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One-Dimensional Diffusion of a Protein along a Single-Stranded Nucleic Acid

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I. Introduction

One-dimensional diffusion can accelerate the formation of site-specific interactions within biopolymers by up to 10^3-fold (Berg *et al.*, 1981). Such facilitated diffusion is used by transcription factors and restriction endonucleases to locate specific sites on double-stranded DNA (von Hippel and Berg, 1989). The backbone of RNA, like that of DNA, could allow for the facilitated diffusion of proteins. Yet, the facilitated diffusion of a protein along RNA (or any single-stranded nucleic acid) has not been demonstrated previously.

Bovine pancreatic ribonuclease A (RNase A; RNA depolymerase; EC 3.1.27.5) is a distributive endoribonuclease that catalyzes the cleavage of the P–$O_{5'}$ bond of RNA on the 3′ side of pyrimidine residues. RNase A binds to polymeric substrates (Imura *et al.*, 1965; Irie *et al.*, 1984; Moussaoui *et al.*, 1995), but the mechanism by which RNase A locates a pyrimidine residue within a polymeric substrate is not known.

Binding to phosphoryl groups is important for the one-dimensional diffusion of proteins along DNA (Winter *et al.*, 1981), and may likewise provide nonspecific interactions necessary to generate one-dimensional diffusion by RNase A. RNase A has three defined phosphoryl group binding subsites, P0, P1, and P2, as well as three base binding subsites, B1, B2, and B3 (Parés *et al.*, 1991). The subsite interactions in the RNase A•RNA complex are shown in Figure 1a. The P0 and P2 subsites interact with phosphoryl groups that remain intact during catalysis; the P1 subsite is the active site.

The B1 subsite is responsible for the pyrimidine specificity of RNase A. RNase A cleaves poly(cytidine) [poly(C)] or poly(uridine) [poly(U)] 10^4-fold faster than poly(adenosine) [poly(A)] as a result of the selectivity of the B1

subsite. In contrast to the B1 subsite, the B2 and B3 subsites prefer to bind purines. Previously, we demonstrated that enlarging the B1 subsite increases the rate of poly(A) cleavage by 10^3-fold (delCardayré and Raines, 1994; delCardayré *et al.*, 1994). This enlargement also converts the distributive mechanism of wild-type RNase A to a processive mechanism when poly(A) is the substrate.

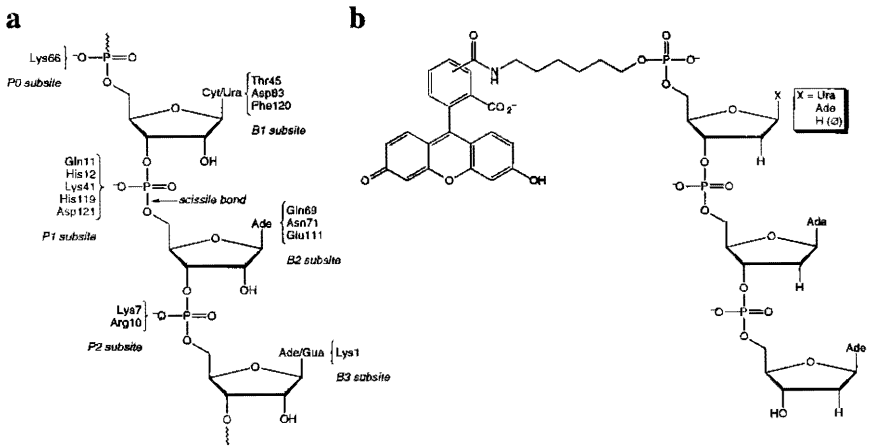

Figure 1. a. Amino acid residues of RNase A that compose the subsites for binding phosphoryl groups (P0, P1, and P2) and bases (B1, B2, and B3) of single-stranded nucleic acids. **b.** Fluorescein-labeled deoxynucleotides used to assess binding to the B1 subsite.

