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INTERACTION OF RIBONUCLEASE T_1 WITH DNA, MONONUCLEOTIDES AND OLIGONUCLEOTIDES

M. K. Campbell, A. M. Lawler, J. C. Cooper and M. C. Dalton

Department of Chemistry, Carr Laboratory
Mount Holyoke College, South Hadley, MA 01075

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The interaction of ribonuclease T_1 with DNA and nucleotides was investigated by fluorescence titration to establish whether or not this enzyme is a helix-destabilizing protein. Binding of the enzyme to DNA, ribonucleotides and oligodeoxyribonucleotides of chain length ten or more leads to enhancement of fluorescence emission of the enzyme as a function of increasing nucleotide/protein ratio. For deoxyribonucleotides of chain length less than ten, only quenching is observed. Energy transfer from the bases is postulated to be the source of the enhancement of fluorescence, while the decrease can be ascribed to changes in the distribution of charged groups in or near the binding site.

The importance of helix-destabilizing proteins in the DNA replication process is well established, making a detailed understanding of their mode of action highly desirable. Bovine pancreatic ribonuclease (RNase A) has been shown to have helix-destabilizing properties (1), as has gene-32 protein from bacteriophage T_4 (2,3). Both bind preferentially to single stranded rather than double stranded DNA. It would be of interest to determine whether RNase T_1, the guanine-specific ribonuclease from Aspergillus oryzae (EC 2.7.7.26), is also a helix-destabilizing protein. In addition to obvious similarities to RNase A, RNase T_1 is, like gene-32 protein, an acidic protein and binds to nucleic acids and nucleotides in spite of gross electrostatic repulsion (2-4).

Helix-destabilizing proteins bind preferentially to A-T rich regions of DNA, so the specificity of RNase T_1 for guanine allows for a comparison of base recognition and conformation recognition. If RNase T_1 is indeed a helix-destabilizing protein, the comparison with RNase A would be of interest. If

Abbreviations used: RNase, ribonuclease; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; MOPS, 3-(N-morpholino/propane sulfonic acid; r, nucleotide/protein ratio.

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RNase T_1 should prove not to be a helix-destabilizing protein, the contrast with RNase A would also be of interest.

The binding of gene-32 protein to DNA and various nucleotides has been elucidated by fluorescence spectroscopy (2,3). This method is particularly well suited for studies on RNase T_1, which contains one tryptophan residue (4). Fluorescence emission in native proteins is normally from tryptophan as a result of energy transfer from tyrosine residues, of which there are nine in RNase T_1 (5). The fact that there is only one tryptophan residue removes the possibility of large variations in the fluorescence emission spectrum due to differences in local environment. It has already been established that there is energy transfer from at least some of the tyrosine residues to the tryptophan (6,7). In this study, the binding to RNase T_1 of native and denatured DNA and of a series of nucleotides was investigated by fluorescence titration.

Materials and Methods

Crystalline RNase T_1 was purchased from Calbiochem. Calf thymus DNA, Escherichia coli DNA and monodeoxyribonucleotides were from Sigma. Oligonucleotides and monoribonucleotides were obtained from P-L Biochemicals. All other materials including buffers were reagent grade. Thermal denaturation of DNA samples was done by heating stock solutions at 100° C for fifteen minutes followed by rapid quenching in ice. Concentrations were determined by absorbance, and final sample concentrations were chosen to produce a total absorbance of less than 0.1. The RNase T_1 concentration was kept constant within the tolerances of weighing and dilution.

Fluorescence measurements were done on a Perkin-Elmer MPF-44B spectrofluorometer equipped with a DCSU-II correction unit. The cell compartment was kept at 25.0 ± 0.1° C. Correction for the inner filter effect due to the absorbance of the added nucleic acids or nucleotides was done by the method of Hélène, Brun and Yaniv (8). All samples were kept at pH 7.5 in Tris buffer except for experiments to investigate the effect of the buffer itself when MOPS and sodium diethylmalonate were used. All results are the average of five measurements, and the error of estimate for individual measurements is less than 5%.

