Action of Dimeric Hybrids of Native and Selectively Alkylated Ribonuclease A on Double-Stranded Ribonucleic Acid

By GIUSEPPE D'ALESSIO, JIŘÍ DOSKOČIL and MASSIMO LIBONATI

*Institute of Organic and Biological Chemistry, University of Naples, Via Mezzocannone 16,
80134 Napoli, Italy,*

*and Institute of Organic Chemistry and Biochemistry,
Czechoslovak Academy of Science, Praha, Czechoslovakia*

(Received 27 March 1974)

The hypothesis that both active sites of dimeric ribonuclease A have to be simultaneously available in order to make the enzyme able to degrade double-stranded RNA is tested with purified hybrid dimers of ribonuclease A and its carboxymethylated derivative. The results obtained support the hypothesis.

It is well known that RNA molecules having a secondary structure stabilized by adequate ionic strength are virtually insensitive to digestion by ribonucleases that function by a phosphotransferase mechanism (Barnard, 1969; Uchida & Egami, 1971). However, nucleases specific for double-stranded RNA and/or the polyribonucleotide strand of a DNA–RNA hybrid have been described (Libonati *et al.*, 1967; Robertson *et al.*, 1968; Hausen & Stein, 1970). Moreover, non-specific enzymes active on both single- and double-stranded RNA are also known (Stern, 1970; Palese & Koch, 1972). Among these is ribonuclease BS-1, isolated from bovine seminal plasma (D'Alessio *et al.*, 1972a) and characterized as a dimeric protein made up of two identical subunits (D'Alessio *et al.*, 1972c). The amino acid sequence of the polypeptide subunit has been determined and found to be strictly homologous to the sequence of bovine pancreatic ribonuclease A (Suzuki *et al.*, 1972). Ribonuclease A (ribonuclease 3'-pyrimidino-oligonucleotidohydrolase, EC 3.1.4.22) is not capable of significantly degrading double-stranded RNA, but acquires this ability when artificially dimerized (Libonati, 1971). This observation and the finding that naturally dimeric ribonuclease BS-1 is active on double-stranded RNA (Libonati & Floridi, 1969) stimulated the hypothesis that degradation of double-stranded RNA becomes possible when two active sites are simultaneously present on one ribonuclease molecule (D'Alessio *et al.*, 1972b). Preliminary results, obtained by studying the action of hybrid dimers prepared by aggregation of ribonuclease A with its carboxymethylhistidine derivative (Crestfield *et al.*, 1963; Crestfield & Fruchter, 1967), were in general agreement with the hypothesis (D'Alessio *et al.*, 1972b). These results, however, could not be considered definitive, as the dimeric enzyme used was a heterogeneous mixture of homologous and hybrid

dimers, namely N:N,* CM:N and CM:CM dimers in the expected proportions 1:2:1 (Crestfield & Fruchter, 1967).

In this paper we describe the isolation of homogeneous CM:N hybrid dimer and its action on double-stranded RNA. These results strongly support the proposed hypothesis.

Methods

Carboxymethylation of ribonuclease A. The procedure used for selective *N*-alkylation with bromoacetate of specific histidine residues of ribonuclease A, based on the studies of Goren & Barnard (1970a,b), has been already described (D'Alessio *et al.*, 1972b). The carboxymethylated derivatives of the protein, isolated by chromatography on CM-cellulose (Goren & Barnard, 1970b), were identified on the basis of their elution volumes and amino acid compositions. Amino acid analyses of the protein that was eluted in the peak corresponding to ribonuclease A selectively alkylated at histidine-119 showed no significant differences in any amino acid, in comparison with ribonuclease A, except for a lower content of histidine (between 2.8 and 3.05 mol/mol of protein, compared with the value of 4 histidine residues/molecule of ribonuclease A), and the presence of *N*-1-carboxymethylhistidine, found in the ratio of 0.78–0.97 mol/mol of protein. The protein derivative thus identified as [*N*-1-carboxymethylhistidine-119]-ribonuclease A showed no enzymic activity when tested with cytidine 2':3'-cyclic phosphate; under the conditions of the assay an activity of 0.5% of that of the native enzyme would have been detected.