Single-stranded DNA is an excellent substrate analog for RNase A, and this analogy is the basis for the work described here. First, we report on the use of DNA oligonucleotides and fluorescence polarization to probe the binding of adenine to the B1 subsite of RNase A. Then, we describe the use of DNA/RNA chimeric oligonucleotides to distinguish between three-dimensional and one-dimensional diffusion mechanisms for catalysis by RNase A. Our results provide a biophysical rationale as well as direct evidence for the diffusion of a protein along a single-stranded nucleic acid.

II. Materials and Methods

A. Oligonucleotide synthesis

DNA and DNA/RNA chimeric oligonucleotides were synthesized with a Model 392 DNA/RNA synthesizer from Applied Biosystems (Foster City, CA) with reagents from Glen Research (Sterling, VA). Oligonucleotides were purified by elution from an acrylamide gel after electrophoresis.

To assess binding to the B1 subsite, we synthesized deoxynucleotides that differ only in the base that interacts with the B1 subsite (Figure 1a). The ligands have a uridine (U), adenosine (A), or abasic (Ø) residue at their 5′ ends, followed by two

adenosine residues to fill the enzymic B2 and B3 subsites. Each deoxynucleotide is labeled with fluorescein (Fl) so that binding can be detected by fluorescence polarization. The products of these syntheses are shown in Figure 1b.

To probe for one-dimensional diffusion, we synthesized DNA/RNA chimeric oligonucleotides. Special precautions were taken to avoid ribonuclease contamination during synthesis, purification, and use of these chimeras. For example, all water was treated with diethylpyrocarbonate before exposure to the chimeras. Ribonucleotide 2′-hydroxyl groups were deprotected with 1 M tetrabutyl ammonium fluoride in dimethyl formamide (Aldrich Chemical; Milwaukee, WI). Purified oligonucleotides were labeled on the 5′ end with [γ-^{32}P]ATP (duPont; Wilmington, DE) by T4 kinase (Promega; Madison, WI), and desalted with a Nick™ gel filtration column (Pharmacia; Uppsala, Sweden).

B. Binding

Fluorescence polarization (like fluorescence anisotropy) can be used to measure the rate of tumbling of a fluorescent molecule (Jameson and Sawyer, 1995; Royer, 1995). A receptor (*e.g.*, RNase A) binding a fluorescent ligand (*e.g.*, a labeled nucleic acid) slows the tumbling of the ligand. Accordingly, fluorescence polarization can reveal the fraction of a nucleic acid that is bound to RNase A.

Fluorescence polarization experiments were performed as described elsewhere (B. M. Templer and R. T. Raines, unpubl. results). Briefly, RNase A (Sigma Chemical; St. Louis, MO) was dialyzed exhaustively at 4 °C against distilled water to remove salts. The enzyme was then lyophilized. The lyophilized enzyme was suspended in 0.90 mL of 0.10 M Mes-HCl buffer, pH 6.0, containing NaCl (0.10 M), such that the concentration was 1 – 2 mM (15 – 30 mg/mL). Fluorescein-labeled deoxynucleotides were dissolved in buffer and added to half of the enzyme solution to a final concentration of 2 – 3 nM. The sample volume was then raised to 1.00 mL with buffer. A blank containing enzyme but not DNA was made by raising the volume of the remaining enzyme solution to 1.00 mL with buffer. The precise concentration of enzyme was determined by assuming that $A = 0.72$ at 277.5 nm for a 1.0 mg/mL solution. At least five repetitive fluorescence polarization readings (with individual blank readings) were made at room temperature with a Beacon™ fluorescence polarization instrument (Panvera; Madison, WI). The average and standard deviations were calculated for the readings. The protein sample was then diluted by removing 0.25 mL and replacing it with buffer containing the same concentration of labeled deoxynucleotide as was in the original protein sample. The blank was diluted with buffer. The data collection and dilution steps were repeated up to thirty times. The resulting data were fit to eq 1 by a non-linear least squares analysis, which was weighted by the standard deviation of each reading.

