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Purification and characterization of the *Thermus thermophilus* HB8 RecX protein

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Abstract

The RecA protein plays a central role in homologous recombination by promoting strand exchange between ssDNA and homologous dsDNA. Since RecA alone can advance this reaction *in vitro*, it is widely used in gene manipulation techniques. The RecX protein down-regulates the function of RecA, indicating that it could be used as an inhibitor to control the activities of RecA *in vitro*. In this study, the RecX protein of the hyper-thermophilic bacterium *Thermus thermophilus* (ttRecX) was over-expressed in *Escherichia coli* and purified by heat treatment and several column chromatography steps. Size-exclusion chromatography indicated that purified ttRecX exists as a monomer in solution. Circular dichroism measurements indicated that the α-helical content of ttRecX is 54% and that it is stable up to 80 °C at neutral pH. In addition, ttRecX inhibited the DNA-dependent ATPase activity of the *T. thermophilus* RecA protein (ttRecA). The stable ttRecX may be applicable for variety of techniques using the ttRecA reaction.

Keywords: Thermostable; RecA; *Thermus thermophilus*; Recombination

The RecA protein[1] plays an important role in genetic recombination and recombinational DNA repair in bacteria [1,2]. RecA assembles on single-stranded DNA (ssDNA) to make a nucleoprotein filament that in its active form has ATPase activity [3]. The active RecA filament promotes *in vitro* DNA strand exchange between ssDNA in the filament and homologous double-stranded DNA (dsDNA) upon ATP hydrolysis [1,2]. Since RecA protein alone can promote DNA strand exchange *in vitro*, it has been useful for gene manipulation techniques [4]. For example, a procedure to cleave DNA at any site has been developed [5]. This method is based on RecA-mediated triple-stranded DNA formation between the targeted DNA site and a homologous synthetic deoxyoligonucleotide, followed by endonucleolytic cleavage of that site. Multiplex PCR, which assesses over a dozen sites in the same reaction mixture, uses the heat-stable RecA protein from *Thermus thermophilus* (ttRecA) [6], which promotes precise priming and minimizes non-specific PCR products. Since ttRecA is very stable, it may be very useful for other applications that require high temperature, high pH and so on.

Recently, it was demonstrated that the *Escherichia coli* RecX protein inhibits RecA-mediated DNA strand exchange and DNA-dependent ATP hydrolysis activities [7,8]. Therefore, we speculated that RecX could be used as an inhibitor to regulate RecA activities *in vitro*. In this study, we have cloned the *T. thermophilus recX* gene and expressed and purified the product. We have characterized the physical properties of ttRecX and examined its effect on ttRecA activity to determine whether it could be used as a ttRecA inhibitor.

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[1] *Abbreviations used:* ttRecX, *Thermus thermophilus* RecX protein; ttRecA, *Thermus thermophilus* RecA protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; CD, circular dichroism; PAGE, polyacrylamide gel electrophoresis.

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Material and methods

Cloning of the T. thermophilus recX gene

Thermus thermophilus HB8 genomic DNA was purchased from Takara Bio. The *T. thermophilus recX* gene was amplified by PCR from genomic DNA with the primers *Nde*-recX (5′-GCGGATCCAT ATGGGGAGCGAG GCTTTGGC-3′) and *Bam*-recX (5′-TAAGGATCCTTAC TACTTATACCCTTCCCC-3′) and *Kod* DNA polymerase (Toyobo). The primers included *Nde*I and *Bam*HI restriction sequences (underlined) for cloning purposes and were based on the *T. thermophilus recX* nucleotide sequence (GenBank Accession No. AB059835). A single DNA fragment of the expected size was obtained. For construction of the expression plasmid, the PCR product was digested by *Nde*I and *Bam*HI, purified and inserted into plasmid pET-3a. The resulting plasmid, pET3a-tt*recX*, was confirmed by sequencing.

