Altering Substrate Specificity and Detecting Processivity in Nucleases

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I. Introduction

The alteration of a natural enzyme in an attempt to create a designer catalyst has become a common goal of protein engineers. Many efforts have focused on the changing of substrate specificity, such that the resulting enzyme catalyzes the same chemical conversion (for example, proteolysis) but with a different preference for substrate (for example, cleavage of the peptide bond of an acidic rather than basic amino acid residue). In addition to changing substrate specificity, mutations to active-site residues can, in theory, also affect the *mechanism* by which an enzyme binds to its substrate. Examples of different mechanisms for binding to substrates are observed in enzymes that modify biopolymers. Some of these enzymes bind a polymeric substrate, catalyze a chemical reaction, and release a polymeric product. In contrast, *processive* enzymes bind a polymeric substrate, catalyze a chemical reaction, and remain associated with the polymer until all reactions of that molecule are complete.

Bovine pancreatic ribonuclease (RNase A; E.C. 3.1.27.5) catalyzes the cleavage of the P–$O_{5'}$ bond of RNA on the 3'-side of pyrimidine nucleosides. Structural data implicate hydrogen bonds formed between threonine 45 and the pyrimidine base as largely mediating this specificity (Wlodawer, 1985; Eftink and Biltonen, 1987). In an attempt to create mutants of RNase A that catalyze the cleavage of RNA on the 3'-side of *purine* nucleosides, codon 45 of the RNase A cDNA was subjected to saturation mutagenesis, and *E. coli* cells expressing the resulting mutant pool were screened for the production of an enzyme able to cleave polyR (where R refers to a puRine nucleotide) efficiently. This rapid screening method identified several mutants of RNase A as having the ability to cleave polyA, the most active of which was T45G

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RNase A. Unlike the cleavage of polyY (where Y refers to a pYrimidine nucleotide) by the wild-type enzyme, the cleavage of polyA by T45G RNase A is processive, that is, cleavage occurs by the one-dimensional progression of the enzyme along the polymeric substrate (delCardayré and Raines, 1993).

II. Materials and Methods

Heterologous Production and Purification of RNase A. The cDNA for RNase A was carried in the bacterial expression plasmid pET22B(+)(Novagen, Madison, WI) such that its transcription was under the control of the T7 RNA polymerase promoter (Studier et al., 1990). Targeting of mature RNase A to the periplasm was directed by the pelB leader peptide. *E. coli* cells harboring this plasmid have been induced to express up to 50 mg/L of recoverable, native RNase A. The details of the production and purification of RNase A in *E. coli* (as well as in *S. cerevisiae*) are presented elsewhere (delCardayré et al., 1993).

Construction of Mutant Library. The cDNA for RNase A was altered by oligonucleotide-mediated site-directed mutagenesis (Kunkel et al., 1987) to generate an unique and translationally silent *Nhe*I site 3' to codon 45, resulting in plasmid pBXR1. The codon for threonine 45 was then randomized by cassette mutagenesis (Reidhard-Olson et al., 1991) of the *Cla*I/*Nhe*I fragment in pBXR1. The resulting construct, pBXR1(T45All), was a library of pBXR1 plasmids in which at least one codon for each of the 20 amino acids was represented at codon 45. This library was transporated into *E. coli* strain BL21(DE3), which had an inducible gene for T7 RNA polymerase (Studier et al., 1990).

Screen for Mutants with Altered Substrate Specificity. A scheme depicting the methods used to isolate RNase A mutants that cleave polyR is shown in Figure 1. *E. coli* BL21(DE3) cells harboring the pBXR1(T45All) library were grown and induced to express the ribonuclease cDNAs by the addition of IPTG. Cells were removed by centrifugation and the culture medium was assayed by zymogram electrophoresis (Blank et al., 1982; Ribó et al., 1991; Kim and Raines, 1993) for an enzyme with the ability to cleave polyR. Briefly, media samples were diluted with SDS-PAGE loading buffer lacking any reducing agent, boiled, and subjected to electrophoresis in an SDS-polyacrylamide gel (Ausubel et al., 1989) containing polyR (0.5 mg/mL). After electrophoresis, the gel was washed with 10 mM Tris-HCl buffer, pH 7.5, containing isopropanol (20% v/v) to extract the SDS and thereby allow the ribonucleases to renature. The gel was then incubated for 40 min in 10 mM Tris-HCl buffer, pH 7.5, to allow for the manifestation of enzymatic activity. The gel was stained with 10 mM Tris-HCl buffer, pH 7.5, containing toluidine blue (0.2% w/v), and then washed extensively with water. Each location in the gel where a ribonuclease had degraded the polyR appeared as a clearing in a blue background.