Results and Discussion

The wavelength of maximum fluorescence emission of RNase T_1 is not changed in the presence of DNA, nor, as shown in Figure 1, is there a loss of fine structure in the spectrum. The most salient point is that the intensity of fluorescence emission of the enzyme shows a consistent pattern of enhancement followed by a decrease and eventual quenching as a function of increasing nucleotide/protein ratio. Figure 2 shows that the same type of behavior is

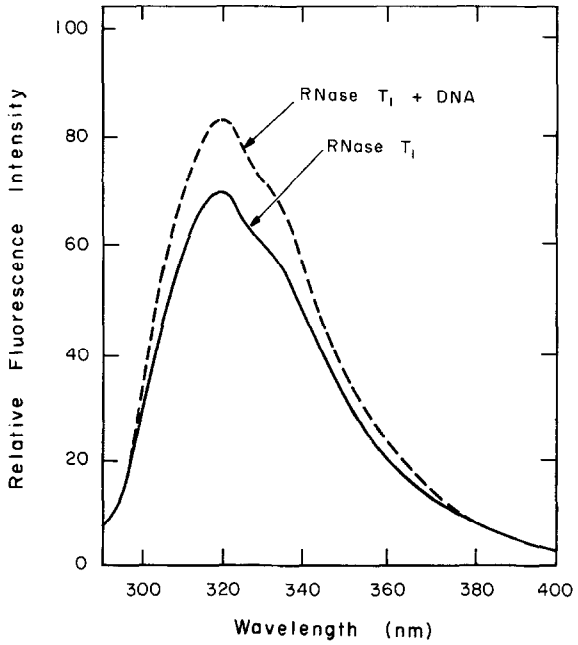

Figure 1. Fluorescence emission spectrum of RNase T_1 in the presence and absence of native calf thymus DNA. Excitation wavelength: 280 nm. Buffer: Tris pH 7.5.

observed for both native and denatured DNA. The maximum value of increase in fluorescence intensity for native calf thymus DNA is 35% at an r (nucleotide/protein ratio) value of 50. For denatured calf thymus DNA, the maximum enhancement of fluorescence intensity is 20% at r = 25. The same behavior is observed with E. coli DNA. The calculated binding site size is 22 nucleotide pairs for the native and 9 nucleotides for the denatured DNA.

A comparison of the fluorescence behavior of RNase T_1 in the presence of ribo- and deoxyribomononucleotides shows that the pattern of enhancement followed by decrease of fluorescence intensity is also observed for guanosine 2'-monophosphate and guanosine 3'-monophosphate, chosen because of the specificity of RNase T_1 for guanylic acid residues. As summarized in Table 1, the maximum enhancement of fluorescence is 15% for Guo-2'-P and 35% for Guo-3'-P, both at r values of 20 to 25. In the deoxy series, quenching alone is observed with the dGuo-3'-P monomer as well as with deoxyguanylic acid oligomers up to a chain length of ten. Quenching increases monotonically with no break points in the titration curve. For $(dG)_{10}$, the fluorescence intensity of RNase T_1 is increased marginally, by 3.5% at r = 50, followed by quenching.

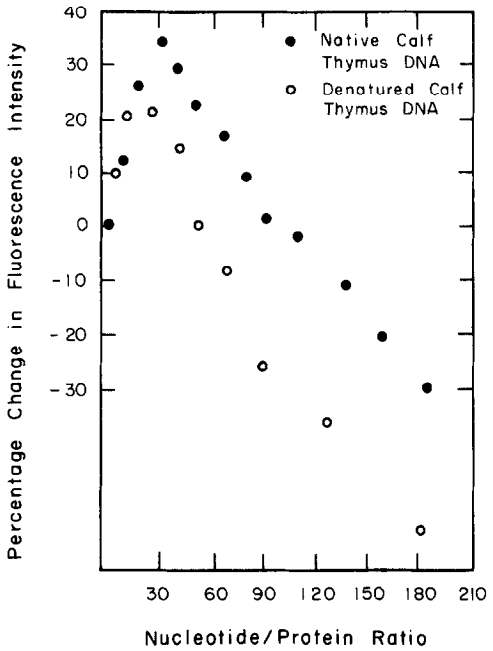

Figure 2. Fluorescence emission intensity of RNase T_1 as a function of nucleotide/protein ratio in presence of native and denatured calf thymus DNA. Excitation wavelength: 280 nm. Emission wavelength 323 nm. Buffer: Tris pH 7.5.

