A unique DNase activity shares the active site with ATPase activity of the RecA/Rad51 homologue (*Pk*-REC) from a hyperthermophilic archaeon

Naeem Rashid[a], Masaaki Morikawa[b], Shigenori Kanaya[b], Haruyuki Atomi[a], Tadayuki Imanaka[a,*]

[a] *Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Yoshida-Honmachi, Sakyo-ku, Kyoto 606-8501, Japan*

[b] *Department of Material and Life Science, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan*

Received 30 October 1998; received in revised form 18 January 1999

Abstract A RecA/Rad51 homologue from *Pyrococcus kodakaraensis* KOD1 (*Pk*-REC) is the smallest protein among various RecA/Rad51 homologues. Nevertheless, *Pk*-Rec is a super multifunctional protein and shows a deoxyribonuclease activity. This deoxyribonuclease activity was inhibited by 3 mM or more ATP, suggesting that the catalytic centers of the ATPase and deoxyribonuclease activities are overlapped. To examine whether these two enzymatic activities share the same active site, a number of site-directed mutations were introduced into *Pk*-REC and the ATPase and deoxyribonuclease activities of the mutant proteins were determined. The mutant enzyme in which double mutations Lys-33 to Ala and Thr-34 to Ala were introduced, fully lost both of these activities, indicating that Lys-33 and/or Thr-34 are important for both ATPase and deoxyribonuclease activities. The mutation of Asp-112 to Ala slightly and almost equally reduced both ATPase and deoxyribonuclease activities. In addition, the mutation of Glu-54 to Gln did not seriously affect the ATPase, deoxyribonuclease, and UV tolerant activities. These results strongly suggest that the active sites of the ATPase and deoxyribonuclease activities of *Pk*-REC are common. It is noted that unlike Glu-96 in *Escherichia coli* RecA, which has been proposed to be a catalytic residue for the ATPase activity, the corresponding residual Glu-54 in *Pk*-REC is not involved in the catalytic function of the protein.

Key words: RecA/Rad51 homologue; Complementation; Mutagenesis; ATPase; DNase; Archaeon

1. Introduction

Bacterial RecA protein plays a critical role in homologous recombination, repair of damaged DNA, and SOS response [1]. Rad51 protein performs similar functions in eukaryotic cells [2]. RecA/Rad51 has DNA-dependent ATP hydrolyzing (ATPase) activity which is essential for the strand exchange reaction. Based on X-ray analysis of *Escherichia coli* RecA crystals, a schematic model explaining the allosteric effect of ATP binding on DNA binding has been proposed [3]. Lys-72, Thr-73, Glu-96, Asp-144 and Ser-145 constitute a catalytic center of ATP hydrolytic reaction, while the flexible L2 loop is responsible for ssDNA binding [4]. The ATP binding triggers proper conformational change of the loop to express ssDNA binding activity, and ssDNA or dsDNA binding also stimulates ATPase activity [5]. Gln-194 has been suggested to be a mediator residue between the ATP binding site and the DNA binding motif [4]. Moreover, the residue has recently been demonstrated to be not only an exclusive mediator, but also an 'on-off' switch required for the general activation of the RecA function [6].

RecA/Rad51 homologues from hyperthermophilic archaea have been designated as RadA due to their higher sequence similarity to Rad51 from *Saccharomyces cerevisiae* than to RecA from bacteria [7]. We have previously cloned the *Pk-rec* gene encoding a Rec protein from *Pyrococcus kodakaraensis* KOD1. This protein, composed of 210 aa, is the smallest RecA/Rad51 homologue so far identified [8]. The amino acid sequence comparison of *Pk*-REC with other RecA/Rad51 proteins demonstrated that ATP binding motifs A and B were well conserved while the region including the L2 loop showed little similarity [8]. Even though the protein comprises only the main central domain of RecA protein with N- and C-terminal truncations, *Pk*-REC is a super multifunctional protein and shows ds- and ss-DNase activities, and also a DNA-independent ATPase activity [9]. Interestingly, the main reaction product of this ATPase is AMP, instead of ADP. In order to examine whether ATPase and deoxyribonuclease (DNase) activities share the same active site or not, several mutations were introduced into the conserved residues which are expected to be responsible for ATP hydrolysis. The effects of each mutation on ATPase, DNase, and UV tolerant activities of the protein were examined.