Once polyR cleavage was detected, 100 individual clones were screened for

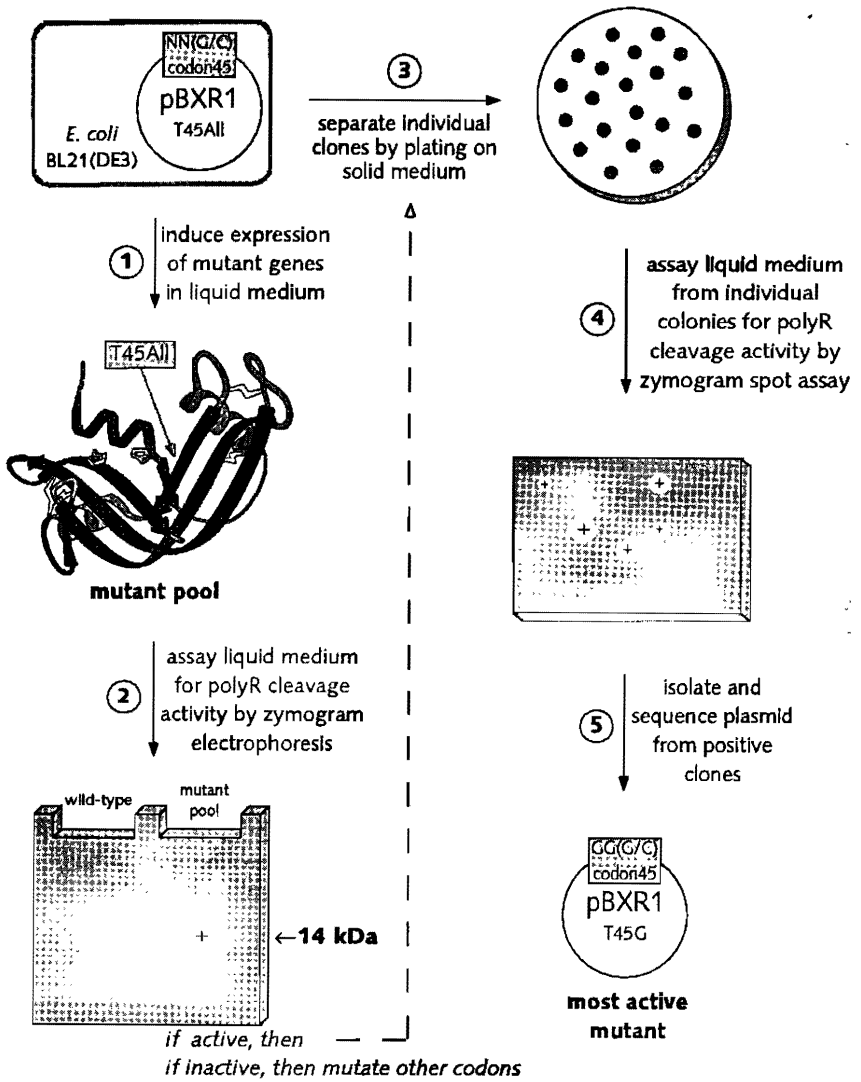


Figure 1. Scheme for the rapid isolation of ribonuclease mutants with altered substrate specificity.

the mutant(s) responsible for the new activity. Individual cultures (100 x 2 mL), each expressing a single copy of pBXR1(T45All), were grown and induced to express their copy of the mutagenized RNase A cDNA. Cells were removed by centrifugation and culture medium from each sample was assayed by a zymogram spot assay. Briefly, samples (100 x 1μL) were spotted on an agarose (1% w/v) gel containing 10 mM Tris-HCl buffer, pH 7.5, and a polyR (0.3 mg/mL). The resulting gel was incubated for 12 h at 37 °C, and then stained and washed as described above. Plasmids from the mutants scoring positive for polyR cleavage were isolated and sequenced, and mutant enzymes were purified.

Assays for Processivity. The analysis of products isolated during the course of a reaction is essential for determining if an enzyme is acting processively. For example, previous workers have used an enzyme to degrade a polymer partially, and then separated the products (for example, by gel filtration chromatography). If the only degradation products were high molecular weight polymer (that is, starting material) and monomer, then the enzyme was considered to act processively (Nossal and Singer, 1968). We have developed two new, facile assays for nuclease processivity that use modern methods of nucleic acid analysis.