$$P = \frac{P_{max}[\text{RNase A}]}{K_d + [\text{RNase A}]} + P_{min} \quad (1)$$

In eq 1, P is the average of the measured fluorescence polarization, P_{min} is the polarization of free deoxynucleotide, and P_{max} is the polarization at deoxynucleotide saturation minus P_{min}. [RNase A] is protein concentration, and K_d is the equilibrium dissociation constant. For Fl-d(AAA) and Fl-d(ØAA), the value of P_{max} was poorly defined but apparently similar to that for Fl-d(UAA); therefore, the P_{max} of Fl-d(UAA) was used to fit the Fl-d(AAA) and Fl-d(ØAA) data.

C. One-dimensional diffusion

Enzymes capable of one-dimensional diffusion should cleave a substrate with a long nonspecific binding region faster than a similar substrate with a short such region (Berg *et al.*, 1981). The substrates used here derive from simpler substrates with long and short nonspecific binding regions (Figure 2a). By merging the simpler substrates into one, evidence for facilitated diffusion can be obtained directly in a single experiment. A conceptually analogous experiment has been performed with *Eco*RI endonuclease (Jeltsch *et al.*, 1994).

a

Simple Substrates

d(AAAAA)Ud(AAAAA)

d(AAAAA)Ud(AAAAAAAAAAAAAAAAAAAAAAAAA)

Composite Substrates

Oligo 1: d(AAAAA)Ud(AAAAA)Ud(AAAAAAAAAAAAAAAAAAAAAAAAA)

Oligo 2: d(AAAAAAAAAAAAAAAAAAAAAAAAA)Ud(AAAAA)Ud(AAAAA)

b

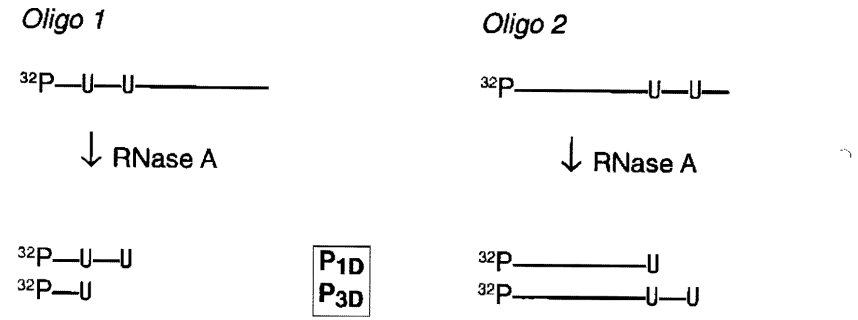

Figure 2. a. DNA/RNA chimeric oligonucleotide substrates used to detect one-dimensional diffusion by RNase A. Oligo 1 and Oligo 2 are circular permutations containing two cleavage sites, one of which is proximal to a long nonspecific binding region. **b.** Products of the cleavage of Oligo 1 and Oligo 2. P_{1D} results from one-dimensional diffusion of RNase A along the long poly(dA) tract.

Oligo 1 and Oligo 2 are chimeric oligonuclotides that contain 35 DNA residu and 2 RNA residues. The RNA residues are uridine nucleotides, and are referr to as the 1D and 3D sites. We chose this naming system because the 1D site closer to the long nonspecific binding region and will be cleaved faster if RNa A uses a one-dimensional diffusion mechanism. In both substrates, the 1D clea age site is flanked on one side by 25 deoxyadenosine residues. The 1D and 3 cleavage sites are separated by 5 deoxyadenosine residues, and 5 mo deoxyadenosine residues separate the 3D site from the end. Oligo 1 has the u dine nucleotides near the 5′ end, whereas Oligo 2 has the uridine nucleotides ne the 3′ end.