2. Materials and methods

2.1. Bacterial strains and plasmids

The hyperthermophilic archaeon *P. kodakaraensis* KOD1 was isolated from a solfataric hot spring in Kagoshima, Japan [10]. *E. coli* strain JM109 was used as a host for subcloning the gene fragments and general DNA manipulations. RecA deficient *E. coli* strain HMS174(DE3)pLysS (F-recA hsdR (rk12-mk12$^+$)RifR(DE3)-pLysS(Cm)) was used as a host to express wild and mutant *Pk-rec* genes by using the pET-8c expression vector (Novagen, Madison, WI, USA).

2.2. Site-specific mutagenesis

Mutagenesis was carried out by the mega primer method utilizing polymerase chain reactions (PCR) [11]. Oligonucleotides were synthesized with Gene Assembler Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). In the preparation of a mutant gene, two mega primers were independently amplified by PCR using respective mutagenic primers. Another round of PCR was then performed using these two mega primers as priming strands as well as template. The fidelity of the mutation with no unexpected mutations was confirmed by DNA sequencing.

2.3. Production and purification of Pk-REC mutants

E. coli strain HMS174(DE3)pLysS carrying the expression plasmid harboring the *Pk-rec* mutant gene was grown overnight at 37°C in NZCYM medium containing ampicillin (50 mg/l). The pre-culture was inoculated (1%) into a fresh NZCYM medium and the cultivation was continued till the optical density at 660 nm reached 0.35. The culture

*Corresponding author. Fax: (81) (75) 753-4703.

PII: S0014-5793(99)00107-6

was then supplied with 1 mM (final concentration) of IPTG and incubated for an additional 4 h at 37°C. Cells were harvested by centrifugation at 6 000×*g* for 10 min and washed with 50 mM sodium phosphate buffer (pH 7.0). The cell pellet was resuspended in the same buffer and cells were disrupted by sonication. Soluble and insoluble fractions were separated by centrifugation (15 000×*g* for 30 min). All of the *Pk*-REC mutant proteins were recovered mainly in the soluble fraction. Most of the host proteins were precipitated by heat treatment at 80°C for 15 min, while *Pk*-REC mutants remained in the soluble fraction. Then the soluble fraction was applied to ion exchange and affinity chromatography. *Pk*-REC mutant proteins were eluted at 0.3–0.4 M NaCl from a MonoQ column (pH 7.0) and at 0.85–0.95 M NaCl from a HiTrap Heparin affinity column (pH 7.0) (Amersham Pharmacia Biotech). The fractions which gave a single band on SDS-PAGE were combined and used for further studies. The protein concentration was determined by bicinchoninic acid protein assay kit (Pierce, Rockford, Ill) according to the manufacturer's instructions using bovine serum albumin as a standard.

2.4. ATPase activity

The ATPase activity of each *Pk*-REC mutant was assayed in a reaction mixture containing 20 mM Tris-HCl (pH 8.0), 2 mM DTT, 0.1 mM ATP, 2 mM $MgCl_2$, 100 μg/ml BSA, 0.1 μg *Pk*-REC and water to give a final volume of 25 μl. Each reaction contained 100 nCi [α-^{32}P] ATP. Reaction mixtures were prepared on ice and incubations were at 55°C for 30 min followed by storage on ice: 1 μl of the mixture was then spotted directly onto Polygram CEL 300 PEI thin layer chromatography plates (Macherey-Nagel, GmbH, Duren, Germany). The substrate and products of the reaction were separated by one dimensional chromatography using 1 M LiCl as a developing solvent.

