Cycling Probe Technology with RNase H Attached to an Oligonucleotide

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INTRODUCTION

RNase H is an enzyme that specifically degrades the RNA portion of DNA/RNA hybrids (3,8). The enzyme does not cleave single-stranded DNA or RNA. This property has allowed the development of cycling probe technology (CPT), a method for detection and quantification of low amounts of target DNA (5,7). The principle of this method, based on RNase H activity and the use of chimeric probes DNA-RNA-DNA, is outlined in Figure 1. The reaction is carried out at a temperature that allows the chimeric probe to anneal to the single-stranded target DNA. RNase H cuts within the RNA portion of the chimeric probe, and the shorter cleaved probe fragments dissociate from the target, thereby regenerating the target for further cycling. The resulting accumulation of probe fragments can subsequently be detected. CPT has potential applications in infectious and genetic disease diagnostics. Since the target DNA is not amplified, CPT exhibits low background. Furthermore, CPT is fast, linear, isothermal and simple compared with other DNA detection methods.

High concentrations of RNase H are required for the detection of low amounts of target DNA. Attachment of the RNase H to nucleic acid is an approach to reduce the amount of enzyme used and to increase the efficiency and specificity of RNase H in CPT. An enzyme conjugate has been previously used to increase the efficiency and specificity of nucleic acid enzymes by covalently attaching an oligodeoxynucleotide to a ribonuclease (2). A similar approach was used to link a nanodeoxynucleotide to RNase H (11).

Another strategy to improve the efficiency and the specificity of RNase is to generate a fusion protein between RNase H and streptavidin. A biotinylated nucleic acid could then be bound to this fusion enzyme. Since CPT is generally performed at 65°C for increased specificity, the thermostable RNase H from Thermus thermophilus (9) was chosen. In this paper, we describe the cloning, purification and the characterization of a streptavidin-RNase H fusion protein. The results show that this fusion protein is active in CPT experiments at very low concentrations when bound to a biotinylated probe. This novel enzyme can be used for detection of low amounts of DNA using CPT, or for the cleavage of RNA in antisense studies.

MATERIALS AND METHODS

Cloning of the Streptavidin-RNase H Gene Fusion

General cloning techniques were as described by Sambrook et al. (16). Unless otherwise indicated, modifying enzymes were from Life Technologies (Gaithersburg, MD, USA). DNA sequencing was performed by the dideoxy termination method (17). The RNase H gene from T. thermophilus (9) was polymerase chain reaction (PCR)-amplified from genomic DNA using primers BC202'5'-CCG AAT TCA ACC CCT TAT GCC TCT TCG TGA-3′ and BC203'5'-CCG AAT TCT ACC CCT CCC CCA GGA-3′. The amplified product was cloned as a blunt-ended fragment into the Smal site of pTZ19R (Pharmacia Biotech, Montreal, QC, Canada) resulting in plasmid pIDB1. Primers FB102'5'-CCG CAT ATG AAC CCC TCC CCC AGG-3′ and BC-202 were used to amplify the corre-
sponding fragment from pIDB1. It was subsequently cloned as a blunt-ended fragment into the SmaI site of pTZ19R. The resultant plasmid was designated pIDB6. The restriction fragment NdeI - EcoRI from pIDB6 was then cloned into pT7-7 (United States Biochemical, Cleveland, OH, USA) to generate pIDB9. This plasmid allows expression of the RNase H in its native form.

To construct the streptavidin-RNase H fusion protein, the sequence encoding RNase H was PCR-amplified from pIDB1 utilizing primers FB102 and BC202. The amplified fragment was phosphorylated and ligated into the vector pTSA-18F (18) that had been previously cut with SmaI and treated with phosphatase. The resulting plasmid was designated pIDB10. The vector pTSA-18F contains a DNA sequence that codes for a truncated form of streptavidin, the expression of which is controlled by the T7 promoter.

Expression and Purification

Native RNase H was purified as described (10) with the following modifications. After the second phosphocellulose column and concentration of the RNase H fractions, the proteins were applied to a Superose® 12 column (Pharmacia Biotech) that had been equilibrated with 20 mM sodium acetate, pH 5.5, and 150 mM NaCl at a flow rate of 0.4 mL/min. The three fractions corresponding to the RNase H peak were pooled and concentrated with a Centriprep® 10 (Amicon, Beverly, MA, USA). RNase H was stored at -20°C in glycerol buffer (20 mM sodium acetate, pH 5.5, 150 mM NaCl, 40% glycerol).

