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Abstract

Thermostable RecA protein (ttRecA) from *Thermus thermophilus* HB8 showed strand exchange activity at 65 °C but not at nucleoprotein complex was observed at both temperatures. ttRecA showed single-stranded DNA (ssDNA)-dependent ATP activity was maximal at 65 °C. The kinetic parameters, K_m and k_{cat}, for adenosine triphosphate (ATP) hydrolysis with poly(0.60 s^{-1} at 65 °C, and 0.34 m M and 0.28 s^{-1} at 37 °C, respectively. Substrate cooperativity was observed at both tempera coefficient was about 2. At 65 °C, all tested ssDNAs were able to stimulate the ATPase activity. The order of ATPase stimu poly(dC) > poly(dT) > M13 ssDNA > poly(dA). Double-stranded DNAs (dsDNA), poly(dT)·poly(dA) and M13 dsDNA, were u enzyme at 65 °C. At 37 °C, however, not only dsDNAs but also poly(dA) and M13 ssDNA showed poor stimulating ability. A M13 ssDNA gave circular dichroism (CD) peaks at around 192 nm, which reflect a particular structure of DNA. The conform an upshift of temperature or binding to *Escherichia coli* RecA protein (ecRecA), but not to ttRecA. The dissociation constan and poly(dA) was estimated to be 44 μM at 25 °C by the change in the CD. These observations suggest that the capability conformation of ssDNA may be different between ttRecA and ecRecA. The specific structure of ssDNA was altered by hea After this alteration, ttRecA and ecRecA can express their activities at each physiological temperature.

Abbreviations

ATP-γ-S
adenosine 5′- *O* -(3-thiotriphosphate)
CD
circular dichroism
ecRecA
E. coli RecA protein
IPTG
isopropyl-β- D(–)-thiogalactopyranoside
ttRecA
T. thermophilus RecA protein
UV
ultraviolet.

RecA protein is essential for homologous recombination and recombinational repair in *Escherichia coli* [1–3]. Many RecA ho isolated not only from prokaryotes [4] but also from eukaryotes [5]. This suggests that the mechanism of DNA recombinatio conserved in most living organisms, and that RecA protein may play a central role in the system. The properties of *E. coli* R have been extensively studied, and it has been shown that this relatively low-molecular-mass protein (38 kDa) has four mark *in vitro* [1–3]. First, RecA monomers aggregate by themselves and polymerize on DNA to form a nucleoprotein helical filam protein promotes a DNA strand exchange reaction. Third, RecA protein has single-stranded DNA (ssDNA)-dependent ATPa protein has coprotease activity to induce an SOS response. The fact that these different activities are all shown by this single

Interestingly, poly(dA) and M13 ssDNA activated the ATPase at 65 °C but not at 37 °C. It has been reported that poly(dA) sh
positive band at around 192 nm by circular dichroism (CD) spectrometry [16]. This CD band in the region of vacuum UV indi
not have a random, but rather a specific, structure. In order to know the relationship between the structure and the ATPase s
measured the CD spectra of ssDNA and showed that poly(dA) and M13 ssDNA have a specific structure whereas poly(dC) a
also showed that the positive CD band decreases as the temperature rises. From these observations, we surmise that the sp
ssDNA prevents activation of ttRecA to express the ATPase activity.

Unlike the case of ttRecA, ecRecA expresses ATPase activity to varying degrees in the presence of various kinds of ssDNAs
suggests that the characteristic of ecRecA for ssDNA differs from that of ttRecA. The temperature dependency of the ATPas
similar to that of the structural alteration of poly(dA) and M13 ssDNA. In order to elucidate whether RecA protein can induce
ssDNA, we carried out CD measurement of poly(dA) in the vacuum UV region in the presence of various concentrations of tt
lower temperatures, and showed that ecRecA was able to change the specific structure of poly(dA) whereas ttRecA could no
observations, we discuss the difference between ecRecA and ttRecA with regard to the capability of modifying the specific s
necessary for the binding.