* Abbreviations: N:N, dimeric ribonuclease A; CM:CM, dimeric carboxymethylated ribonuclease A; CM:N, dimeric hybrid of ribonuclease A and its carboxymethylated derivative; SSC, 0.15M-NaCl–0.015M-sodium citrate, pH 7; CmHis (in Fig. 1), *N*-carboxymethylhistidine.

Preparation of the hybrid dimer. Aggregation of ribonuclease A and carboxymethyl-ribonuclease A was performed as described by Crestfield *et al.* (1962) by freeze-drying a 5ml solution of 40% (v/v) acetic acid containing 50mg of each protein. Dimeric aggregates were completely separated from unchanged monomeric protein and from higher aggregates by gel filtration through a column (2.5cm×80cm) of Sephadex G-75 (Pharmacia; superfine grade) equilibrated with 0.04M-Tris-HCl buffer, pH7.8. The peak of dimeric protein, identified on the basis of its elution volume, was directly applied to a column of CM-cellulose and fractionated with the chromatographic system described in the preceding paragraph (Goren & Barnard, 1970b).

Preparation of double-stranded RNA. ³H-labelled double-stranded RNA was isolated from non-permissive *Escherichia coli* K38 cells infected with f2 sus11 bacteriophage (Lodish & Zinder, 1966). The bacteria were grown in three Erlenmeyer flasks on a Dubnoff shaker, each flask containing 100ml of low-phosphate medium containing 0.25mM-phosphate (Pačes *et al.*, 1968). When a density of 2×10^8 cells/ml was attained, the cultures were infected at a multiplicity of 10. After 35min, 1mCi of [5-³H]-uridine (Institute of Research and Application of Radioisotopes, Praha, Czechoslovakia; specific radioactivity 23Ci/mmol) was added, together with non-labelled uridine to a final concentration of 61.5 μ M.

Bacteria were harvested 40min later. Double-stranded RNA was isolated as described by Billeter & Weissmann (1966), the final step of chromatography on methylated serum albumin being omitted. The yield was 4mg of double-stranded RNA, with a specific radioactivity of about 6500c.p.m./ μ g. In $0.1 \times$ SSC the 'melting' temperature (T_m) was 86°C and the hyperchromicity at 260nm was 24.9%. No change in absorbance was observed on heating from 25° to 72°C. The sedimentation coefficient of double-stranded RNA was 8.1S in SSC.

Enzyme assay on double-stranded RNA. The assay mixture (total volume 1ml) contained 0.082M-NaCl, 0.0082M-sodium citrate, 0.042M-sodium phosphate and ³H-labelled double-stranded RNA (1.9×10^4 c.p.m.); final pH was 6.4 and ionic strength 0.152mol/litre. Incubations were carried out at 25°C, and stopped at 0°C, by addition of 0.1vol. of chilled 60% (w/v) trichloroacetic acid. Precipitates were collected on Millipore membranes, and radioactivity was measured, as described by D'Alessio *et al.* (1972b).

Other methods. Amino acid analyses were performed as described elsewhere (D'Alessio *et al.*, 1972a). Enzymic activity on cytidine 2':3'-cyclic phosphate (Sigma, St. Louis, Mo., U.S.A.) was measured as previously described (Floridi *et al.*, 1972).

Fraction	Putative species	His (residues/molecule)		CmHis (residues/molecule)		Activity (%) on cytidine 2':3'-cyclic phosphate	
		Calc.	Obs.	Calc.	Obs.	Calc.	Obs.
C-1	CM:CM I	6	5.76	2	1.60	0	0
C-2	CM:N I	7	6.90	1	—	50	36
C-3	CM:CM II	6	5.74	2	1.92	0	0
C-4	N:N I	8	—	0	—	100	—
C-5	CM:N II	7	6.88	1	1.11	50	43
C-6	N:N II	8	7.70	0	0	100	80

Fig. 1. Elution profiles obtained from CM-cellulose chromatography of dimeric aggregates of ribonuclease A and characterization of the isolated species

Samples were: (a) dimeric ribonuclease A; (b) dimeric carboxymethylated ribonuclease A; (c) dimers obtained from an equimolar mixture of ribonuclease A and its carboxymethylated derivative. The chromatographic system used is indicated in the text. The tracings recorded with an automatic monitor (Uvicord, LKB) are shown. Elution was performed at a flow rate of 10ml/h.

