to a published procedure (17). Uniformly

tritiated ssDNA was prepared by an established procedure (8) and a specific activity of 10~50 Ci/mol. The molar concentration of DNA's stock solutions were determined by absorbance at 260 nm, using 36 and 50 µg/ml A_{260} for ssDNA and duplex DNA as conversion factors, respectively. The concentrations of DNA were expressed in terms of nucleotide. The duplex DNA of M13mp18 was linearized by digesting with *Eco*RI or *Pst*I restriction endonuclease. Restriction enzymes were purchased from New England BioLabs. All enzymes or ATP regenerating system and the other reagents were obtained from Sigma.

Strand exchange reaction

Unless indicated otherwise, reactions were carried out at 37°C in 25 mM Tris-Cl (pH 7.5), 1 mM DTT, 5% glycerol, 10 mM MgCl$_2$, 1 mM ATP, and ATP regenerating system (2.4 U/ml pyruvate kinase, 2.4 mM phosphoenolpyruvate, 0.44 mM KCl) and at indicated concentration of DNA, recA protein, and SSB. The reaction was started by adding of ATP and SSB after preincubation of all other components for 5 min at 37°C. When changing the salt concentration of the reaction mixture, salts were added before preincubation.

Measurements of product molecule

The product formation of strand exchange was measured by using the agarose gel electrophoresis and S1 nuclease assay method (3). For the gel assay, after indicated time aliquots (25 ml) of raction mixture were removed, SDS (5%) and gel loading buffer (25% glycerol, 5 mM EDTA, 0.005% bromophenolblue) were added. These samples were subjected to electrophoresis on 0.8% agarose gel. For the S1 nuclease assay, aliquots (50 ml) of the raction mixture containing M13mp18 ssDNA which is uniformly labeled with [^{3}H] were deproteinized with 10% SDS (5 µl) and incubated with 80 units of S1 nuclease in 620 µl digestion buffer containing 500 mM NaCl, 50 mM sodium acetate (pH 4.6), 1 mM zinc acetate, and 32 mg/ml of heat-denatured calf thymus DNA, for 30 min at 37°C. The reaction was stopped by adding 10% trichloroacetic acid, then the samples were filtered through Whatman GF-C filter discs. Filters were washed with 3 ml of cold 10% trichloroacetic acid, and then dried and assayed for radioactivity by liquid scintillation. To determine the total acid precipitable counts (100%), an aliquot (50 µl) of the reaction mixture was treated the same as indicated above, except no S1 nuclease was added. The counts of control experiment which contained no duplex DNA were less than 5% of total counts and were subtracted from

all data. The first method separated the substrate from the product (RF II) by gel electrophoresis. The second method employed the S1 nuclease assay, which measures conversion of S1 nuclease-sensitive ^{3}H-labeled ssDNA into S1 nuclease-resistant heteroduplex DNA. Only the ^{3}H-labeled ssDNA that is converted into heteroduplex DNA becomes acid insoluble after S1 nuclease treatment.

ATPase assay

ATPase activity was measured by spectrophotometric coupled assay as indicated (13). Reactions were carried out in 25 mM Tris-HCl (pH 7.5), 1 mM DTT, 5% glycerol, 10 mM MgCl$_2$, 1 mM ATP, and ATP coupling system and indicated concentration of DNA, recA protein and SSB in a volume of 0.5 ml in 0.5 cm path length at 37°C. The reaction was initiated by adding ATP and SSB after preincubation of all other components for 5 min at 37°C. Reaction progress was measured by monitoring ATP hydrolysis which is coupled to the ATP regenerating system. Regeneration of ATP from ADP and phosphoenolpyruvate is coupled with oxidation of NADH, which can be monitored by a decrease in absorbance at 380 nm. A system lag due to the coupling system was approximately 1 min. An extinction coefficient of 1,210 M^{-1}cm^{-1} at 380 nm for NADH was used to calculate the rate of ATP hydrolysis. Adding more regeneration enzymes does not increase the rate, implying that coupling system is never limiting. Absorbances were continuously measured over a period of 1 h.

Results

The following experiments were performed to study the effect of salts on DNA-dependent ATPase activity and the efficiency of ATP hydrolysis during the recA-mediated strand exchange reaction.