The use of composite substrates could complicate data interpretation because the possibility of multiple catalytic events on the same substrate. Of course, diff sion in one dimension, like diffusion in three dimensions, cannot be direction (von Hippel and Berg, 1989). Thus, RNase A bound to the long nonspecific bin ing region should cleave the 1D site faster than the 3D site regardless of the site proximity to the 5′ or 3′ end. Thus, comparing the *initial* rates of cleavage Oligo 1 and Oligo 2 resolves the complications incurred from the consolidati of substrates.

Only two detectable products are formed from the degradation of Oligo 1 Oligo 2 because only a 5′ ^{32}P label is used for detection (Figure 2b). RNase cleavage at the 1D site produces a detectable product, P_{1D}. Cleavage at the 3 site forms a detectable product, P_{3D}, of a different length. For Oligo 1, P_{1D} is nt and P_{3D} is 6 nt. For Oligo 2, P_{1D} and P_{3D} are 26 and 32 nt, respectively. T ratio $[P_{1D}]/[P_{3D}]$ is approximately equal to the ratio of the initial rates of cleava at the 1D (k_{1D}) and 3D (k_{3D}) sites (*i.e.*, $[P_{1D}]/[P_{3D}] \cong k_{1D}/k_{3D}$). This ratio is indicator of one-dimensional diffusion of RNase A along Oligo 1 and Oligo 2. ratio of $[P_{1D}]/[P_{3D}] > 1$ is indicative of one-dimensional diffusion; $[P_{1D}]/[P_{3D}] = 1$ is indicative of three-dimensional diffusion.

Assays for one-dimensional diffusion were performed as follows. Reactio were initiated at room temperature by the addition of substrate. The reaction mi ture consisted of 0.050 M Mes-HCl buffer, pH 6.0, containing RNase A (1 fmo 0.1 pmol), NaCl (0.025, 0.12, or 1.0 M), and substrate (0.4 – 0.8 μM). Aliquots μL) of the reaction were quenched at various times by the addition to an equ volume of formamide (95% v/v) containing EDTA (20 mM), xylene cyan (0.05% w/v), and bromophenol blue (0.05% w/v). Less than 10% of the substra was cleaved during the course of an experiment. Reaction products were sep rated by electrophoresis on a denaturing 18% (w/v) acrylamide gel. To preve shattering, these gels were soaked in an aqueous solution of acetic acid (7% v/ and methanol (7% v/v), then in methanol before drying under reduced pressu (Thomas *et al.*, 1992). Detection and quantification of cleavage products we made using a PhosphorImager™ radioisotope imaging system from Molecu Dynamics (Sunnyvale, CA).

III. Results

A. *Binding*

Fluorescence polarization data for the binding of RNase A to Fl-d(UAA), Fl-d(AAA) and Fl-d(ØAA) are shown in Figure 3. RNase A binds Fl-d(UAA) approximately 20-fold more tightly than Fl-d(AAA) or Fl-d(ØAA), demonstrating that the B1 subsite has affinity for a pyrimidine base. The similarity in binding affinity for Fl-d(AAA) and Fl-d(ØAA) indicates that the B1 subsite of RNase A does not bind adenine significantly, but does not discriminate against it.

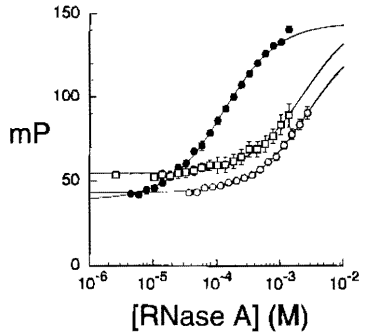

Figure 3. Binding of RNase A to Fl-d(UAA) (●), Fl-d(AAA) (○), and Fl-d(ØAA) (□) as assessed by changes in fluoresence polarization (mP). Data were obtained in 0.10 M Mes-HCl buffer, pH 6.0, containing NaCl (0.10 M). Data were fit to eq 1, yielding K_d values of 0.13 mM, 3.3 mM, and 2.5 mM for Fl-d(UAA), Fl-d(AAA), and Fl-d(ØAA), respectively.

