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Note

Coupling of proteolytic quenching and high-performance liquid chromatography to enzyme reactions

Application to bovine pancreatic ribonuclease

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The elucidation of an enzyme mechanism implies the identification and temporal resolution of the reaction intermediates. Many systems can be studied by convenient optical methods (*e.g.*, stopped-flow), but not all give a signal and even if there is one it may be difficult to assign it to a given chemical species.

High-performance liquid chromatography (HPLC) is a powerful analytical technique which has been much used to identify chemically the components of enzyme reaction mixtures. In such studies the investigator is faced with three problems. First, the conditions must be found under which the reaction mixture can be stopped ("quenched", *e.g.*, by acid) without introducing artifacts. Second, the enzyme must not be reactivated during the subsequent treatment (*e.g.*, pH adjustment) often necessary for HPLC. These problems have been discussed[1]. Finally, the chemical species of interest must be cleanly separated by the HPLC method used.

It was our aim to extend previous studies[2,3] on bovine pancreatic ribonuclease (RNase) with cytidine 2′:3′-cyclic phosphate (C>p) as the substrate to a detailed chemical analysis of the two reactions of this system: synthesis to cytidylyl-3′:5′-cytidine 2′:3′-cyclic phosphate (CpC>p) and hydrolysis to 3′-CMP. In particular, we wished to study the transient kinetics of the RNase–C>p systems with the aim of detecting any relationship between the synthetic and hydrolytic pathways. Such a study necessitates the sampling of reaction mixtures milliseconds old, which can be carried out by the rapid flow quench method[4,5].

Here we report an HPLC procedure for the rapid separation and determination of the two products of RNase in the presence of very high concentrations of the substrate C>p. The success of this analysis depends on an effective way of immedi-

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ately stopping the reaction and destroying the enzyme without decomposing the labile substrate and product CpC>p. With RNase this was a problem. Thus, by decreasing the pH to 2 the reaction is stopped but on increasing it for the subsequent HPLC analysis the enzyme is reactivated. It is not possible to use a stronger acid as this decomposes C>p and CpC>p. Finally, it was found that the RNase is destroyed by adding pepsin to the reaction mixture at pH 2. The pH could now be increased and the mixture then analysed by HPLC. By this procedure, which we term proteolytic quenching, we were able to obtain progress curves for the formation of CpC>p and 3′-CMP at high substrate concentrations. We propose that proteolytic quenching could be of use with other systems involving robust enzymes.

EXPERIMENTAL

Materials

Bovine pancreatic ribonuclease (twice crystallized) was purchased from Biozyme (Blaenavon, U.K.). The RNase A component was obtained as described previously[6]. The enzyme concentration was calculated by using $\varepsilon_{278} = 9800$ l mol^{-1} cm^{-1} (ref. 7).

C>p was synthesized from the isomeric mixture of cytidine 2′- and 3′-phosphates (Sigma) according to the method of Szer and Shugar[8] and its purity checked as described[9]. Concentrations of C>p were calculated by using $\varepsilon_{268} = 6850$ l mol^{-1} cm^{-1} (ref. 10).

Pepsin from porcine stomach mucosa was purchased from Sigma. Ammonia and acetic acid (HPLC grade) were obtained from Scharlau (FEROSA, Barcelona, Spain). All other reagents were of analytical-reagent grade.

A Nucleosil 10 SB anion-exchange column (300 mm × 8 mm O.D. × 4 mm I.D.) and Vydac-310SB precolumn stationary phase were purchased from Macherey, Nagel & Co. (Düren, F.R.G.).

Apparatus

All the HPLC experiments were carried out with a modular HPLC apparatus (LKB, Bromma, Sweden) consisting of a pump (Model 2150) controlled by an automated gradient controller (Model 2152) and a sample injector with a 20-μl sample loop (Model 7125; Rheodyne, Cotati, CA, U.S.A.). Nucleotides were detected by monitoring the effluent at 254 nm with an absorbance detector (Uvicord SD, 2158) and an integrator–recorder unit (Chromatopac C-R3A; Shimadzu, Kyoto, Japan).

^{31}P NMR measurements were carried out on a Bruker AM 500 NMR spectrometer with a resonance frequency of 202.46 MHz for the phosphorus nucleus.

Reaction conditions

All reactions were carried out at constant temperature (25°C), pH (5.5) and ionic strength (0.2). Both the enzyme and substrate were dissolved in 0.2 *M* acetic acid–sodium acetate buffer (pH 5.5). Typically, the reaction mixture contained 25 μl of substrate dissolved in the buffer to which 5 μl of RNase solution was added.

Identification of nucleotides by means of ^{31}P NMR

The samples to be assayed were obtained by HPLC separation. Both samples

and standards were dissolved in 0.1 *M* Tris–acetic acid (pH 7.0). Phosphoric acid was used as an external standard and was assigned a chemical shift value of 0 ppm.