2.5. DNase activity

DNase reaction mixture (30 μl), containing double (pUC18 vector plasmid) or single (M13 mp19) stranded, circular DNA (5 μg) and purified recombinant *Pk*-REC protein (0.1 μg), was incubated at 60°C for 10 min in 30 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$. An appropriate amount of ATP was also added to the reaction mixture when the inhibitory effect of ATP on DNase activity was examined. For each reaction mixture, 5 μl of sample loading buffer (40 mM Tris-acetate (pH 8.0), 1 mM EDTA, 50% glycerol and a trace of bromophenol blue) was added and stored on ice to stop the reaction and then analyzed by 1% agarose gel electrophoresis in Tris-acetate/EDTA buffer. DNA was visualized under UV with ethidium bromide staining. For the kinetic study of DNase activity, the liberated acid soluble oligo- or mono-nucleotides were quantitatively measured [9]. The reaction mixture contained 20 μg calf thymus genomic DNA, 1 μg *Pk*-REC, 30 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$ and sterilized water to give a total volume of 50 μl. The reaction was carried out at 60°C and stopped after 30 min by the addition of 50 μl of acid lanthanum reagent composed of 0.02 M $La(NO_3)_3$ in 0.2 N HCl. The precipitates were removed by centrifugation (16 000×*g* for 15 min) and the absorbance of the supernatant solution was measured by a model Ultrospec 3000 spectrophotometer (Amersham Pharmacia Biotech) at 260 nm against a blank sample incubated without enzyme.

2.6. UV tolerant (DNA repairing) activity in vivo

Tolerance against UV light irradiation of the transformants of *E. coli recA* null mutant strain HMS174 (DE3)pLysS with plasmids harboring the mutated *Pk-rec* genes were examined as follows. Fresh single colonies were grown to the mid log phase in NZCYM medium containing ampicillin. The gene expression was induced by the addition of 1 mM IPTG. After incubation for 4 h, the cells were exposed to UV light for various periods, diluted, and plated onto LB plates containing ampicillin to determine the number of colony forming units [12]. The UV dose was measured with an ultraviolet intensity meter J225 (UVP, Upland, CA, USA).

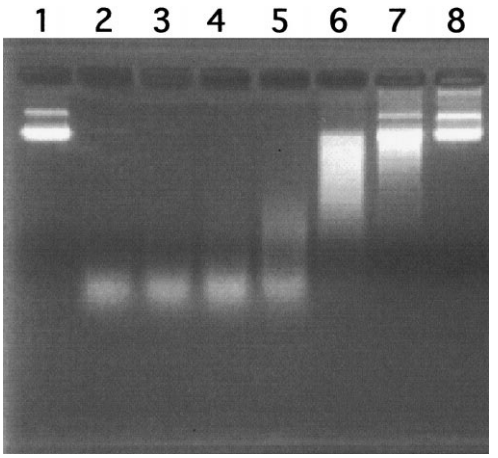

Fig. 1. Inhibitory effect of ATP on the DNase activity of wild type *Pk*-REC. pUC18 vector DNA was used as a substrate. Lane 1, control reaction from which both *Pk*-REC and ATP were omitted; lane 2, only ATP was omitted; lanes 3–8, 0.5, 1, 2, 3, 4 and 5 mM ATP was added in the reaction mixture along with *Pk*-REC (0.1 μg).

3. Results and discussion

3.1. Inhibitory effect of ATP on the DNase activity of Pk-REC

To examine whether the ATPase and DNase activities of *Pk*-REC are competitive with each other, DNase activity was measured in the presence of various concentrations of ATP (Fig. 1). DNase activity was clearly inhibited in the presence of 3 mM or higher concentrations of ATP. We have previously shown that ATPase activity was apparently inhibited by DNA as well [9]. Therefore, it seems likely that the active centers of ATPase and DNase activities are closely related to each other. This observation encouraged us to perform site-directed mutagenesis studies to identify the active site residues for these activities of *Pk*-REC.