B. *Facilitated diffusion*

A typical time-course for the degradation of Oligo 1 and Oligo 2 by RNase A in the presence of 25 mM NaCl is shown in Figure 4. The concentration of P_{1D} exceeds that of P_{3D} at all times for both Oligo 1 and Oligo 2. These data provide evidence that RNase A uses one-dimensional diffusion to locate pyrimidine nucleotides within a polymeric substrate.

The one-dimensional diffusion of RNase A is diminished by added NaCl. The ratio $[P_{1D}]/[P_{3D}]$ for Oligo 1 and Oligo 2 at three concentrations of NaCl is shown in Figure 5. RNase A displays no indication of facilitated diffusion at high NaCl concentration, where $[P_{1D}]/[P_{3D}] \cong 1$. At 0.12 M NaCl concentration, $[P_{1D}]/[P_{3D}] > 1$, indicating that RNase A can use one-dimensional diffusion at NaCl concentrations close to physiological. At 0.025 M NaCl, $[P_{1D}]/[P_{3D}]$ is even greater, consistent with a facilitated diffusion mechanism that relies on the nonspecific binding to the phosphoryl group of poly(dA). Under these low-salt conditions, RNase A also shows the slowest turnover of substrate. As shown in Figure 4, the cleavage occurs in a burst but is then inhibited by products. The size of this burst increases with enzyme concentration (data not shown).

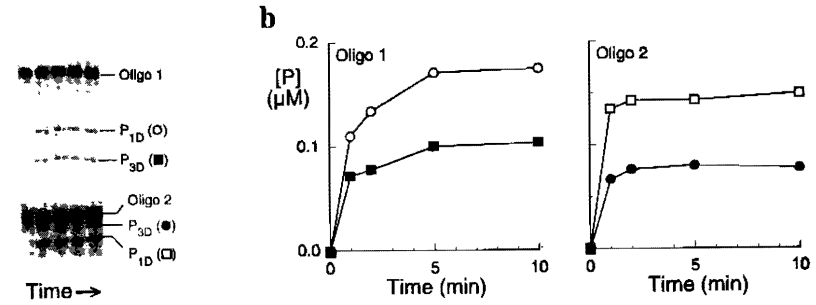

Figure 4. a. Reaction products 0, 1, 2, 5, and 10 min after addition of RNase A to Oligo 1 and Oligo 2. Reactions were performed in 0.050 M Mes-HCl buffer, pH 6.0, containing NaCl (0.025 M). **b.** Plots of product formation versus time for Oligo 1 and Oligo 2.

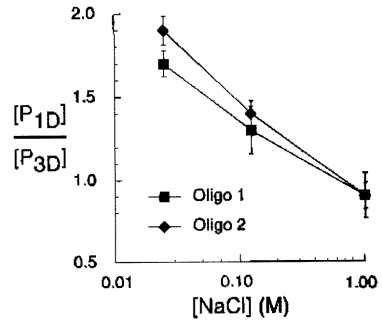

Figure 5. The $[P_{1D}]/[P_{3D}]$ ratio versus the log of the concentration of NaCl. Data were obtained in 0.050 M Mes-HCl buffer, pH 6.0, containing NaCl (0.025, 0.12, or 1.0 M).

IV. Conclusions

RNase A can use one-dimensional diffusion along a poly(dA) tract to accelerate the location of a uridine substrate. Use of this mechanism depends on the concentration of NaCl, as expected if the enzyme were binding to the nucleic acid by nonspecific interactions with phosphoryl groups. Binding of the enzymic active site to adenosine residues is 20-fold weaker than to uridine residues, which could enhance the ability of the enzyme to slide along the poly(dA) tract.

A facilitated diffusion mechanism may have evolved for a sinister purpose. Some homologs of RNase A are cytotoxic because they are able to deliver ribonucleolytic activity to the cytosol of mammalian cells (Youle *et al.*, 1993). Facilitated diffusion may enable these cytotoxic ribonucleases to use the poly(A) tail of mammalian mRNA's as a runway leading to substrates in the indispensable coding region.