RESULTS AND DISCUSSION

Proteolytic quenching

The RNase reactions are readily stopped by the addition of acid but even with acids of relatively low strenght, such as 4% trichloroacetic acid or 0.2 *M* HCl, the 2′:3′-cyclic phosphodiester bond is cleaved to the 2′- and 3′-phosphomonoesters. Finally, it was found that 0.2 *M* H_3PO_4 in 0.1 *M* HCl (pH 1.8) added to an equal volume of the reaction mixture (0.2 *M* acetic acid–sodium acetate, pH 5.5) gave a solution with a final pH of 2. At this pH the acid hydrolysis of the substrate and the product CpC>p is low and the reactions are stopped.

Separate studies showed that the substrate and reaction products of the RNase–C>p system could be separated by ion-exchange HPLC. This requires the adjustment of the pH of the mixture to that of the original reaction mixture (5.5), which almost completely reactivated the enzyme, making the analysis of reaction mixtures a few minutes old impossible.

Furthermore, active enzyme is adsorbed on the HPLC precolumn, where its accumulation causes problems with succeeding samples. Means of irreversibly destroying the acid-quenched reaction mixture were therefore sought.

The irreversible inactivation of RNase at pH 2 was finally achieved by incubating it with pepsin. This protease cleaves the Phe 120–Asp 121 bond in RNase, giving a derivative which is known as "pepsin-inactivated RNase". This material is inactive[11].

The quenching procedure finally adopted was as follows. RNase reaction mixture (30 μl; see Figs 1 and 2 for details) were incubated for the desired times and quenched by the addition of 25 μl of 0.2 *M* H_3PO_4 in 0.1 *M* HCl. The final pH was 2. This was followed by the immediate addition of 5 μl of a pepsin solution (3.6 mg/ml) and the mixture was left for 15 min at 25°C. Under these conditions, complete and irreversible inactivation of the RNase was obtained. The mixture was either analysed immediately or kept at −20°C. Immediately before injecting the sample on to the HPLC column, the pH was increased to 5.5 by the addition of 4 μl of 2 *M* NaOH. Storage of the samples for several months at −20°C did not affect the reproducibility of the results provided that adequate blanks were carried out.

HPLC analysis

The assay method is based on the fast chromatographic separation of nucleotides by an anion-exchange HPLC column at pH 5.5. The conditions were as follows: 20 μl of the sample at pH 5.5 were injected on to the column, previously equilibrated with 0.1 *M* ammonium acetate solution (pH 5.5). Elution was carried out immediately at a flow-rate of 1 ml/min with a linear salt gradient from 0.1 to 0.6 *M* ammonium acetate solution (pH 5.5). To prevent clogging of the HPLC column with pepsin and digested RNase, a precolumn was used which was reconditioned after approximately 20 runs. Fig. 1 shows a typical chromatogram. Although the hydrolytic action of RNase A on C>p produces only 3′-CMP[3], a small amount of 2′-CMP appeared in the chromatograms. Both monophosphates are produced by the hydrolysis of C>p in the acid quench.

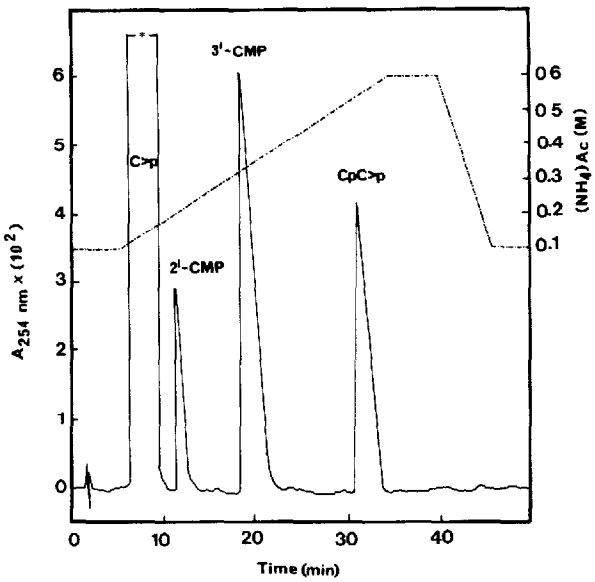

Fig. 1. Chromatography of the components of a reaction mixture (C>p = 40 m*M*, RNase A = 0.6 μM) incubated for 5 min. The reaction was stopped by addition of H_3PO_4–HCl and then pepsin and the products were separated on an anion-exchange HPLC column. Ac = Acetate.