Expression and purification of proteins

ttRecA was prepared as previously described [6]. ttRecX was purified as follows. The *E. coli* strain Rosetta (DE3) (Novagen) was transformed with pET3a-tt*recX*. Cells were grown to an OD_{600} of 0.6 and treated with isopropyl β-D-thiogalactopyranoside at a final concentration of 0.4 mM for 4 h. The harvested cells (1 g) were resuspended in 5 ml buffer containing 50 mM Tris–HCl (pH 7.5), 2 mM EDTA and 10 mM β-mercaptoethanol, and sonicated. After the removal of debris by centrifugation (60,000*g* for 60 min), the supernatant was heated for 20 min at 75 °C and centrifuged (60,000*g* for 60 min) to remove the precipitate. The supernatant was applied to an SP Sepharose 6 Fast Flow column (Amersham Biosciences, 5 ml) equilibrated with buffer containing 25 mM Tris–HCl (pH 7.5), 1 mM EDTA and 5 mM β-mercaptoethanol. ttRecX was eluted with a linear gradient of KCl (0.1–1.0 M). Fractions containing ttRecX, detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), were collected and ammonium sulfate was added to a final concentration of 80%. The solution was centrifuged (20,000*g* for 60 min), and the precipitate was suspended in 200 μl buffer containing 25 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5 mM β-mercaptoethanol and 500 mM KCl, applied to a Superdex75 10/300GL column (Amersham Biosciences) that had been equilibrated with the same buffer and eluted at a flow rate of 0.5 ml/min. Fractions containing ttRecX were collected and concentrated. Equal amounts of glycerol were added, and the samples were stored at −20 °C until use. The concentration of ttRecX was determined using a molar absorption coefficient of 15,530 $M^{-1}\,cm^{-1}$ at 280 nm.

Gel electrophoresis of ttRecX

Protein samples were heated for 3 min at 85 °C before being loaded onto the gel. Proteins were electrophoresed on a 15% polyacrylamide gel containing 0.1% SDS under reducing conditions and visualized by staining with Coomassie Brilliant Blue R-250 (CBB). The purity of ttRecX in each lane was estimated using the CS Analyzer (ATTO).

Size-exclusion chromatography

Fifty microliter ttRecX (40 μM) was applied to a Superdex-75 HR 10/300GL column (Amersham Biosciences) and eluted at a flow rate of 0.5 ml/min in buffer containing 25 mM Tris–HCl (pH 7.5), 1 mM EDTA, 5 mM β-mercaptoethanol and 500 mM KCl.

CD spectroscopy

Circular dichroism (CD) measurements were carried out on a Jasco spectropolarimeter, model J720-W. The mean residue ellipticity, [*q*], was obtained as previously described [9].

ATP hydrolysis assays

The ssDNA-dependent ATP hydrolysis activity of ttRecA was measured as previously described [3] with minor modifications. Reaction mixtures contained 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 1.5 mM phosphoenolpyruvate, 1 mM ATP, 320 μM NADH, 10 U/ml pyruvate kinase, 10 U/ml lactate dehydrogenase, 500 μM ssDNA (heat denatured DNA from calf thymus), 1 μM ttRecA and the indicated concentration of ttRecX in 200 μl. The kinetics of ATP hydrolysis at 37 °C was followed by measuring the absorption of NADH at 340 nm using an Ultrospec 4300 pro spectrometer (Amersham Pharmacia). The amount of hydrolyzed ATP was calculated from A_{340} per minute data (5–30 min) using an ε340 value of $6.22 \times 10^3\,M^{-1}\,cm^{-1}$ for NADH.

Results and discussion

Purification of ttRecX

ttRecX was prepared as described in Materials and methods. Each step of the purification was monitored by 15% SDS–PAGE with CBB staining (Fig. 1). Almost all *E. coli* proteins could be removed by heat treatment, but some heat-stable proteins persisted. In general, this process is very useful for preparing *T. thermophilus* proteins expressed in *E. coli*. ttRecX was purified to homogeneity through two additional steps, ion exchange and size-exclusion chromatography. The purity at each step was determined by densitometric analysis, in which the density of the band corresponding to ttRecX in the SDS–polyacrylamide gel was compared with that of other bands in the same lane (Table 1). The purity of the ttRecX obtained as a final product was estimated at over 99.5%. Purified ttRecX was confirmed by N-terminal sequence analysis and mass spectrometry (date not shown). The overall yield was about 1.5 mg ttRecX from 1 g cells.