^{31}P NMR Assay for Processivity. The chemical states of the phosphoryl group during RNA cleavage (that is, acyclic diester → cyclic diester → monoester) can be distinguished by ^{31}P NMR spectroscopy (Cozzone and Jardetzky, 1977; Thompson et al., 1993). Further, the relative molecular weights (strand length) of each of these species can be inferred from the ^{31}P NMR chemical shift of the peaks within a certain species, as low molecular weight species have a greater downfield shift than do high molecular weight species. To determine whether a ribonuclease was cleaving polyRNA processively, the chemical state of the phosphoryl group during the cleavage of polyRNA by the ribonuclease was monitored. High molecular weight polyC and polyA were prepared by ethanol precipitation of commercial materials. Reactions were performed in Mes/NaCl buffer containing polyC or polyA (2 mg/mL) and sufficient ribonuclease to degrade the polymer completely in about 50 min. [Mes/NaCl buffer was 0.1 M Mes buffer, pH 6.0, containing NaCl (0.1 M).] Spectra were recorded in 10-mm NMR tubes having D_2O inserts. Free induction decays were obtained at 25 °C on a Bruker AM400 spectrophotometer by using the parameters: spectral width, 4854 Hz; pulse width, 18.1 μs; acquisition time, 1.69 s; relaxation delay, 3.2 s; number of scans, 64. The free induction decays were subjected to Fourier transformation with a line broadening of 5 Hz, and the resulting spectra were phased with the program FELIX (Hare Research; Bothell, WA). Chemical shift values were recorded relative to aqueous H_3PO_4 (100 mM).

If the ribonuclease were cleaving the polyRNA processively, then the spectra would show little accumulation of high molecular weight polymer containing a 3'-terminal cyclic diester (oligo>p). Instead, the high molecular weight polymer would

be completely converted to a monomeric cyclic diester (N>p). A sizable accumulation of the oligo>p would, for example, be expected to arise in the degradation of polyC, which is not cleaved processively.

Distraction Assay for Processivity. If a ribonuclease were acting processively on polyRNA, then once the enzyme had bound to a single strand, it would be temporarily unable to bind to another strand added after that initial association. This *distraction* would continue until the enzyme had finished degrading the original strand. To test whether a ribonuclease was cleaving polyA processively, the following order-of-addition experiment was performed. Unlabeled polyA in Mes/NaCl buffer was incubated with the ribonuclease for a period of time, t', to allow any processive complex to form. Then, [^{32}P]polyA was added and the mixture was incubated for an additional time, t, before being quenched with a stop solution containing formamide (95% v/v) and xylene cyanol (0.05% w/v). A control reaction was also performed in which the labeled and unlabeled polyA were pre-mixed and then exposed to the ribonuclease for the same time, t, used in the distraction reaction. These reaction products were separated on an acrylamide (7.5% w/v in 1 x TBE) gel containing urea (8 M), and visualized by autoradiography (Ausubel et al., 1989). If the ribonuclease were cleaving polyA processively, then pre-incubation with unlabeled polyA should distract it from degrading labeled polyA.

III. Results

Screen for Mutants. Zymogram electrophoresis of supernatant from *E. coli* cells induced to express the pBXR1(T45All) library demonstrated the presence of an enzyme able to cleave polyA efficiently. Zymogram spot assay of individual transformants from this library identified those clones responsible for this new activity. Analysis of the enzyme and DNA from these transformants indicated that the most active mutant had a glycine residue at position 45. Analysis of whole cell extract producing T45G RNase A confirmed that this mutation had increased dramatically the ability of RNase A to cleave polyA, as shown in Figure 2.

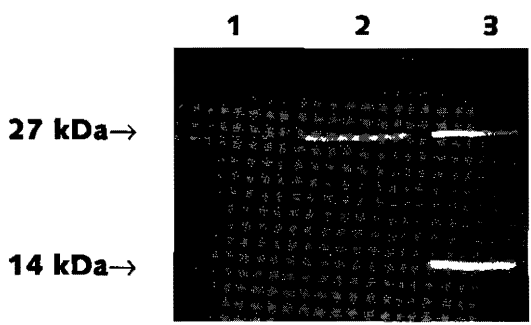


Figure 2. Zymogram electrophoresis on a polyA gel of *E. coli* BL21(DE3) cells (lane 1) and IPTG-induced *E. coli* BL21(DE3) cells carrying pBXR1 (lane 2) or pBXR1(T45G)(lane 3). The bands are from *E. coli* RNase I (27 kDa) and T45G RNase A (14 kDa).