The peaks C>p, 2'-CMP and 3'-CMP in the chromatogram were identified with the use of respective standards. The identity of CpC>p (retention time 34 min), the product of the synthetic activity of RNase, was ascertained in two ways: by digestion with RNase and by ^{31}P NMR. The material with a retention time of 34 min was incubated with RNase and subjected to HPLC analysis. After incubation for 2 min only a peak corresponding to C>p was observed. Longer incubation times

TABLE I

REPRODUCIBILITY OF THE ANALYSIS OF RNase REACTION MIXTURES BY HPLC

Reaction mixtures with initial C>p concentrations of (A) 40 m*M* or (B) 10 m*M* and 0.6 μM RNase were incubated for the times shown, quenched by acid proteolysis and analysed by HPLC. Each point is the average of three determinations. For further details, see the text and Fig. 1.

	Nucleotide	*Reaction time (min)*				
		0	*1*	*5*	*10*	*15*
A	C>p (m*M*)	36.7±1	35.1±1	34.6±1	33.1±1	32.8±1
	2'-CMP (μM)	111±15	114±18	115±20	102±14	106±17
	3'-CMP (μM)	161±16	243±19	374±19	462±21	582±20
	CpC>p (μM)	0	121±12	301±14	312±12	296±15
B	C>p (m*M*)	10.5±0.4	9.80±0.4	9.65±0.40	9.55±0.45	9.03±0.35
	2'-CMP (μM)	29±1	27±4	24±2	27±2	23±4
	3'-CMP (μM)	27±2	69±5	138±5	192±2	244±7
	CpC>p (μM)	0	14±3	14±2	13±5	15±3

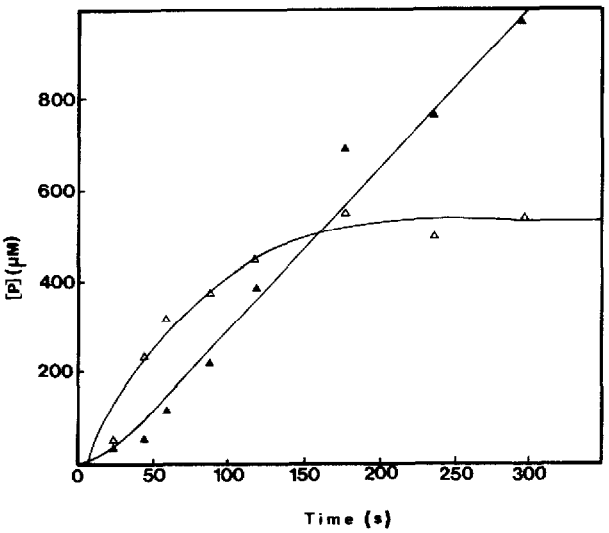

Fig. 2. Progress curves of the products of the RNase reaction obtained with acid proteolytic quenching. The reaction mixtures (50 m*M* C>p plus 0.55 μM RNase A) were incubated for the times shown, quenched with H_3PO_4–HCl and pepsin and separated by HPLC. ▲ = 3′-CMP; △ = CpC>p.

resulted in the progressive appearance of 3′-CMP with the concomitant disappearance of C>p. This is what would be expected from the hydrolysis by RNase of a dinucleotide of cytidine ending in a 2′:3′-cyclic phosphate.

Second, the structure of the dinucleotide was confirmed by ^{31}P NMR spectroscopy, which unambiguously showed the presence of both a 3′–5′-phosphodiester bond and a terminal 2′:3′-cyclic phosphate as judged by a resonance found at 20.576 ppm ascribed to the cyclic phosphate of the dinucleotide and another at −0.560 ppm ascribed to 3′–5′ internucleotide phosphodiester bond with reference to 3′–5′-CpC.

The reproducibility of the HPLC analysis of acid protease-quenched RNase reaction mixtures at two concentrations of C>p is assessed in Table I.

Kinetic analysis of the synthetic and hydrolytic reactions of RNase

By using the procedure described above, we obtained progress curves for CpC>p and 3′-CMP formation by RNase at a concentration of C>p of 50 m*M* (Fig. 2). The curves have the following features.

First, CpC>p synthesis is initially more rapid than 3′-CMP production, but a plateau is reached within 3 min. The size of this plateau increased with increasing C>p concentration, but it was independent of RNase concentration. Bernfield[12,13] also studied the synthetic reactions of RNase and obtained plateaux, but his conditions were different from ours (pH 7 and a time scale of several hours).

Second, there is a lag in the production of 3′-CMP before the steady state (k_{cat} = 3.2 s^{-1}) was attained. It is possible that there is a correlation between this lag and the burst phase observed in the dinucleotide formation. Hence there could be a relationship between the synthetic and hydrolytic pathways; this possibility is under further study.

In conclusion, proteolytic quenching coupled with a convenient HPLC analysis provides a means of studying the mechanism of RNase.

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