Experimental procedures

Enzymes and chemicals

The sources of enzymes and reagents were as follows: DNA modification enzymes including restriction enzymes were from
Gene, Toyobo, Japan and New England Biolabs (USA); isopropyl-β- D(–)-thiogalactopyranoside (IPTG) was from Wako Pure
phosphocellulose (type P11) and DEAE-cellulose (type DE52) were from Whatman Biochemicals (Germany); plastic-backed
cellulose sheets (MN-Polygram CEL300PEI/UV) were from Machery and Nagel (England); [α-^{32}P]ATP was from ICN (USA);
kinase (type II) was from Sigma (USA); pig heart lactate dehydrogenase (grade II) was from Toyobo; adenosine 5′- *O* -(3-thio
was from Boehringer Mannheim (Germany); poly(dT), poly(dC), poly(dA) and poly(dA)·poly(dT) were from Pharmacia (USA)
and M13 double-stranded DNA (dsDNA) were provided by T. Mikawa (Osaka University).

Media, bacterial strains, plasmids and DNA manipulation

The *E. coli* strains used were DH5α[18] for plasmid DNA preparation and BL21(DE3) harboring the pLysE plasmid [19] for
ttRecA. They were grown on Terrific broth or LB medium at 37 °C [18]. The plasmids used were pTA3, which contains the en
recA gene [9] in the pET3a expression vector [19]. DNA manipulation was carried out by standard procedures [18].

Determination of nucleotide and polydeoxyribonucleic acid concentrations

The concentration of ATP or ATP-γ-S was determined using the molar absorption coefficient of ε_{259} = 15 400 $M^{-1}\cdot cm^{-1}$ or ε_2
respectively [20]. The residue molar concentrations of polydeoxyribonucleic acids were determined using the following mola
ε_{264} = 8520 $M^{-1}\cdot cm^{-1}$ for poly(dT) [21]; ε_{268} = 7400 $M^{-1}\cdot cm^{-1}$ for poly(dC) [21]; ε_{257} = 8600 $M^{-1}\cdot cm^{-1}$ for poly(dA) [22]; ε_{264}
poly(dA)·poly(dT) [22].

Overexpression of the *T. thermophilus recA* gene in *E. coli*

To create a *Nde* I restriction enzyme site in the first ATG codon of the *T. thermophilus recA* gene, a pair of DNA primers wa
(MilliGen/Biosearch , USA, Cyclone Plus DNA synthesizer). These were 5′-CCTGAGAGGTG CATATGGACGAGAG-3′ and 5
GAACTCCTTCTCA-3′, the underlining indicating the *Nde* I site and the *Xmn* I site, respectively. A DNA fragment of about 8
polymerase chain reaction under the same conditions as those described previously [9] using pTA3 as a template. The amp
the N-terminal side was cut with *Nde* I and *Xmn* I. The rest of the DNA fragment containing almost all of the *T. thermophilus*
prepared by cutting pTA3 with *Xmn* I and *Bam* HI. The pET3a vector was cut with *Nde* I and *Bam* HI. These three DNA frag
to construct the *T. thermophilus recA* gene under the regulation of the T7 promoter, and the resulting plasmid was named p
sequence of the amplified region in pEA1 was confirmed by dideoxy sequencing using a ‘Taq Dye Terminator Cycle Sequen

procedure as that described previously [24].

ATPase assay

At 37 °C, hydrolysis of ATP by ttRecA was measured by an enzyme coupling method [25, 26] at pH 7.5. A total of 200 μL of contained 50 m M Tris/HCl, 10 m M $MgCl_2$, 100 m M KCl, 1 m M DTT, 10 m M phosphoenolpyruvate, 2 m M NADH, 25 U·mL^{-1} 25 units·mL^{-1} lactate dehydrogenase, DNA, ATP and ttRecA as indicated. The activity was measured from the decrease in a 0.1-cm cell using a Hitachi spectrophotometer, model U-3000. Because the coupling enzymes are heat-inactivated at high a thin-layer chromatography (TLC) method [17] to measure ATPase activity at 65 °C. The procedure was described in the pr Analysis of the ATPase activity was performed using the Hill equation (28):

where v and V_{max} are the initial and maximal initial ATPase reaction velocities, [S] is the concentration of substrate (ATP), dissociation constant and n is the Hill coefficient. Eqn (1) was rearranged to give

Kinetic parameters were determined by linear fitting to Eqn (2) at various concentrations of ATP.