Effect of salts on ssDNA-dependent ATP hydrolysis

The recA binding to DNA is mediated by the interactions with sugar phosphate backbones of DNA (9). The DNA-dependent ATPase activity could be affected by ionic strength of the reaction mixtures, primarily by virtue of effects on DNA binding. DNA-dependent ATPase activity showed different sensitivity to anion concentrations. In low salt concentration range, the ssDNA-dependent ATP hydrolytic reaction was relatively resistant to salt, 20% inhibited at 50 mM NaCl. Over 100 mM NaCl, reaction was completely inhibited by NaCl (Fig. 2). Less inhibition was observed in the presence of sodium
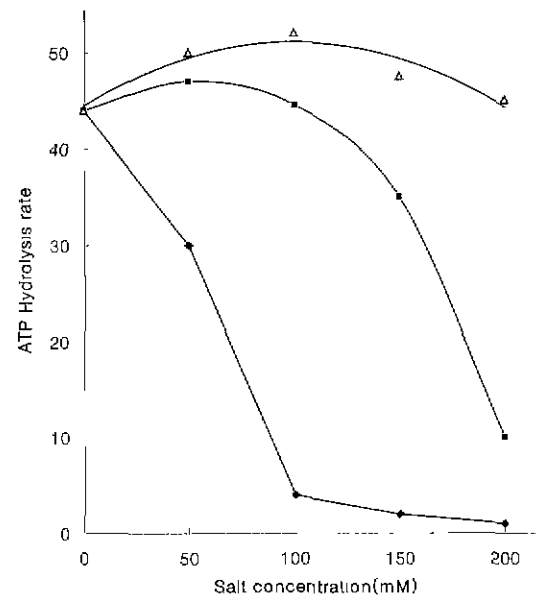

Fig. 2. The Effect of salts on ssDNA-dependent ATP hydrolytic reaction Reactions contained 5 μM M13mp18 ssDNA, 2 μM recA protein, 1 mM ATP and 0.5 μM SSB. Salts were added directly to the mixture before preincubation; NaCl (◆), sodium-acetate (■), and sodium glutamate (△).

acetate or sodium glutamate. The ATP hydrolysis rate was unaffected by sodium acetate up to 100 mM concentration. Inhibition was not observed in reactions with glutamate concentrations up to 200 mM. Rather, glutamate exhibited a stimulatory effect on ssDNA-dependent ATP hydrolysis.

Effect of salts on strand exchange reaction

DNA strand exchange was monitored by two methods. Product formation was qualitatively determined by gel electrophoresis, and heteroduplex formation was estimated by S1 nuclease assay. Joint molecules of ssDNA with linear duplex DNA, which migrated slower than linear duplex (designated as F III DNA), were produced for the first 10 min. As the reaction progressed, regions of heteroduplex in joint molecules extended. A nicked circular duplex DNA (F II DNA) was produced as the final product within 20 min. After that, accumulation of F II DNA was observed in agarose gel electrophoresis (Fig. 3). But with this assay, the extents of reactions were not quantitatively evaluated. The extent of the strand exchange (in terms of total heteroduplex base pairs formed) varied somewhat at reaction conditions. As Fig. 4 shows, 50% of ssDNA ended up with heteroduplex in an hour. And the addition of 50 mM Na-glutamate or Na-acetate to the reaction condition stimulated strand exchange to a degree. The enhanced extent and increased rate of heteroduplex formation were
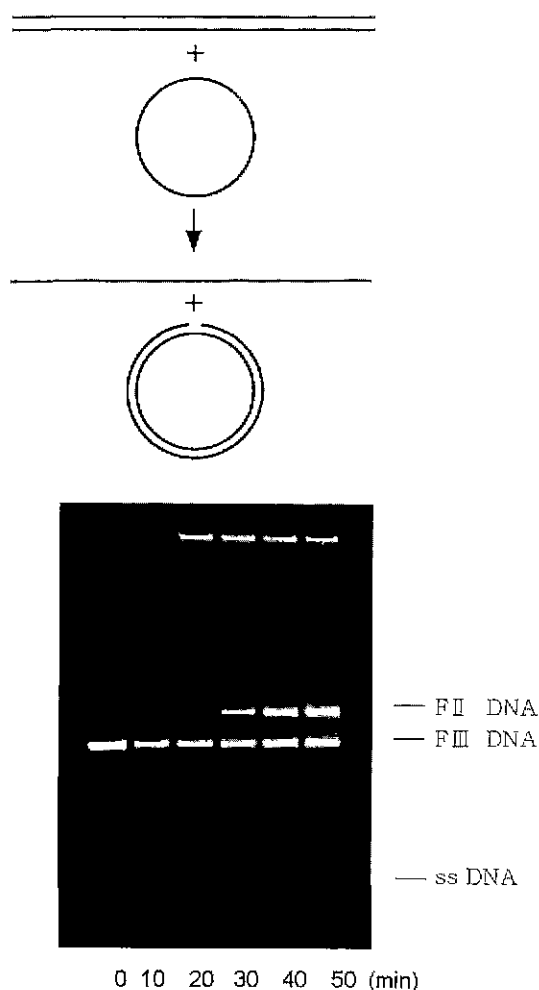
Fig. 3. Strand exchange reactions measured by agarose gel electrophoresis. Reactions contained 5 µM M13mp18 ssDNA, 8 µM M13mp18 linear dsDNA, 2 µM recA protein, 1 mM ATP and 0.5 µM SSB in a reaction buffer.