Plasmid pIDB10 was transformed into the bacterial strain NM522 (6). T7 RNA polymerase was supplied by infecting the recombinant Escherichia coli with an M13 phage containing the T7 polymerase gene under control of the lac UV5 promoter (22,23). NM522 cells containing pIDB10 were grown at 37°C in 1 L of 2YT medium (2YT = 10 g yeast extract, 16 g Bacto-tryptone, 5 g NaCl/L, pH 7.0) containing 0.05 mg/mL ampicillin. When the culture reached an OD$_{600}$ of 0.3–0.4, isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM to induce the expression of the T7 polymerase gene. After 1 h, 10 mL of M13 phage (ca. 5 × 10$^9$ plaque-forming units/mL) were added, and the infected cells were further grown for 4 h. Cells

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**Figure 1. Principle of the CPT reaction.** Single-stranded target (I) serves as a catalyst for CPT. In the presence of excess chimeric probe (DNA-RNA-DNA, the DNA portion is indicated by a straight line and the RNA portion by a serrated line) (II) and RNase H (III), the RNA portion of the resulting probe-target complex (IV) is cleaved by RNase H at 65°C. The shorter cleaved probe fragments dissociate from the target, thereby regenerating the target DNA for further cycling (V). The released fragments are resolved from the uncut probe and detected (VI).
were harvested by centrifugation at 2900 × g for 15 min at 4°C. Cell pellets were resuspended in 30 mL of lysis buffer (1 M Tris-HCl, pH 7.4, 1 mM EDTA) and stored frozen at -70°C. After thawing, the cells were lysed on ice using a sonicator (Model 450 Sonifier; Branson Ultrasonic, Danbury, CT, USA) and centrifuged at 39000 × g for 15 min at 4°C. The pellet was resuspended in 20 mL of urea buffer (20 mM sodium acetate, pH 5.5, 8 M urea), homogenized using a 20.5-gauge needle and syringe, and incubated overnight at 4°C. The sample was again clarified by centrifugation at 39000 × g for 15 min. The protein solution was applied to a 2-mL phosphocellulose column connected to an FPLC (Pharmacia Biotech), which had been pre-equilibrated with loading buffer. The column was washed with 8 M urea, 0.2 M NaCl acetate, pH 4.0, and 0.1 mM EDTA. The eluted protein fractions were pooled and applied to a PD-10 desalting column (Pharmacia Biotech) equilibrated with 20 mM sodium acetate, pH 5.5, and 150 mM NaCl. Protein eluted from the desalting column was concentrated using a Centricon filter (Amicon) and stored in the same loading buffer, and the protein was concentrated with a Centricon (Millipore) and stored frozen at -70°C. Af-

### Oligonucleotide Preparation

Chimeric probes (DNA-RNA-DNA) were synthesized using an automated Applied Biosystems 394 DNA/RNA synthesizer (Perkin-Elmer/ Applied Biosystems Division, Foster City, CA, USA) according to the manufacturer's protocols (ABI User Bulletin #79). The regular 1.0 µmol cyanoethyl synthesis cycle was modified to give a 10-min wait step for the coupling reaction when ribonucleoside phosphoramidites were accessed. Base labile Expedite™ deoxyribonucleoside and ribonucleoside phosphoramidites and Expedite Cap A solution were obtained from Millipore (Bedford, MA, USA). Cold aqueous ammonia solution was used to cleave the oligonucleotides from the support, to remove the cyanoethyl phosphate protecting groups and to remove the protecting group on the bases. Triethylamine trihydrofluoride (Aldrich Chemical, Milwaukee, WI, USA) was used to remove the ribosyl protecting group on each 2’ hydroxyl group of the RNA portion of the probe (21). Two chimeric probes were used in this study, ARK2, 5’-d(GTC AGA CCC) r(AAAA) d(CCC CGA GAG GG)-3’, and ARK2B, (biotinylated ARK2) 5’-d(GTC GTG AGA CCC) r(AAAA) d(CCC CGA GAG GG) S12-B-3’, where B is a biotin and S a spacer Phosphoramadite 9 (Glen Research, Sterling, VA, USA). The oligonucleotides were purified by C18 reverse-phase HPLC. ARK2T is the synthetic target DNA complementary to ARK2 and ARK2B.

