ACTION OF DIMERIC RIBONUCLEASES ON DOUBLE-STRANDED RNA

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Received 12 June 1972

1. Introduction

Ribonucleases characterized by a phosphotransferase mode of action are virtually inactive towards double-stranded RNA [1, 2], provided the ionic strength of the incubation mixture is high enough to stabilize the secondary structure of the nucleic acid.

The only example so far known of a nuclease specific for double-helical polyribonucleotides is RNAase III from *E. coli* [3–5]; a similar activity has also been reported to occur in animal serum [6].

Recently, however, it has been shown [7] that double-stranded RNA and the poly(A)· poly(U) complex are degraded by RNAase BS-1, a ribonuclease isolated from bovine seminal plasma [8, 9], which has been found to be composed of two identical subunits, each containing the structural elements of RNAase A active site [10, 11].

The finding [12] that RNAase A in the dimeric form acquires the ability to significantly degrade double-stranded RNA, its catalytic activity on single-stranded RNA remaining unchanged, would be consistent with the hypothesis that degradation of double-stranded polyribonucleotide is dependant on the presence of two active sites on the enzyme molecule.

Abbreviations:

SSC, sodium chloride 0.15 M, sodium citrate 0.015 M, pH 7.
TCA, trichloroacetic acid.
N, native RNAase A.
CM or CM-His-119-RNAase A, RNAase A alkylated at histidine 119.
N:N, dimeric RNAase A.
CM:CM, dimeric CM-His-119-RNAase A.
CM:N, hybrid dimers of CM-His-119-RNAase A and native RNAase A.

This hypothesis has been tested by studying the effect of the selective inactivation of one of the two active centers of dimeric RNAase A on the catalytic action of the enzyme towards double-helical polyribonucleotides. The experimental results, to be described in the following sections, are in agreement with the hypothesis.

2. Materials and methods

2.1. *Substrates*

Double-stranded RNA, labeled with ^{14}C or ^{3}H, was prepared from *E. coli* Hfr 3000 infected with MS2 phage (Miles Lab., Inc., Kankakee, Ill.), according to Billeter and Weissmann [13], using [^{14}C] uracil (61 mCi/mmole, The Radiochemical Centre, Amersham or [^{3}H] uridine (20 Ci/mmole, NEN GMbH, Dreieichenhain bei Frankfurt/Main) as precursors. Under conditions of the standard ribonuclease assay [13], SSC at pH 7, 0.187 ionic strength, the double-stranded RNA was 95–96% resistant to digestion, whereas, after heat denaturation, only about 0.5% of the radioactivity remained RNAase A-resistant. The specific activity was 160 cpm/μg for the ^{14}C-labeled and 510 cpm/μg for the ^{3}H-labeled double-stranded RNA.

The poly(A)·poly(U) complex [14] was prepared as previously described [7] by mixing equivalent amounts of poly(A) and poly(U) (Sigma Chem. Co., St. Louis, Mo.) in the presence of SSC.

2.2. *Alkylation of RNAase A*

A procedure derived from the studies of Goren and Barnard [15, 16] was employed. After purification of RNAase A (Sigma Type XII-A) on a carboxymethyl-

cellulose chromatographic system [16], the protein was desalted by gel filtration on Biogel P-2 (Bio-Rad, Richmond), deionized by passage through a column of AG 501-X8 resin (Bio-Rad) and alkylated with recrystallized bromoacetic acid for 37 min at 35° in 0.033 M sodium acetate, pH 5.5 [15]. CM-His-119-RNAase A was then isolated by a second chromatography on carboxymethyl-cellulose and identified by its chromatographic properties and amino acid analysis. Its activity towards cytidine 2′:3′-cyclic phosphate [9] was less than 0.2% that of native RNAase A.

2.3. *Preparation of hybrid dimers*

Hybrid dimers of RNAase A (N) and carboxymethylated RNAase A (CM) were prepared as described by Crestfield and Fruchter [17] by aggregation of equimolar amounts of RNAase A and CM-His-119-RNAase A. Monomeric and dimeric species were then isolated by gel-filtration through Sephadex G-75, and characterized by amino acid analysis and enzymatic activity towards cytidine 2′:3′-cyclic phosphate.