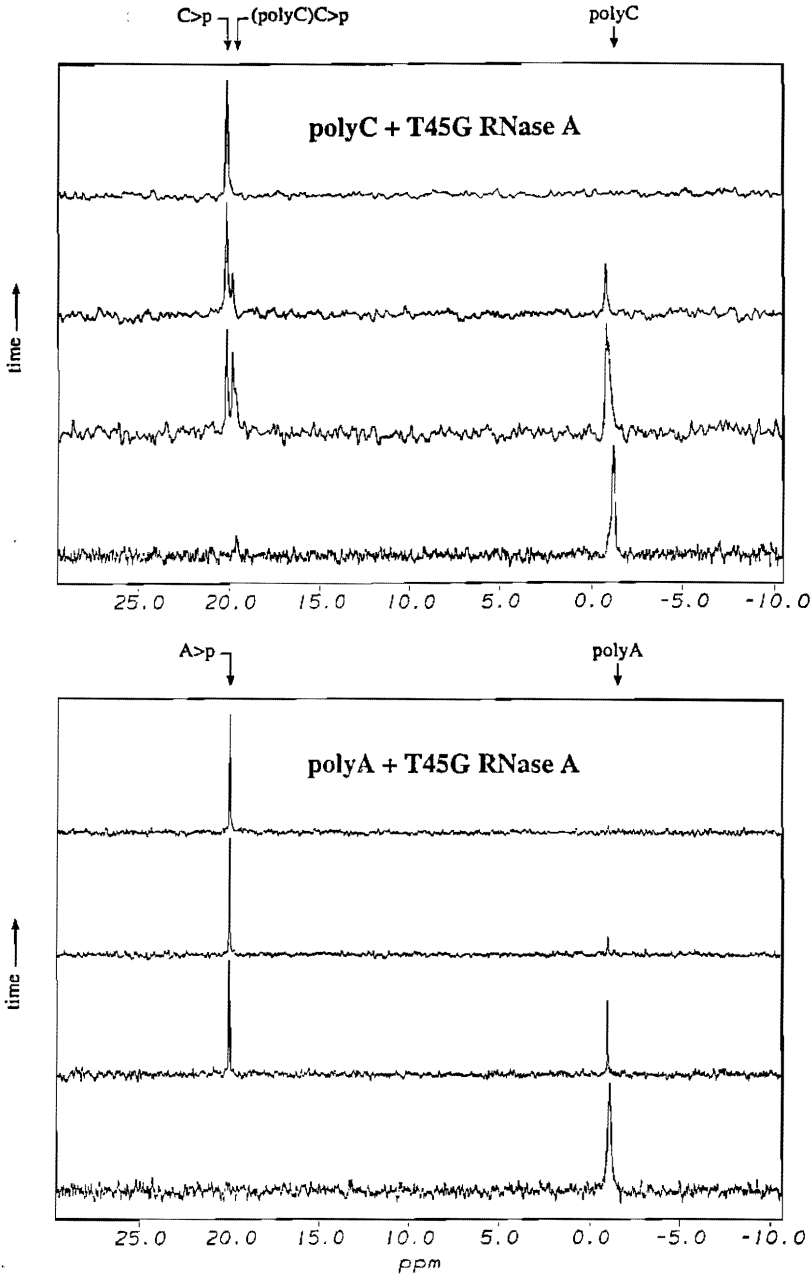
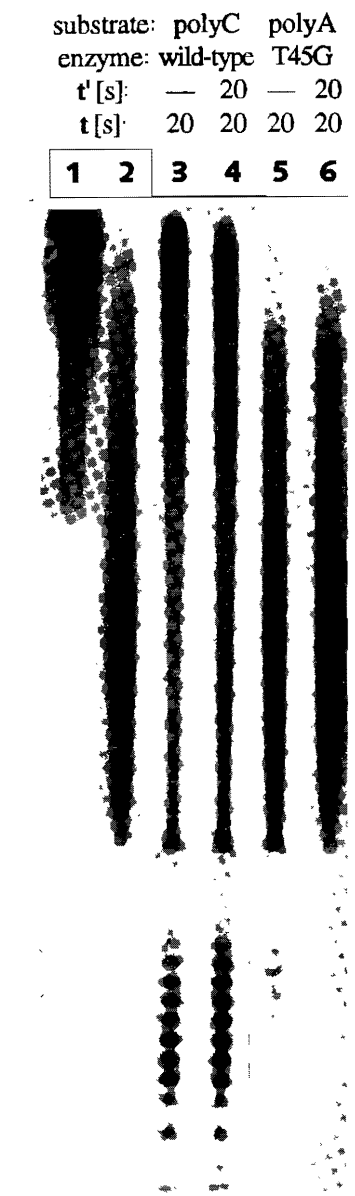


Figure 3. ^{31}P NMR spectra showing the time-course of the degradation of polyC (top panel) or polyA (bottom panel) by T45G RNase A.