References

Berg, O. G., Winter, R. B., and von Hippel, P. H. (1981). *Biochemistry* **20**, 6929-6948.
delCardayré, S. B., and Raines, R. T. (1994). *Biochemistry* **33**, 6031-6037.
delCardayré, S. B., Thompson, J. E., and Raines, R. T. (1994). *In* "Techniques in Protein Chemistry V" (Crabb, J. W., ed.) pp. 313-320, Academic Press, New York.
Imura, N., Irie, M., and Ukita, T. (1965). *J. Biochem.* **58**, 264-272.
Irie, M., Mikami, F., Monma, K., Ohgi, K., Watanabe, H., Yamaguchi, R., and Nagase, H. (1984). *J. Biochem. (Tokyo)* **96**, 89-96.
Jameson, D. M., and Sawyer, W. H. (1995). *Methods Enzymol.* **246**, 283-300.
Jeltsch, A., Alves, J., Wolfes, H., Maass, G., and Pingoud, A. (1994). *Biochemistry* **33**, 10215-10219.
Jensen, D. E., and von Hippel, P. H. (1976). *J. Biol. Chem.* **251**, 7198-7214.
Moussaoui, M., Guasch, A., Boix, E., Cuchillo, C. M., and Nogués, M. V. (1995). *J. Biol. Chem.* **271**, 4687-3692.
Parés, X., Nogués, M. V., de Llorens, R., and Cuchillo, C. M. (1991). *Essays Biochem.* **26**, 89-103.
Royer, C. A. (1995). *Methods Molec. Biol.* **40**, 65-89.
Thomas, M., Abedi, H., Farzaneh, F. (1992). *Biotechniques* **13**, 533.
von Hippel, P. H., and Berg, O. G. (1989). *J. Biol. Chem.* **264**, 675-678.
Winter, R. B., Berg, O. G., and von Hippel, P. H. (1981). *Biochemistry* **20**, 6961-6977.
Youle, R. J., Newton, D., Wu, Y.-N., Gadina, M., and Rybak, S. M. (1993). *Crit. Rev. Therapeutic Drug Carrier Systems* **10**, 1-28

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Metal-dependent Structure and Self Association of the RAG1 Zinc-Binding Domain

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I. Introduction

Structural zinc-binding domains are often characterized by the requirement of zinc coordination for proper protein folding [1]. One specific class of zinc-binding motif that will be discussed here is the zinc C_3HC_4 motif, also known as the RING finger [2]. To date at least eighty proteins include a sequence of approximately 50 residues consistent with a RING finger motif. This conserved sequence, with minor variations in some cases, is defined as follows: C-X_2-C-loopI-C-X-H-X_2-C-X_2-C-loopII-C-X_2-C, where X represents any amino acid. A common function attributable to the RING finger module has remained elusive, although a role in protein-protein interactions has been speculated [2].

One of the first RING finger sequences was identified in RAG1, a protein expressed in developing lymphocytes by recombination activating gene-1 [3]. RAG1, along with RAG2, is an essential component of the V(D)J recombination reaction, which produces the genetic sequence encoding for the variable regions of the T cell receptor and immunoglobulin chains. Briefly, V(D)J recombination is accomplished via selection and assembly of gene segments known as variable (V), joining (J), and sometimes diversity (D) in an ordered and precisely regulated process (for a review see [4]). The RING finger sequence of RAG1 is present within the N-terminal third of the protein, which contains a total of 1040 residues in the murine form.

Besides the RING finger sequence, we have recently identified the presence of two C_2H_2 zinc finger sequences within RAG1 [5]. A domain in RAG1 containing one of the zinc finger modules plus the RING finger forms a highly specific dimer, as characterized by a variety of biophysical techniques [5]. The dimerization of this zinc-binding domain provides further support for the participation of RING fingers in protein-protein interactions. This dimerization