### Streptavidin Binding Activity

Streptavidin binding activity to 32P-labeled probes was estimated by gel electrophoresis. The fusion enzyme was incubated with ARK2 or ARK2B in CPT buffer (8 mM MgCl2, 50 mM Tris-HCl, pH 8.1, 0.025% Triton® X-100) containing 1 M NaCl. SDS-PAGE loading buffer (120 mM Tris-HCl, pH 8.1, 0.025% Triton® X-100) containing 1 M NaCl. SDS-PAGE loading buffer (120 mM Tris-HCl, pH 8.1, 0.025% Triton® X-100) containing 1 M NaCl.

![Figure 2. Details of the streptavidin fusion cloning vector pTSA-18F (A) and the streptavidin-RNase H fusion construct (B).](image)

![Figure 3. SDS-PAGE analysis of the effect of heat treatment of the streptavidin-RNase H fusion protein. Molecular weight standards in kDa (lane 1). Treatment for 20 min in gel loading buffer at 25°C (lane 2), 37°C (lane 3), 65°C (lane 4) and 100°C (lane 5).](image)
6.8, 4% SDS, 20% glycerol, 0.01% bromophenol blue) was added to the protein oligonucleotide mixture, and it was subjected to gel electrophoresis using the PhastSystem™ (Pharmacia Biotech). Gels were analyzed by a PhosphorImager™ utilizing ImageQuant™ software (Molecular Dynamics, Sunnyvale, CA, USA). The percentage of probe that was bound to the fusion protein was estimated by integration of the bands corresponding to unbound and bound probe.

**RNase H Assays**

The acid-soluble counts assay was based on a previously published method (4). Briefly, the single-stranded substrate M13 phage DNA was transcribed using 3H-UTP to create a labeled RNA/DNA hybrid (12). RNase H enzyme (0.1 – 0.02 ng) was added to a reaction mixture containing 10 mM Tris-HCl, pH 9.0, 1 mM MgCl₂, 0.005% bovine serum albumin (BSA), 1 mM β-mercaptoethanol and 1–3 µM labeled substrate M13 and then incubated for 5–10 min at 65°C. The reaction was then stopped by the addition of 50 µL of 0.5 mg/mL carrier tRNA plus 150 µL of 20% trichloroacetic acid (TCA) and placed on ice for 5–10 min. The sample was centrifuged at 14 000×g for 5 min at 4°C. Supernatant aliquots of 50 µL were added to 5 mL of liquid scintillant for counting.

Cycling conditions were modified from a previously published method (5). The two chimeric probes used in this study were 5’-labeled with 32P-ATP (16) using RTG kinase (Pharmacia Biotech) and purified by gel electrophoresis. Prior to CPT reactions, the enzyme was incubated with the biotinylated probe in CPT buffer containing 1 M NaCl. Reactions containing native enzyme and/or non-biotinylated probe were used as controls. CPT reactions with different ratios of probe:enzyme (a 1:1 ratio is calculated to contain one streptavidin-RNase H fusion

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**Figure 4. SDS-PAGE analysis of the binding of the fusion streptavidin-RNase H to biotinylated probe ARK2B.** Constant amounts of probe (1 fmol) were incubated with no streptavidin-RNase H (lane 1), 1 fmol of streptavidin-RNase H (lane 2), 50 fmol of streptavidin-RNase H (lane 3) and 5000 fmol of streptavidin-RNase H (lane 4).
subunit and one probe) were carried out in 20-µL volumes in CPT buffer containing 50 mM NaCl for 30 min at 65°C. The reaction was stopped by adding 20 µL of urea loading buffer (8 M urea, 100 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) on ice. The products of CPT were resolved on a 20% acrylamide, 7 M urea gel at 500 V. The gel was analyzed by a PhosphorImager, and the amount of cyclized probe was estimated by integration of the bands corresponding to intact and cut probe.

RESULTS

Cloning and Enzyme Purification

The 24-nucleotide junction between the truncated streptavidin gene and the RNase H gene in pIDB10 was confirmed by sequencing. The expressed fusion protein contains 293 amino acid (aa) residues. It consists of streptavidin aa residues 16 to 133, followed by the polylinker (8 aa residues) and the full-length RNase H protein (166 aa residues) (Figure 2). The fusion protein was induced by the addition of M13/T7 phage, and the expected polypeptide of approximately 32 kDa was obtained when analyzed by SDS-PAGE. The fusion protein was purified to apparent homogeneity with a yield of approximately 1 mg/L of culture.