3.2. Constructions of mutant enzymes

The amino acid residues in *Pk*-REC which were identical to

Table 1
Comparison of ATPase and DNase activities of each *Pk*-REC mutant enzyme

Mutant enzyme	DNase activity[a]			ATPase activity[b]
	k_{cat} (*OD*/5 min)	K_m (μg)	k_{cat}/K_m	
K33A/T34A	0.015	0.10	0.15	<10
D112A	0.18	0.22	0.82	60
E54Q	0.22	0.15	1.4	40
Wild-type	0.3	0.15	2.0	100 (AMP)

[a]k_{cat} and K_m values were determined for DNase activity (see Section 2). k_{cat} value has the unit of increase in OD_{260}/1μg enzyme in 5 min reaction. Since calf thymus genomic DNA was used as a substrate, the unit for K_m is μg DNA in 30 μl reaction mixture.
[b]ATPase activity was estimated by the intensity densitograph of the remaining ATP spot on the TLC plate. Relative activities to wild-type*Pk*-REC are shown. Wild-type *Pk*-REC produced AMP as a reaction product, while other mutant *Pk*-RECs produced ADP. No decrease in ATP was confirmed in the control reaction (no enzyme) [5].

the residues of the active site of *E. coli* RecA were chosen as targets for mutagenesis. The alignment of the amino acid sequences of various RecA/Rad51 homologues in the well conserved regions are shown in Fig. 2. According to the crystal structure of the RecA-ADP complex [3], Lys-72 and Thr-73 in motif A contribute to the ATP hydrolytic reaction by holding β- and γ-phosphate of substrate ATP directly or through a Mg^{2+} bridge (Fig. 2a). These two successive residues are well conserved in *Pk*-REC also (Lys-33 and Thr-34, respectively). Asp-144 in RecA motif B (Asp-112 in *Pk*-REC) constitutes the active center for ATP hydrolysis (Fig. 2b). This residue provides a ligand for the Mg^{2+} binding through a water molecule. Glu-96 in RecA in the third conserved region (Glu-54 in *Pk*-REC) is shown to be a catalytic residue and suggested to function as a general base which activates a water molecule for an in line attack on the γ-phosphate of ATP (Fig. 2c). In order to examine the function of the above described residues in *Pk*-REC, site-directed mutations were introduced. The functional group of the side chain of each

a

Pk-REC	27	G	P	Y	A	S	G	K	T	T	L	A	L	Q	39
RecA	66	G	P	E	S	S	G	K	T	T	L	T	L	Q	78
RadA	114	G	E	F	G	S	G	K	T	Q	L	C	H	Q	126
Dmc1	121	G	E	F	R	C	G	K	T	Q	M	S	H	T	133
Rad51	185	G	E	F	R	T	G	K	S	Q	L	C	H	T	197
K33A,T34A	27	G	P	Y	A	S	G	A	A	T	L	A	L	Q	39

b

Pk-REC	108	L	V	V	V	D	S	I	T	A	H	117
RecA	140	V	I	V	V	D	S	V	A	A	L	149
RadA	206	L	I	V	V	D	S	V	T	S	H	215
Dmc1	212	L	I	V	V	D	S	I	M	A	N	221
Rad51	276	L	I	V	V	D	S	V	M	A	L	285
D112A	108	L	V	V	V	A	S	I	T	A	H	117

c

Pk-REC	50	Y	V	D	T	E	G	G	F	S	P	59
RecA	92	F	I	D	A	E	H	A	L	D	P	101
RadA	146	Y	I	D	T	E	G	T	F	R	W	155
Dmc1	153	Y	I	D	T	E	G	T	F	R	P	162
Rad51	217	Y	I	D	T	E	G	T	F	R	P	226
E54Q	50	Y	V	D	T	Q	G	G	F	S	P	59

Fig. 2. Comparison of members of the RecA-like protein family. (a) And (b) are ATP binding motifs A and B, respectively. (c), The conserved region around E96 in *E. coli* RecA. Abbreviations: RecA, RecA from *E. coli*; RadA, RadA from *Sulfolobus solfataricus*; Dmc 1 and Rad 51, recombination proteins from *Saccharomyces cerevisiae*. Sequences of mutant proteins in the present study are also shown.