Fig. 4. Strand exchange reactions measured by S1 nuclease assay. (A) Effect of Na-glutamate on DNA strand exchange. Reactions contained 5 µM M13mp18 ssDNA, 8 µM M13mp18 linear dsDNA, 2 µM recA protein, 1 mM ATP and 0.5 µM SSB in a reaction buffer containing 10 mM $MgCl_2$; 0 mM (O), 50 mM (△), 75 mM (●) and 100 mM (□) (B) Effect of sodium-acetate on DNA strand exchange. Reactions were done under the condition same as in (A); 0mM (O), 50mM (△), and 75 mM(●).(C)Effect of sodiumglutamate on DNA strand exchange.Reaction were done in a reaction buffer including 10 mM acetate magnesium instead of 10 mM $MgCl_2$; 0 mM (O), 50 mM (△), and 75 mM (●). Extents of reaction of reaction were normalized to the condition, which is set at 1.p using 10 mM $MgCl_2$. (D) Strand exchange reactions in the presence of sodium-glutamate. Reactions were carried out same as in (A) for the indicated times 10, 30 50min.; (1) 50 mM (2) 75 mM (3) 100 mM.

observed in the reactions with sodium glutamate or sodium acetate, and more heteroduplex DNA formed in the presence of glutamate than with acetate (Fig. 4A, B), and magnesium acetate worked better than $MgCl_2$ as magnesium source (Fig. 4A, C). When strand exchange reactions were assayed by agarose gel electrophoresis (Fig. 4 D), the reactions seemed to be unaffected by sodium glutamate up to 100 mM. However, in the reactions with NaCl, strand exchange was inhibited. Complete inhibition was achieved by 100 mM NaCl (data not shown). It is also demonstrated that strand exchange is strongly inhibited by 50 mM NaCl (18). This observation of relieved inhibition is compatible with the results reported by Menetski (12), in which recA protein binds more tightly to DNA in a buffer containing acetate ions than in those with chloride ions. From this data, the optimal con-
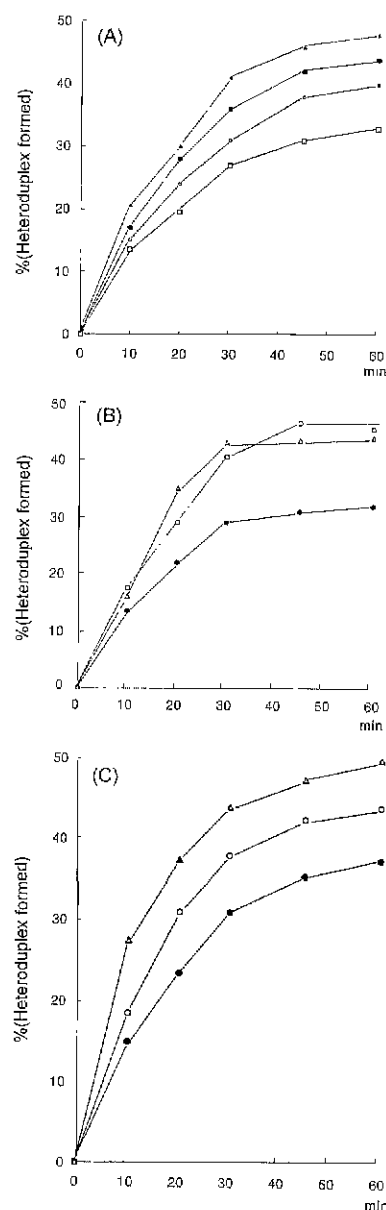
dition for strand exchange seems to be the inclusion of 50 mM sodiumglutamate.