Structure and Binding Activity of Streptavidin-RNase H

Thermostability studies of the fusion protein at different temperatures indicated that the molecular mass was approximately 128 kDa from 25°C to 65°C and 32 kDa at 100°C by SDS-PAGE (Figure 3). These results suggest that the fusion enzyme is comprised of 4 subunits, which are stable at temperatures up to at least 65°C but are dissociated at 100°C. The fusion protein retained its biotin binding properties since it was purified by an iminobiotin affinity column. Experiments to test the binding of the fusion protein to a constant amount of probe were performed by incubating the fusion enzyme with either a biotinylated (ARK2B) or non-biotinylated probe (ARK2) and subjecting the products to SDS-PAGE as described in Materials and Methods.

Figure 5. Analysis of the thermal stability of native RNase H vs. the streptavidin-RNase H fusion. Enzymes were pretreated at different temperatures (0, 25°, 37°, 55°, 80° and 96°C) for 10 min before submitting to the acid-soluble counts assay at 65°C.

Figure 6. Comparison of the fusion streptavidin-RNase H (Panel A) to the native RNase H (Panel B) in the CPT assay using ARK2B probe and different ratios of probe to enzyme. The autoradiogram shows the uncleaved probe (upper band) and the cleaved probe (lower band). Control lanes 1, 5 and 9: probe and enzyme without target. Reaction lanes 2, 6 and 10: 0.01 fmol target. Reaction lanes 3, 7 and 11: 0.1 fmol target. Reaction lanes 4, 8 and 12: 1 fmol target. C1 is a control with probe but without enzyme and target.
Analysis of the products resulting from the incubation of ARK2 with the fusion enzyme showed no high molecular weight bands (data not shown). Analysis of ARK2B without enzyme also showed no high molecular weight bands (Figure 4, lane 1). Five bands corresponding to high molecular weight species were observed after incubation of ARK2B and the fusion enzyme (Figure 4, lanes 2, 3 and 4), indicating that the biotinylated probe was bound to the fusion enzyme and was retarded during gel migration. The highest molecular weight species is likely due to an aggregation of the protein probe conjugate. The presence of the four remaining bands (a, b, c and d from higher to lower molecular weight, respectively) are dependent on the ratio probe:enzyme. Bands a and b are observed only with a 1:1 ratio probe:enzyme, bands c and d with a 1:50 ratio and band d with both 1:50 and 1:5000 ratios. These results suggest that with a 1:1 ratio, more than two probes are bound to each streptavidin-RNase H fusion enzyme, and with a 1:5000 ratio there is one probe bound per fusion enzyme. Approximately 10% of the probe failed to bind to the enzyme at 1:50 and 1:5000 ratios. This may be due to alteration or absence of the biotin component.

**RNase H Activity**

The RNase H activity of the fusion enzyme was compared to the activity of the native RNase H using an acid-soluble counts assay with M13 DNA/RNA substrate. The results showed that the specific activity of the fusion enzyme was approximately 12% of that of the native enzyme (data not shown).

A heat-stability study was performed to compare the fusion enzyme to the native enzyme. The enzymes were diluted in glycerol buffer and incubated at different temperatures. Figure 5 shows that while the native enzyme was more stable than the fusion enzyme, the fusion streptavidin-RNase H was substantially active (approximately 70% of the unheated control) after a 10-min treatment at 65°C.

In CPT experiments, the fusion streptavidin-RNase H was compared with the native enzyme using three ratios of ARK2B probe:enzyme; 1:1, 1:50 and 1:5000 (Figure 6). At a ratio of 1:1 and 1 fmol of target, streptavidin-RNase H was active, whereas the native RNase H was not. At a ratio of 1:50, both enzymes were active with 1 fmol of target; however, only the fusion enzyme was active with 0.1 fmol of target. At a ratio of 1:5000 both enzymes were active. Control reactions (lanes 1, 5 and 9) containing enzyme plus probe in the absence of target showed no probe cleavage (Figure 6). This experiment shows that the fusion streptavidin-RNase H was more efficient than the native enzyme at low concentrations.