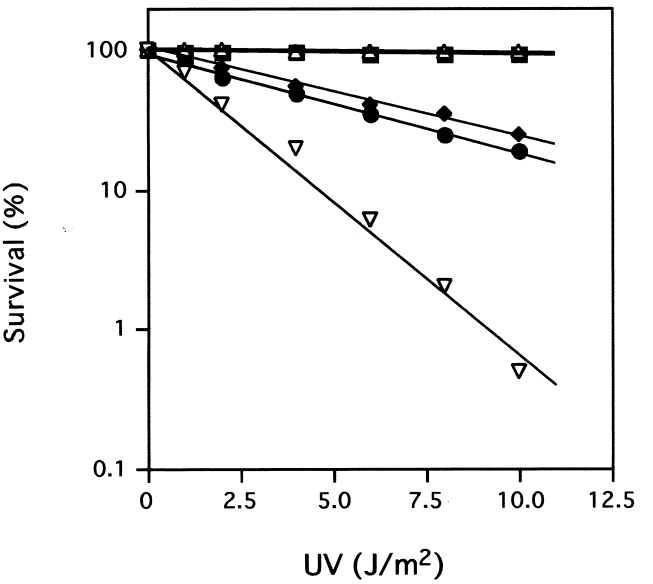

Fig. 3. UV survival of *E. coli recA* mutant strain HMS174 (DE3) pLysS harboring *Pk-rec* mutant and wild-type genes. The data are average values of three independent experiments. *E. coli* HMS174 (DE3) pLysS harboring the *Pk-rec* wild-type gene (△), the D112A mutant gene (●), the K33A and T34A double mutant gene (♦), the E54Q mutant gene (■) and the pET-8c vector (▽) were examined.

amino acid residue was eliminated by replacing the residue with alanine. The double mutant protein K33A/T34A, in which both Lys-33 and Thr-34 were replaced by Ala, was constructed. Asp-112 was also replaced by Ala to create the mutant protein D112A. Glu-54 was replaced by Gln because Gln is nearly identical with Glu in size and polarity but cannot function as a general base. We have constructed the mutant protein E54A as well, in which Glu-54 was replaced by Ala. However, the structure of this mutant protein was found to be altered dramatically from that of the wild-type protein (CD spectrum chart is not shown). Therefore, the mutant protein E54A was not analyzed further.

3.3. ATPase activity

All mutant *Pk*-REC proteins had lower ATP hydrolytic activities than that of the wild-type protein. While the mutant protein K33A/T34A showed a poor ATPase activity and produced a very small amount of ADP only when excess amount of the protein was used for assay, the mutant proteins E54Q and D112A retain 40 and 60% of the ATP hydrolytic activity of wild-type protein, respectively (Table 1). The optimum pH for ATPase activity of the mutant protein E54Q was identical with that of the wild-type protein (pH 8.0–8.5, data not shown). These results indicate that Glu-54 and Asp-112 are not directly involved in the catalytic reaction. None of the mutant proteins was able to hydrolyze ATP to AMP as did the wild-type protein. Relative spatial positions of the active site residues and ATP may be changed by these mutations. Unlike wild-type *Pk*-REC, which exhibited higher ATPase activity in the presence of Zn^{2+} than in the presence of Mg^{2+}, all these mutant proteins preferred Mg^{2+} over Zn^{2+}.