(D) (1) (2) (3)

10 30 50

Fig. 4. Continued.

Fig. 5. Effect of salt on ATP hydrolysis during strand exchange. Standard ATPase assay condition including 5 µM ssDNA, 8 µM linear dsDNA, 2 µM recA protein, 1 mM ATP and 0.5 µM SSB in a reaction buffer containing 10 mM magnesium acetate was used; NaCl (□), sodium acetate (○), and sodium glutamate (▲).
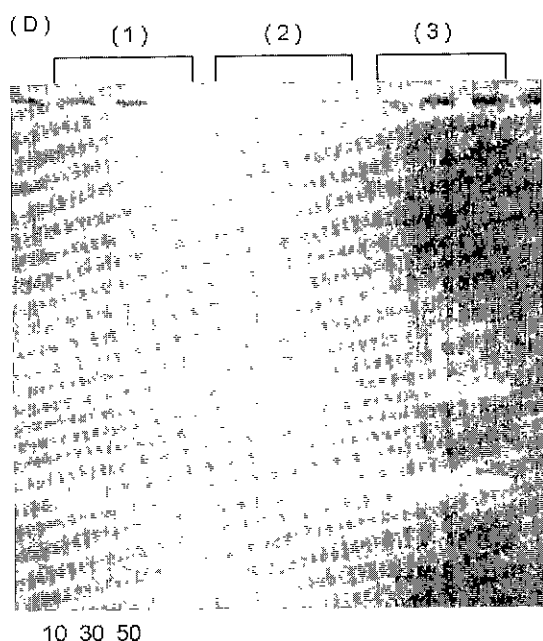
Effect of salts on ATP hydrolysis during DNA strand exchange

Reactions were set up in which the only variable was the salt concentration. When strand exchange was initiated by the homologous pairing of the duplex DNA with the presynaptic recA-ssDNA complex, addition of homologous duplex DNA resulted in an immediate decrease by 33% in the rate of ATP hydrolysis relative to the rate measured in the presence of ssDNA alone (4). The subsequent rate of ATP hydrolysis remained constant throughout DNA strand exchange. The decrease in ATP hydrolysis rate is associated with formation of paranemic joints between ssDNA molecules and duplex DNA. In Fig. 4, the rates of ATP hydrolysis after addition of duplex DNA was measured during strand exchange. The decrease in ATP hydrolysis rate was observed in the presence of 50 mM sodinum glutamate or sodium acetate. Sodium glutamate and sodium acetate did not strongly affect the ATP hydrolysis during strand exchange up to 150 mM and 100 mM, re- spectively. Under the conditions containing NaCl, the ATP hydrolysis rate during strand exchange decreased with increasing salt concentrations. Strand exchange was completely inhibited at 75 mM NaCl (data not shown). Disassembly of recA protein from DNA seemed to result in the decrease of ATP hydrolysis rates in the presence of NaCl. With sodium glutamate, however, the strand exchange reaction was not inhibited until the concentration exceeded 100 mM (Fig. 4D).
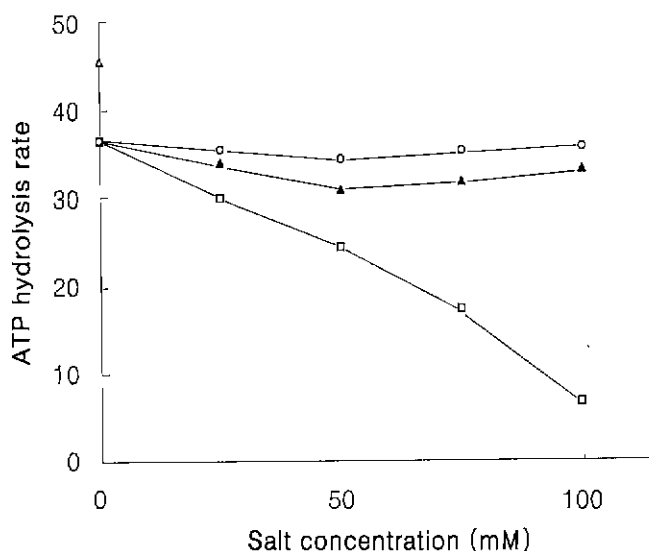
As judged by ATP hydrolysis, strand exchange is an inefficient process. More than 1,000 ATPs are hydrolyzed per base pair of heteroduplex formed in the absence of SSB (3). The addition of SSB improves the efficiency to 100-200 ATPs hydrolyzed per heteroduplex base pairs (5). The initial rate of heteroduplex formation was stimulated by 50 mM sodium glutamate (Fig. 4 A, C). A smaller degree of stimulation of the initial rate of heteroduplex formation was observed at 75 mM sodium glutamate. The initial rate of heteroduplex formation decreased by Na-acetate or over 100 mM sodium glutamate (Fig. 4 A). The efficiency of ATP hydrolysis during strand exchange was estimated from the initial rates of heteroduplex formation and ATP hydrolysis (Fig. 4 and 5). Under the standard reaction conditions with 1 mM ATP, 160 ± 20 ATPs were hydrolyzed for each base pair of heteroduplex formed. Addition of sodium glutamate to the standard reaction condition increased the apparent efficiency of ATP hydrolysis during strand ex- change. Maximum efficiency was obtained at 50 mM sodium glutamate, where 7020 ATPs were hydrolyzed per base pair of heteroduplex formed. The efficiency of ATP hydrolysis at 75 mM and 100 mM sodium glutamate was 100 ± 10 and 120 ± 20 ATPs hydrolyzed per base pair of hetero- duplex formed, respectively. Also the extent of decrease in ATP hydrolysis rates after addition of duplex DNA (Fig. 5) relative to the rates measured in the presence of ssDNA alone (Fig. 2) was the greatest at 50 mM sodium glutamate. This can imply that the effi-