Apparent dissociation constants between ttRecA and DNA

The concentration dependence of various kinds of DNA on the ATPase activity of ttRecA was measured. ATPase activity at the TLC method with 2.5 μM of ttRecA. The activity at 37 °C was measured by the enzyme coupling method with 5 μM of ttR intensity of binding between ttRecA and DNAs, we calculated the apparent dissociation constant from Eqn (3):

where E, L and EL are ttRecA, DNA, and their complex, respectively. K' is the dissociation constant defined by

where parentheses represent respective concentrations.

The total concentrations of ttRecA ($[E]_0$) and DNA ($[L]_0$) are expressed as follows:

The concentration of ttRecA-DNA complex is obtained from Eqns (4)–(6),

The apparent velocity of the reaction, v, is expressed as:

where k is a constant. The kinetic parameters were determined by fitting Eqn (8) to the data. A computer program, Igor (Wa for calculation of the parameters.

Strand exchange assay

A total of 20 μL of reaction mixture contained 10 m M Tris/HCl (pH 7.5), 10 m M $MgCl_2$, 30 m M KCl, 20 m M ATP, 1 m M DTT, ssDNA, 30 μM M13mp18 dsDNA linearized by *Bam* HI digestion, and various concentrations of ttRecA. It also contained an system (10 m M phosphoenolpyruvate, 25 U·mL^{-1} pyruvate kinase). The mixture was incubated at 37 °C or 65 °C for 30 min was stopped by addition of a solution to the final concentration of 10 m M EDTA, 1% SDS and 0.2 mg·mL^{-1} proteinase K. A

mass of about 36 kDa, which agreed with that calculated from the amino acid sequence translated from its nucleotide seque
20 residues were M-D-E-S-K-R-K-A-L-E-N-A-L-K-A-I-E-K-E-F, which coincided with the sequence translated from the first A
T. thermophilus recA gene. To confirm the accurate molecular mass of ttRecA, the purified protein was analyzed by ion-spra
spectrometry. The determined value, 36 385, agreed well with the calculated one, 36 384.

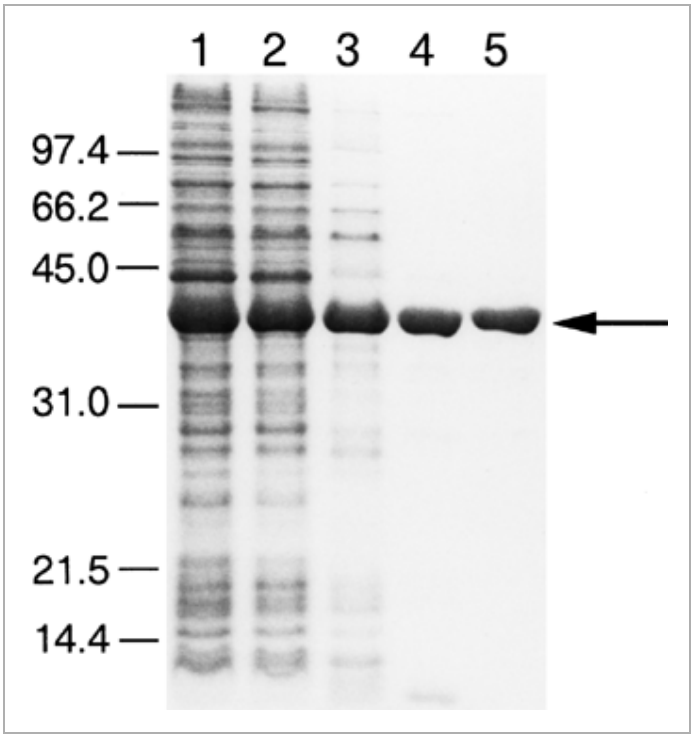

Figure 1.

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Overproduction and purification of ttRecA. The fractions obtained during the purification of ttRecA were electrophore
SDS/polyacrylamide (12.5%) gel [46] and stained with Coomassie Brilliant Blue. Molecular mass markers (in kilodaltons
the left. Lane 1, total cell extract; lane 2, cleared lysate; lane 3, post-heat treatment at 65 °C; lane 4, post-phosphocellu
DEAE-cellulose. The arrow indicates the ttRecA. About 1–10 μg of protein was loaded into each lane of the gel.