2.4. *Enzyme assay*

The assay mixture for double-stranded RNA was composed by 0.082 M sodium chloride, 0.0082 M sodium citrate, 0.042 M sodium phosphate buffer, with a final pH of 6.4 and an ionic strength of 0.152. Under these conditions, which are within the limits usually defined for stabilization of nucleic acid secondary structure [18, 19], control experiments showed that both the ^{14}C- and the ^{3}H-labeled substrates were about 90% resistant to digestion by RNAase A (50 μg/ml) after 30 min at 25°. Incubations were stopped by TCA addition, at 0°, to a final concentration of 7%, and the precipitates were collected on Millipore membranes (HAWP00010, Millipore Filter Co., Bedford, Mass.), washed exhaustively with chilled 6% TCA and dried (10–15 min at 100°). Radioactivity was determined as described [7].

Activity towards the poly(A) · poly(U) complex was assayed at 24° with 54 μg of substrate per ml of SSC, pH 7, following the increase in absorbance at 260 nm with a Zeiss PMQII spectrophotometer. One unit of activity was defined as an increase in A_{260} of 0.001.

RNAase A concentration was determined on the basis of $A^{1\%}_{280} = 6.95$ [20] or with the method of Lowry et al. [21].

3. Results and discussion

RNAase A dimers hydrolyze in 30 min more than 30% of double-stranded RNA under conditions in which degradation by RNAase A is limited to about 10%

While this 10% degradation by native RNAase A (N) can be accounted for by the presence of non-helical regions, presumably at the ends of the double-stranded RNA, the ability of RNAase A′dimers and of the dimeric RNAase BS-1 [7] to degrade double-stranded RNA would appear to depend on the availability of two active sites on the enzymic dimeric structure.

The monomer fraction, isolated from the products of aggregation of equimolar amounts of RNAase A (N) and CM-His-119-RNAase A (CM), and containing equal amounts of N and CM, is only 5% active (in 30 min) towards double-stranded RNA (fig. 1).

The dimer fraction, on the other hand, includes, according to Crestfield and Fruchter [17], the following molecular species: 25% of fully inactive, alkylated dimers, CM:CM, 25% of 'native' dimers, N:N, and 50% of hybrid dimers, CM:N, in which only one of the two 'subunits' contains a carboxymethylated His-119. As these hybrid dimers contain only one active site, they should behave, according to the hypothesis, like RNAase A. The activity of the heterogeneous mixture, containing the CM:N + N:N + CM:CM species, should then amount to the sum of the fractional activities of N:N and of CM:N components, that is to a calculated 42%, assuming as 100% the degradation of double-stranded RNA effected by N:N dimers.

When tested towards double-helical MS2 RNA (see fig. 1), it was found that this mixture gave a value of about 60% degradation. The discrepancy between the expected and the observed values may be explained by the presence, in the mixture as tested, of 25% of the fully active N:N dimers. Their action would produce fragments of double-stranded RNA, thus producing new free ends available for degradation by the CM:N component, whose activity has to be equated to that of monomeric RNAase A (N). It should be mentioned, in this respect, that a limited digestion with RNAase III makes double-stranded RNA susceptible to degradation by RNAase A [3, 22].