ciency of ATP hydrolysis during the homologous pairing and during the subsequent strand exchange was the maximum under the condition containing 50 mM sodium glutamate.

Discussion

The basic conclusion from this work is that strand exchange occurs under physiological conditions, with the rate and efficiency comparable to conditions previously determined to be optimal. The fundamental properties of the recA system are unaffected by addition of 100 mM Na-glutamate. In contrast, a significant inhibition in reactions with NaCl is noted. The increased rate of ATP hydrolysis on ssDNA might result from the stabilization effect of glutamate on the recA-ssDNA complex and/or a direct effect on ATPase activity. However, chloride ion (Cl⁻) is present at low concentrations in *E. coli* cells. The total concentration of organic anion is generally higher than that of chloride anion, the dominant inorganic anion (20). The dominant organic anion in *E. coli* is glutamate (20). It appears that the glutamate concentration substantially exceeds the chloride anion in *E. coli* (10). The physiological concentration of glutamate in *E. coli* cytoplasm is between the range of 30~250 mM (16).

The strand exchange reactions are inhibited by 50 mM NaCl or by the presence of ADP. DNA binding studies have shown that the binding of recA protein to DNA is strongly inhibited by NaCl (18). Previous work has demonstrated that there may exist a correlation between the stability of recA-ssDNA complex and the rate or extent of the subsequent strand exchange reaction (5, 19). From the result of this work, the signifcant effect of salt on recA protein activities might not be a general ionic strength effect, but rather ion specific effects. It has been shown that the binding of recA protein to DNA is mainly electrostatic interaction to the phosphate backbone of DNA (6). The effect of salts on protein tertiary structure was discussed in earlier studies (1, 10, 16). Salts can act as competitors or effectors of DNA binding, and of protein-protein interactions. Inhibition of ATP hydrolytic activities was not associated with increasing salt concentration, but with interference with DNA binding. This can be inferred from the observation that recA has a strong DNA-independent ATPase activity at very high salt concentrations and by observed effects on DNA binding (15). A consideration of DNA binding is not required for the high salt activation of recA protein. Previous results have shown that acetate anion activates ATPase of recA protein better than

glutamate anion. However, the DNA-dependent reaction was less efficient in acetate solution than in glutamate solution. Therefore, glutamate might not be as stronger effector than as acetate on ATP hydrolytic event itself. The overall stability of presynaptic complex of recA protein with ssDNA seems to be enhanced in the presence of glutamate anion. Tighter ssDNA binding observed in glutamate solution may allow recA protein to be unaffected by the increasing ionic strength. Glutamate has the tendency to be excluded from the protein (1). This exclusion causes the protein to be more compact and preferentially hydrated. This nature of glutamate might enhance the interaction of recA protein with ssDNA, which is dependent on the bulk salt concentration, by stabilizing the recA complex with DNA. The condition including 50~100 mM sodium glutamate might be similar to the physiological condition.

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