The fusion enzyme was further tested for activity in CPT experiments using a biotinylated probe (ARK2B) or a non-biotinylated probe (ARK2) (Figure 7). At a ratio of 1:1 of probe:enzyme and 1 fmol of target, the fusion enzyme was active with ARK2B but not with ARK2. At a ratio of 1:50, the fusion enzyme was more active with ARK2B than ARK2. However at a 1:5000 ratio, the fusion enzyme was more active with ARK2 than ARK2B. This experiment shows that the fusion enzyme at low concentrations was more efficient with a biotinylated probe compared with a non-biotinylated probe.

**DISCUSSION**

The streptavidin-RNase H fusion was shown to be thermostable with an apparent quaternary structure composed of 4 subunits. These properties are similar to those of the native streptavidin (1). The streptavidin-RNase H fusion had both biotin binding and RNase H activities. Similarly, a fusion streptavidin-metalloprotein had been produced and was also shown to have an affinity for both biotin and heavy-metal ions (19). The binding activity of the fusion streptavidin-RNase H to a biotinylated oligonucleotide was shown to be a function of the probe:enzyme ratio. The streptavidin-RNase H fusion protein is comprised of four subunits, each of which can bind one biotinylated probe. Our results suggest that the number of probes bound to the fusion enzyme varied from 1 to 4 and that the fusion enzyme became saturated with probe at low enzyme concentrations. The band pattern observed in gel retardation assays is not due to a dissociation of the protein, since the fusion protein was shown to be one band when stained with silver (data not shown). Furthermore, the quaternary structure of the streptavidin protein has
been shown to be stable in the presence of high SDS concentrations (20). These results would indicate that the number of probes bound to the fusion protein could be controlled by manipulating the ratio of enzyme to probe concentrations. For example, RNase H activity may be affected if four biotinylated probes are bound to one streptavidin-RNase H protein. An equilibrium of one probe per streptavidin-RNase H protein might then be preferable.

The fusion enzyme was shown to have lower activity in an acid-soluble counts assay when compared with the native enzyme. This decrease in activity may be due to a change in the conformation of the active site imposed by the attachment of the streptavidin protein. It may also be due to electrostatic repulsion of targets caused by close proximity of the four active sites of a fusion protein or steric hindrance caused by streptavidin subunit association. Similar results were previously observed with E. coli RNase H covalently attached to an oligonucleotide (11,14). Although the fusion enzyme was less active than the native enzyme in an acid-soluble counts assay, the activities of both enzymes were comparable in CPT assays using non-biotinylated probes (data not shown). The distinction between the two types of assays may be explained by differences in the structure of the substrate and/or the amounts of the enzyme and substrate used in each of the two different reactions. Indeed, within a molecule of substrate, the acid-soluble counts assay requires many cleavage events in close proximity to give a positive activity measurement, whereas CPT requires only one cleavage event.

With a high ratio of 1:5000 probe:enzyme, the fusion was more active with a non-biotinylated probe compared with a biotinylated probe. It is possible the RNase H enzyme activity is reduced because of attachment of the probe to the fusion enzyme. However, with a ratio of 1:1 probe:enzyme, the amount of fusion enzyme required for activity in CPT reactions containing a biotinylated probe was significantly less than that required for activity with a non-biotinylated probe. Therefore, the streptavidin-RNase H when linked to the biotinylated probe maintains a close proximity of the enzyme to the substrate and consequently increases the efficiency of the reaction.

One disadvantage of the structure of the fusion enzyme described is that the enzyme becomes nonfunctional in subsequent cycling reactions upon cleavage of the attached probe. However, if the fusion enzyme was attached to a biotinylated target, that enzyme might participate in multiple rounds of probe cleavage. Alternatively, the turnover rate may be increased if the fusion
enzyme is bound to a biotinylated oligomer complementary to a sequence that flanks the target DNA sequence for the probe. In this configuration the enzyme will be maintained in close proximity to the target and would catalyze the conversion of multiple probes. In addition, this approach would provide a second order of specificity to the cycling reaction since two hybridization events (probe and the flanking sequence with the target DNA strand) must occur to achieve catalytic turnover of the probe. Experiments are under way to examine this approach.

In addition to the increase of the efficiency of CPT, the fusion enzyme should prove useful as a model for structural and functional studies of RNase H and its interaction with substrate. RNase H fusion enzyme could also be used in antisense studies to prevent gene expression. If the fusion enzyme were bound to an oligonucleotide sequence complementary to the message, the RNase H enzyme could cleave the mRNA of interest (15).

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REFERENCES


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