Kinetic parameters for ATPase activity of ttRecA

Including ecRecA, many proteins which have Walker's A-type nucleotide binding motif, GXXXXGK$^{T}/_{S}$[29], show ATPase act
consensus sequence [9, 10] and ssDNA-dependent ATPase activity [10]. We studied the temperature dependence of ttRec
activity with poly(dT) as a ssDNA was measured using a TLC method. Its activity appeared maximal at 65 °C. At 37 °C, whic
temperature for many mesophilic organisms, the activity was about half that at the maximum. At over 90 °C and below 15 °C
observed. The optimum temperature of 65 °C for ttRecA activity is consistent with the fact that *T. thermophilus* can grow be
[8].

Although some RecA proteins have been isolated from thermophiles [10, 30], no kinetic studies of their activities have been
measured the ATPase activity of ttRecA at various concentrations of ATP to determine its kinetic parameters. For measurem
the TLC method is advantageous when the reaction temperature is high, but disadvantageous for following the reaction in re
hand, the enzyme coupling method, which is now used widely for measuring ATPase activity, is useful for analysis of steady-
the reaction can be followed spectroscopically in real time. However, the enzyme coupling method cannot be used at high te
coupling enzymes used here, lactate dehydrogenase (from pig heart) and pyruvate kinase (from rabbit muscle), are not ther
ATPase activity at 65 °C was measured using the TLC method. In the presence of poly(dT) as a ssDNA, the amount of ATP

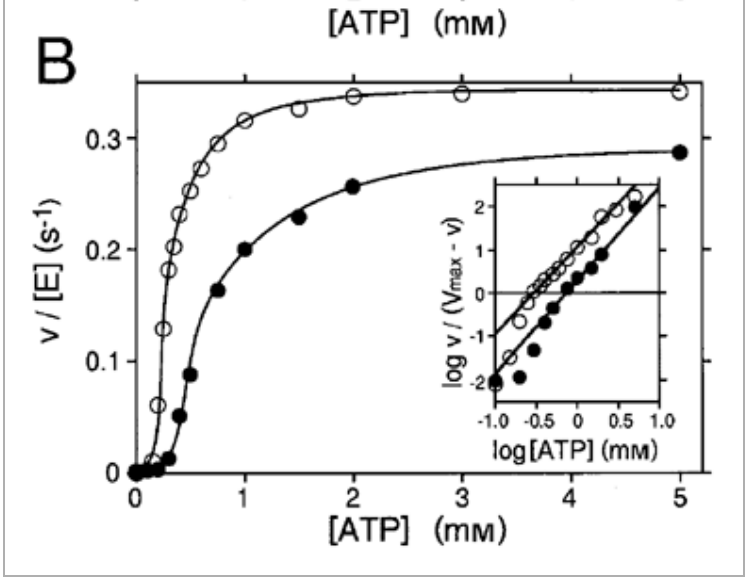

Figure 2.

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ATPase activities of ttRecA under various conditions. (A) ATPase assays were performed by the TLC method at 65
Experimental procedures. The reaction mixture contained 2.5 μM ttRecA and 100 μM poly(dT). (B) ATPase assays were
enzyme coupling method at 37 °C as described in Experimental procedures. The reaction mixture contained 5 μM ttRec
poly(dT) (solid circles) or poly(dC) (clear circles). Substrate dissociation constant (K), turnover number (k_{cat}) and Hill
hydrolysis of ATP were determined from the insets in the figures (see text for details).

Table 1. Steady-state kinetic parameters for ATP hydrolysis catalyzed by RecA protein.

	Temperature (°C)	ssDNA	K [a] (m M)	k_{cat} (s^{-1})	n [b]	k_{cat}/K ($s^{-1} \cdot M^{-1}$)
ttRecA	65	poly(dT)	1.4	0.60	1.9	4.3×10^2
	37	poly(dT)	0.34	0.28	2.1	8.2×10^2
	37	poly(dC)	0.29	0.34	2.0	1.2×10^3
ecRecA	37	poly(dT)	0.10	0.35	6.6	3.5×10^3
	37	φX174 ssDNA	0.038	0.17	3.3	4.5×10^3

[a] K, Substrate dissociation constant. [b] n, Hill coefficient. [c] All parameters for ttRecA protein were determined from Fig. 2.

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