Results

Isolation and characterization of homogeneous hybrid dimer. The fractionation pattern of the dimeric aggregates obtained from an equimolar mixture of ribonuclease A and its carboxymethylated derivative is shown in Fig. 1. The chromatographic system used for this fractionation was originally developed by Goren & Barnard (1970b) for separating ribonuclease A and its carboxymethylated derivatives. Preliminary trials had shown that good yields of homogeneous dimers could not be obtained with the systems previously used (Crestfield & Fruchter, 1967) to fractionate the complex mixture of dimers. The hybrid dimer in fact, as well as the homologous dimer of carboxymethylated ribonuclease A, are resolved by ion-exchange chromatography, just like the dimer of native protein (Fruchter & Crestfield, 1965a), into two isomeric forms (Crestfield & Fruchter, 1967). Thus a total of six components, two forms I and II for each of the three dimeric species [the nomenclature proposed by Fruchter & Crestfield (1965a) is adopted here], are present in the freeze-dried mixture of native and carboxymethylated ribonuclease A.

In Fig. 1 are also shown the chromatographic patterns obtained in control runs with the homologous dimers, prepared by freeze-drying of native ribonuclease A (Fig. 1a) and of carboxymethylated protein (Fig. 1b). A comparison of the three chromato-

graphic patterns indicated that peaks C-2 and C-5 had elution volumes different from those of any homologous dimer. Further, these two peaks comprised about 50% of the total fractionated protein.

Positive identification of the proteins present in peaks C-2 and C-5 as the two isomeric forms I and II of the hybrid dimer of native and selectively alkylated ribonuclease A was then obtained from the data of the amino acid compositions and the enzymic activities of the various isolated components of the dimeric mixture. These results are summarized in Fig. 1.

Activity of the hybrid dimer on double-stranded RNA. Fig. 2 shows the results of one of several experiments performed to compare the activity on double-stranded RNA of the purified CM:N hybrid dimer and the homogeneous N:N and CM:CM dimers. A control assay with native ribonuclease A is included.

The results obtained from assays carried out with two different concentrations of protein (Figs. 2a and 2b) agree in showing that the hybrid CM:N aggregate behaves essentially as ribonuclease A. Whereas the homogeneous N:N dimer in 60 min degrades about 24% (Fig. 2a) or 55% (Fig. 2b) of the substrate, degradation of double-stranded RNA by the CM:N dimer is significantly less, and very close to that obtained by digestion with ribonuclease A. The

Fig. 2. Action of dimeric aggregates of ribonuclease A on double-stranded RNA

Protein concentration was 25 µg/ml in (a) and 50 µg/ml in (b). □, N:N; ●, CM:N; △, CM:CM; ○, monomeric ribonuclease A. Protein concentration was determined as described by D'Alessio *et al.* (1972b). Other experimental details are indicated in the text.

CM:CM aggregate does not show any activity on double-stranded RNA.

Discussion

Dimeric ribonuclease A is considered to be composed of two molecules of monomeric protein non-covalently bound to each other and forming, through the displacement and exchange of their *N*-terminal ends, two composite active sites (Crestfield *et al.*, 1963; Fruchter & Crestfield, 1965*b*). A novel property acquired by ribonuclease A when artificially dimerized is its ability to degrade double-stranded RNA (Libonati, 1971), whereas its activities on cyclic nucleotides (Crestfield *et al.*, 1962) and single-stranded RNA (Libonati, 1971) remain unchanged.

