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RESEARCH NOTE

EXAMINATION OF TYR-264 FOR ATPase ACTIVE SITE IN *E.coli* RecA PROTEIN BY SITE-DIRECTED MUTAGENESIS

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Abstract — Site directed mutagenesis has been introduced to determine active site(s) and molecular structure of *E.coli* RecA protein. Recombinant DNAs were constructed by point mutation of Tyr-264 to Phe which assumed active site for binding and hydrolysis of ATP. RecA proteins were purified from recombinants containing wild type and mutant genes and analyzed for ATPase activity assay. Result suggests that Tyr-264 is involved in ATP binding rather than ATP hydrolysis.

INTRODUCTION

RecA protein is an essential factor for homologous genetic recombination and post-replicational repair of DNA damage in *E.coli*.[1,2] It acts as a regulatory factor for the expression of *din* genes by DNA damages (SOS response).[3] It also involves in mutagenesis, cell division and λ phage induction.[3] Most of these reactions require single stranded DNA dependent binding or hydrolysis of ATP by the RecA protein.[1-4] *In vivo* and *in vitro* studies with recA mutants suggest that the *RecA* protein may be composed of several distinct functional and structural domains.[3,5] For the ATP binding and/or hydrolysis, a region between Ser-70 to Thr-73 was predicted from an amino acid sequence comparison of several ATP binding enzymes,[6] while involvement of Cys-90 and Cys-129 was proposed on the basis of chemical modification of cysteine residues in the RecA protein.[7] Photoaffinity labeling with 8-azidoATP[8,9] or modification by 5'-*p*-fluorosulfonylbenzoyl-adenosine(FSBA)[10] convincingly identified Tyr-264 as a site of ATP binding. On the basis of these information, we changed Tyr-264 to Phe by site directed mutagenesis and examined activities of binding and hydrolysis of ATP *in vitro*.

MATERIALS AND METHODS

Site directed mutagenesis. Site directed mutagenesis was performed by the method of Kunkel *et al.*(1987).[11] Subcloned plasmid in M13mp19 was transformed into *E.coli* CJ236 (*dut*⁻/*ung*⁻) and single stranded DNA containing uracil was isolated. Anealing with a mutagenic primer (Fig.1), double stranded DNA was synthesized with T_4DNA polymerase and lygase, and then transformed into *E.coli* JM109 where the mismatched bases were repaired. Single stranded DNA was isolated and sequenced to confirm nucleotide sequences of wild type and mutant *recA* genes (Fig.2).

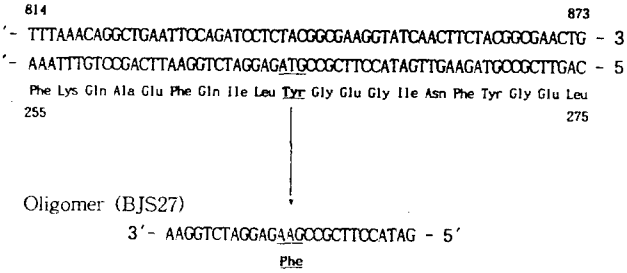

Figure 1. Nucleotide sequence for the region of mutagenesis in *E. coli* recA gene and mutagenic primer (BJS27).

Synthesis of a mutagenic primer. A mutagenic primer (BJS27) was synthesized to change Tyr-264 (GTA) to Phe (GAA) which is very similar in structure but no hydroxyl group. The sequence of the mutagenic primer was shown in Figure 1.

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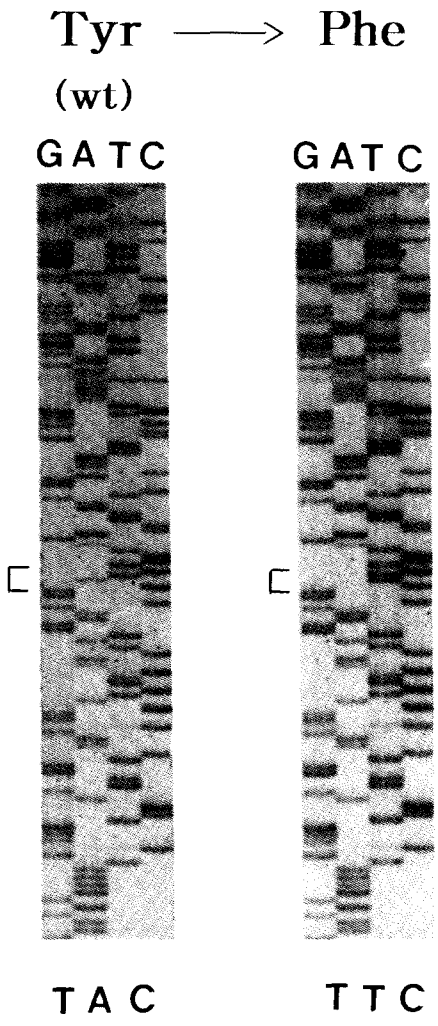

Figure 2. Dideoxy nucleotide sequence analysis of wild type and mutant *recA* genes.