3.4. DNase activity

Like the ATPase activity, the DNase activities of all mutant proteins also decreased to some extent as compared to that of

the wild-type protein (Table 1). The mutant protein D112A and E54Q retained the DNase activity, which was comparable to that of the wild-type protein. On the other hand, the mutant protein K33A/T34A had a much lower DNase activity than the wild-type protein. These correlations between the ATPase and DNase activities strongly suggest that the ATPase and DNase activities share a common active site in which Lys-33 and/or Thr-34 are involved. This is also supported by the following data. First, although *Pk*-REC has no RNA hydrolytic activity, it hydrolyzes the ester bond between α and β phosphate of ATP to produce AMP as the main product, which is the same cleavage site observed in DNase activity [5]. Secondly, both ATPase and DNase activities require divalent cations. Although these activities did not show exactly the same preferences for divalent cations, both activities utilize Mg^{2+} effectively [5].

Since the K_m value of the mutant protein E54Q (0.15 μg) was identical with that of the wild-type protein, and its k_{cat} value was reduced only by 37%, it is clear that Glu-54 is not directly involved in the catalytic function of the protein. Asp-112 also does not seem to be a member of the active site residues. Further mutagenesis studies, as well as X-ray diffraction analysis, will be required to identify the catalytic residue(s) of this enzyme.

3.5. UV light tolerant (DNA repairing) activity in vivo

The mutant *Pk*-REC proteins were examined whether they express DNA repairing activity in *recA* null mutant *E. coli* cells as does wild-type *Pk*-REC. The activity was assayed by the UV light tolerance of cells carrying mutant *Pk-rec* genes in pET-8c multicopy expression vector. Although the mutant protein E54Q had lower ATPase and DNase activities than the wild-type protein, it completely suppressed the UV sensitive phenotype of *recA* null mutant *E. coli* cells (Fig. 3). On the other hand, the mutant proteins K33A/T34A and D112A only partially suppressed the UV sensitive phenotype of the host cells. Thus, the enzymatic activities of the mutant proteins seem to be correlated with their UV tolerant activities. However, the mutant protein K33A/T34A, which showed the lowest enzymatic activity among the mutant proteins constructed in this study, had comparable UV tolerant activity to the mutant protein D112A (Fig. 3). The reason why the mutant protein K33A/T34A has similar UV tolerant activity to the mutant protein D112A is not clear at this point. This may reflect the complexity of the DNA repairing reaction against UV irradiation damage.

References

[1] Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D. and Rehrauer, W.M. (1994) Microbiol. Rev. 58, 538–602.
[2] Shinohara, A., Ogawa, H. and Ogawa, T. (1992) Cell 69, 457–470.
[3] Story, R.M., Weber, I.T. and Steitz, T.A. (1992) Nature 355, 318–325.
[4] Story, R.M. and Steitz, A. (1992) Nature 355, 374–376.
[5] Weinstock, G.M., McEntee, K. and Lehman, I.R. (1981) J. Biol. Chem. 256, 8829–8834.
[6] Kelley, J.A. and Knight, K.L. (1997) J. Biol. Chem. 272, 25778–25782.
[7] Sandler, S.J., Satin, L.H., Hardeep, S.S. and Clark, A.J. (1996) Nucleic Acids Res. 24, 2125–2132.
[8] Rashid, N., Morikawa, M. and Imanaka, T. (1996) Mol. Gen. Genet. 253, 397–400.
[9] Rashid, N., Morikawa, M., Nagahisa, K., Kanaya, S. and Imanaka, T. (1997) Nucleic Acids Res. 25, 719–726.
[10] Morikawa, M., Izawa, Y., Rashid, N., Hoaki, T. and Imanaka, T. (1994) Appl. Environ. Microbiol. 60, 4559–4566.
[11] Sarkar, G. and Sommer, S.S. (1990) Biotechniques 8, 404–407.
[12] Kato, R. and Kuramitsu, S. (1993) J. Biochem. 114, 926–929.