When one of the two active sites present on dimeric ribonuclease A is selectively inactivated, the second site is still functional (Crestfield & Fruchter, 1967). The experiments reported in the present paper show that the availability of a single active site on the dimeric enzyme is not sufficient for action on double-stranded RNA. The activity towards this substrate of the dimeric hybrid, composed of native ribonuclease A and its derivative selectively carboxymethylated at histidine-119, is virtually nil, as it does not differ significantly from that of monomeric ribonuclease A. Thus the hypothesis previously advanced (D'Alessio *et al.*, 1972*b*), that the two active sites of dimeric ribonuclease A have to be simultaneously available for action on double-stranded polyribonucleotides, is confirmed.

What remains to be established is if degradation of polyribonucleotides having a secondary structure, by the action of different nucleases, as, for instance, dimeric ribonuclease BS-1, may rest on the same principle. This possibility can be tested by determining the action on double-stranded RNA of a monomeric derivative of ribonuclease BS-1.

We are grateful to Dr. A. Parente for the amino acid analyses, and to Dr. R. N. Perham for critical reading of the manuscript. Financial support by the Consiglio Nazionale delle Ricerche, Italy (grant no. 73.00620.04), is also acknowledged.

- Barnard, E. A. (1969) *Annu. Rev. Biochem.* **38**, 677-732
- Billeter, M. A. & Weissmann, C. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L. & Davies, D. R., eds.), vol. 1, pp. 498-512, Harper and Row, New York and London
- Crestfield, A. M. & Fruchter, R. G. (1967) *J. Biol. Chem.* **242**, 3279-3284
- Crestfield, A. M., Stein, W. H. & Moore, S. (1962) *Arch. Biochem. Biophys. Suppl.* **1**, 217-222
- Crestfield, A. M., Stein, W. H. & Moore, S. (1963) *J. Biol. Chem.* **238**, 2421-2428
- D'Alessio, G., Floridi, A., De Prisco, R., Pignero, A. & Leone, E. (1972*a*) *Eur. J. Biochem.* **26**, 153-161
- D'Alessio, G., Zofra, S. & Libonati, M. (1972*b*) *FEBS Lett.* **24**, 355-358
- D'Alessio, G., Parente, A., Guida, C. & Leone, E. (1972*c*) *FEBS Lett.* **27**, 285-288
- Floridi, A., D'Alessio, G. & Leone, E. (1972) *Eur. J. Biochem.* **26**, 162-167
- Fruchter, R. G. & Crestfield, A. M. (1965*a*) *J. Biol. Chem.* **240**, 3868-3874
- Fruchter, R. G. & Crestfield, A. M. (1965*b*) *J. Biol. Chem.* **240**, 3875-3882
- Goren, H. J. & Barnard, E. A. (1970*a*) *Biochemistry* **9**, 959-973
- Goren, H. J. & Barnard, E. A. (1970*b*) *Biochemistry* **9**, 974-983
- Hausen, P. & Stein, H. (1970) *Eur. J. Biochem.* **14**, 278-283
- Libonati, M. (1971) *Biochim. Biophys. Acta* **228**, 440-445
- Libonati, M. & Floridi, A. (1969) *Eur. J. Biochem.* **8**, 81-87
- Libonati, M., Viñuela, E. & Weissmann, C. (1967) *Abstr. FEBS Meet.* 4th, p. 144
- Lodish, H. F. & Zinder, N. D. (1966) *J. Mol. Biol.* **19**, 333-348
- Pačes, V., Doskočil, J. & Šorm, F. (1968) *Biochim. Biophys. Acta* **161**, 352-360
- Palese, P. & Koch, G. (1972) *Proc. Nat. Acad. Sci. U.S.A.* **69**, 698-701
- Robertson, H. D., Webster, R. E. & Zinder, N. D. (1968) *J. Biol. Chem.* **243**, 82-91
- Stern, R. (1970) *Biochem. Biophys. Res. Commun.* **41**, 608-614
- Suzuki, H., Greco, L., Parente, A., Farina, B., La Montagna, R. & Leone, E. (1972) *Acta Vitamin. Enzymol.* **26**, 213-214
- Uchida, T. & Egami, F. (1971) *Enzymes*, 3rd edn., **4**, 205-250