For a sample of mixed oligomers of chain length 12 to 18, there is a 10% increase in fluorescence intensity at r = 10, an interesting comparison with the binding site size for denatured DNA.

Table 1

Fluorescence Titration Parameters for RNase T_1

Substance Added	% Maximum Enhancement	Nucleotide/ Protein Ratio at Maximum
native DNA	35	50
denatured DNA	20	25
Guo-2'-P	15	20-25
Guo-3'-P	35	20-25
dGuo-3'-P	Quenching only	—
$(dG)_2$, $(dG)_4$, $(dG)_6$, $(dG)_8$	Quenching only	—
$(dG)_{10}$	3.5	50
$(dG)_{12}$-$(dG)_{18}$ mixture	10	10

The comparison between ribo- and deoxyribonucleotides indicates that interaction at the sugar binding site of RNase T_1 affects the observed fluorescence behavior. It has been suggested that the 2'-hydroxyl group of the ribose interacts with glutamate-58 of RNase T_1 in the course of RNA hydrolysis (9). Local conformational changes in the active site of the enzyme on binding could well affect observed fluorescence, since the emitting tryptophan is residue 59. The comparison between native and denatured DNA also indicates a question of accessibility of substrate to the binding site. The significance of any binding constant that might be calculated is dubious until the mechanism of binding is elucidated, but the binding site sizes may indicate easier accessibility and tighter binding for the single stranded species.

The stability of protein-nucleic acid binding of this type arises from electrostatic attraction between the phosphate group and centers of positive charge on the enzyme, while specific recognition arises from intercalation and similar interactions (10). Most of these experiments were done with a cationic buffer (Tris), and the question of buffer binding to phosphate groups arises. If, however, typical runs with DNA are repeated with an anionic buffer (diethylmalonate) or a zwitterionic buffer (MOPS), the results are the same within experimental error. The more specific types of interactions present a more complex situation.

It is clear from the fact that there is no wavelength shift or loss in fine structure in the emission spectrum that an exciplex, or excited state complex, is not formed when RNase T_1 binds to DNA or to nucleotides. There is, however, a possibility of long range energy transfer by the Förster mechanism (11). The enhancement of fluorescence emission could be caused by transfer of energy from the bases in the found DNA or nucleotide to the tryptophan either directly or, less possibly, through intermediate transfer to a tyrosine. Fluorescence emission is not normally observed in nucleotides or nucleic acids at room temperature at neutral pH, but in the case of binding to RNase T_1, new mechanisms for deactivation of the excited state of the bases, including energy transfer, becomes available. The point is being investigated in more detail.

Quenching of RNase T_1 fluorescence by bound nucleotides has previously been observed (6), but the earlier studies did not cover so wide a range of nucleotide/protein ratios as this work. Quenching of the tryptophanyl emission of RNase T_1 can be ascribed to the greater proximity of charged groups such as imidazolium or carboxylate ions resulting from the nucleotide binding, a local rather than a gross conformational charge.

Base stacking even on the mononucleotide level is well established, especially for purine nucleotides (12). It is possible that the stacking of bases in the substrate is retained in the complex with RNase T_1. It is not possible to determine from these results whether there is any cooperativity in the binding process, since the basic nature of the processes which give rise to the enhancement and subsequent decrease of fluorescence of RNase T_1 has yet to be determined. The question of whether or not RNase T_1 is a helix-destabilizing protein also remains to be determined. Clearly the binding process is not a simple one, but elucidation of the processes involved can be expected to shed new light on questions of protein nucleic-acid interaction.

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