Purification of RecA protein. RecA protein was purified by the method of Cox *et al.* (1981)[12] with minor modification. *E.coli* strain KM4104 containing recombinant plasmids carrying wild type and mutant *recA* genes, respectively, was grown and treated with nalidixic acid. The protein was prepared through polymin P precipitation, ammonium sulfate fractionation, hydroxy apatite and ssDNA-agarose chromatographies. RecA protein was eluted from the DNA agarose column by the addition of ATP, precipitated with ammonium sulfate, dialyzed against 20 m*M* Tris-HCl, pH 7.5, 20% glycerol, 1m*M* dithiothreitol, and 0.1 m*M* EDTA, and stored at -70°C.

Assays. ATPase activity was measured by the method of Kowalczykowski and Krupp (1987).[13] Assay buffer contains 20 m*M* Tris, pH 7.5, 0.1 m*M* dithiothreitol, 0.5 m*M* ATP, 2 m*M* phosphoenolpyruvate, 4 m*M* $MgCl_2$, 0.1 mg NADH, 12.5 U pyruvate kinase and 12.5 U lactate dehydrogenase. After incubation at 37°C for 2 min, 3 m*M* ssDNA M13mp8 and 0.26 μM RecA protein were added to make total volume of 1.0 mL and then ATP hydrolysis was measured by decrease of absorbance at 340 nm.

RESULTS AND DISCUSSION

E.coli RecA protein promotes the homologous pairing and strand exchange of DNA substrates. ATP is required as a cofactor and hydrolyzed to ADP and Pi in these processes. The exact role of ATP is not clear since RecA protein could promote homologous pairing and strand exchange in the absence of ATP hydrolysis.[3] Using a nonhydrolyzable ATP analog, ATP[γ]S, RecA could produce significant amounts of heteroduplex DNA at low Mg^{++} concentrations.[15] Thus it has been suggested that ATP hydrolysis leads ATP bound high-affinity DNA-binding state to ADP bound low-affinity DNA-binding state to result in the release of heteroduplex DNA.[3]

To elucidate ATP binding site of the RecA protein photoaffinity labelling with 8-azidoadenosine-5'-triphosphate[8, 9] and chemical modification with 5'-p-fluorosulfonylbenzoyladenosine[10] were conducted. Results suggested that Tyr-264 is the exclusive site of

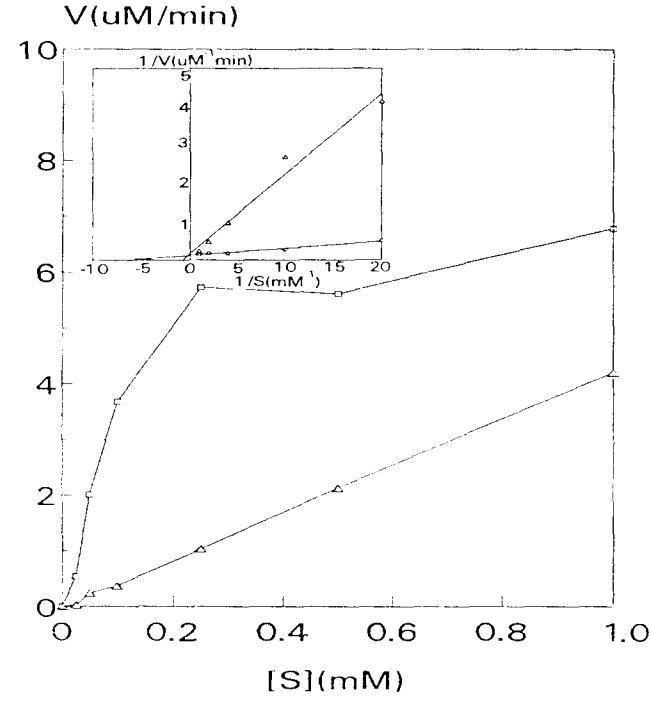

Figure 3. Substrate dependent ATPase activities of wild type and mutant RecA proteins. The inset shows Lineweaver-Berk plots of the ATPase activities of wild type (▦) mutant (△) RecA proteins.

On the basis of these observations site-directed mutagenesis was employed to change Tyr-264 to Phe (Fig.1-2) to minimize structural perturbation by the changed amino acid residue. RecA proteins were purified and examined for the activity of ATP hydrolysis.

Figure 3 shows ATP hydrolysis activities of wild type and mutant RecA proteins in the presence of single stranded DNA as a function of substrate concentrations, indicating that two proteins follow typical Michaelis-Menten type kinetics and shows significant activity differences between wild type and mutant RecA proteins. The calculated Vm and Km values were 8.4 μM/min and 152 μM for wild type, and 6.5 μM/min and 469 μM for mutant RecA proteins, respectively, indicating that change of Tyr-264 to Phe gives slight decrease of ATPase catalytic activity, whereas the change causes dramatic decrease of binding affinity of ATP to RecA protein. Recent data on the structure of RecA protein elucidated by X-ray crystallography suggest that Tyr-264 lies near the ATP binding site.[16,17]

Tyr-264 is located at the pyrimidine base of the nucleotide but does not show any specific contacts.[17] Present data is good agreement with the X-ray results that Tyr-264 is not involved in catalytic reaction but plays important role for specific binding of ATP. Further experiments with other specific residues such as Lys-72[16] and Glu-96[17] by site directed mutagenesis are in progress.

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