^{31}P NMR Assay. The spectra resulting from the degradation of polyC and polyA by T45G RNase A are shown in Figure 3. During the degradation of polyC, the resonance from the acyclic diester shifted downfield from –1.30 ppm to –0.90 ppm. (This shift is characteristic of a decrease in the strand length of the acyclic diester.) Concurrent with this shift was the appearance of resonances for two cyclic diesters: one at 19.7 ppm (from oligoC>p), and another at 20.1 ppm (from C>p). In contrast, during the degradation of polyA, the resonance from the acyclic diester remained at –1.03 ppm, and a single cyclic diester resonance appeared at 19.9 ppm (from A>p). These data indicate that catalysis by T45G RNase A produces monomeric cyclic diesters but not oligo>p species. This behavior is that expected of a processive nuclease.

Distraction Assay. Autoradiograms from the distraction assay for processivity are shown in Figure 4. The precipitated polyA and polyC had no detectable contamination from strands of 10 or fewer nucleotides. Pre-incubation with unlabeled polyC did not prevent labeled polyC from being degraded rapidly by wild-type RNase A to strands of 10 or fewer nucleotides. In contrast, pre-incubation with unlabeled polyA prevented labeled polyA from being degraded rapidly by T45G RNase A. These results indicate that T45G RNase A releases individual strands of polyA slowly, as would be expected of an enzyme that degrades polyA processively.

Figure 4. Autoradiograms from the distraction assay for processivity. Intact [^{32}P]polyC (lane 1); intact [^{32}P]polyA (lane 2). Other lanes show the effect of pre-incubation (for 20 s) with unlabeled substrate on the degradation (for 20 s) of either [^{32}P]polyC by wild-type RNase A (lanes 3 and 4) or [^{32}P]polyA by T45G RNase A (lanes 5 and 6).

IV. Conclusions

A rapid screening procedure that coupled saturation mutagenesis with the highly sensitive zymogram technology was used to identify mutants of RNase A that had altered substrate specificity. The most active mutant, T45G RNase A, cleaved polyA efficiently. This mutant also appeared to cleave polyA processively. This unusual property was demonstrated by a ^{31}P NMR assay that monitored the appearance of various nucleotide species during catalysis and by an order-of-addition experiment that tested the ability of one substrate to distract the enzyme from cleaving another. The techniques described should be useful for altering the specificity of nucleases in general as well as for the determination of nuclease processivity.

Acknowledgments

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References

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K. (1989) *Current Protocols in Molecular Biology*, Wiley, New York.

Blank, A., Sugiyama, R. H., & Dekker, C. A. (1982) *Anal. Biochem. 120*, 267–275.

Cozzone, P. J., & Jardetzky, O. (1977) *FEBS Lett. 73*, 77–79.

delCardayré, S. B., & Raines, R. T. (1993), manuscript in preparation.

delCardayré, S. B., Ribó, M., Quirk, D. J., Rutter, W. J., & Raines, R. T. (1993), manuscript in preparation.

Eftink, M. R., & Biltonen, R. L. (1987) in *Hydrolytic Enzymes* (Neuberger, A., & Brocklehurst, K., Eds.) pp 333–375, Elsevier, New York.

Kim, J.- S., & Raines, R. T. (1993) *Protein Science 2*, 348–356.

Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.

Nossal, N. G., & Singer, M. F. (1968) *J. Biol. Chem. 243*, 913–922.

Reidhard-Olson, J. F., Bowie, J. U., Breyer, R. M., Hu, J. C., Knight, K. L., Lim, W. A., Mossing, M. C., Parsell, D. A., Shoemaker, K. R., & Sauer, R. T. (1991) *Methods Enzymol. 208*, 564–586.

Ribó, M., Fernández, E., Bravo, J., Osset, M., Fallon, M. J. M., de Llorens, R., & Cuchillo, C. M. (1991) in *Structure, Mechanism and Function of Ribonucleases* (de Llorens, R., Cuchillo, C. M., Nogués, M. V. & Parés, X., Eds.) pp 157–162, Universitat Autónoma de Barcelona, Bellaterra, Spain.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60–89.

Thompson, J. E., Venegas, F. D., & Raines, R. T. (1993), manuscript in preparation.

Wlodawer, A. (1985) in *Biological Macromolecules and Assemplies, Vol II: Nueclic Acids and Interactive Proteins* (Jurnak., F. A., & McPherson, A., Eds.) pp 393–439, Wiley, New York.