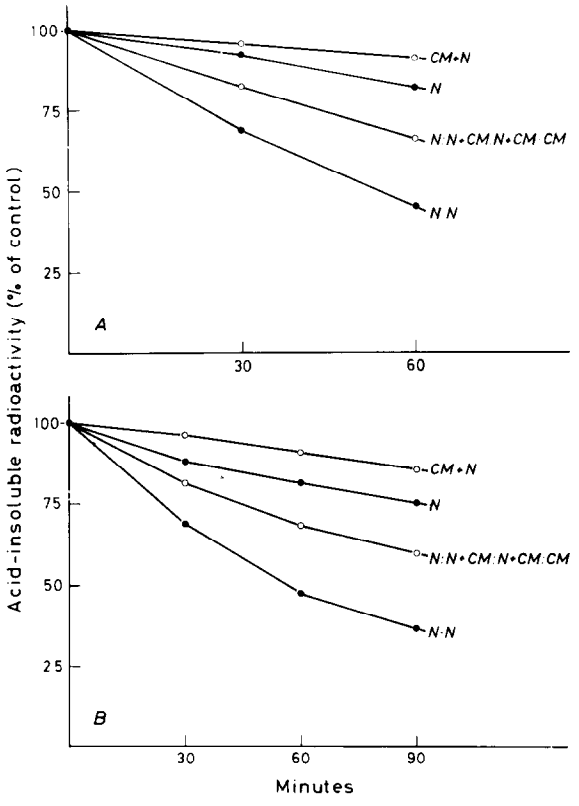

Fig. 1. Degradation of double-stranded MS2 RNA incubated with: RNAase A (N); a mixture (CM + N) containing equimolar amounts of RNAase A and CM-RNAase A; dimeric RNAase A (N:N); a mixture (CM:CM + CM:N + N:N) containing homologous and hybrid dimers of RNAase A and CM-RNAase A. A), ^{3}H-labeled double-stranded RNA, 0.73 μg; B) ^{14}C-labeled double-stranded RNA, 1.35 μg. Protein concentration, 50 μg in a final volume of 1 ml (for details, see text).

On the other hand, in the case of the poly(A)·poly(U) complex, the relatively high resistance of the poly(A) strand to RNAase A (N) digestion would minimize that effect. Degradation of this substrate by the mixture of homologous and hybrid dimers was found, in fact, in several repeated experiments, to be within the expected value of 42% of the activity shown by 'native' dimers (N:N). A typical experiment is shown in fig. 2. In the figure are also included the kinetics of degradation of the poly(A)·poly(U) complex by the equimolar mixture

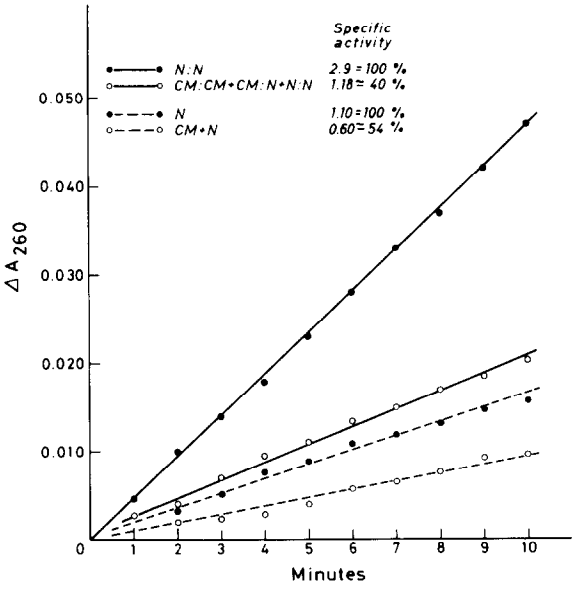

Fig. 2. Degradation of the poly(A)·poly(U) complex, 54 μg/ml, incubated with: 1.5 μg/ml RNAase A (N); 1.5 μg/ml of a mixture (CM + N) containing equimolar amounts of RNAase A and CM-RNAase A; 1.6 μg/ml dimeric RNAase A; 1.8 μg/ml of a mixture (CM:CM + CM:N + N:N) containing homologous and hybrid dimers of RNAase A and CM-RNAase A. Final volume, 2 ml SSC, pH 7. Temperature, 24°.

of CM and N monomers and by native RNAase A (N).

Work is now in progress for the isolation of the hybrid CM:N species, which would be the most appropriate tool to confirm the hypothesis as proposed above.

Acknowledgements

We are indebted to Prof. E. Leone for his constant interest, and to Dr. A. Parente for having kindly performed the amino acid analysis.

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