Studies on Trypsin Covalently Bound to RNA

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Bovine trypsin (EC 3.4.4.4) was bound to RNA in the presence of water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide], and the enzymic properties of this complex were compared with those of free trypsin. RNA bound trypsin does not lose its activity in the presence of 0.5% SDS, and this is consistent with the fact that the intensity of the fluorescence spectrum of this complex does not change when SDS is added. The RNA-enzyme complex seems suitable for studies on the conformation of immobilized enzyme.

There have been many reports on immobilized enzymes, but little is yet known about the chemical and physicochemical properties of these enzymes. Here we define immobilized enzymes as enzymes bound to carriers, even when the resulting complexes are soluble. Therefore we studied the physicochemical characters of immobilized trypsin using yeast RNA as a soluble carrier. RNA is a suitable carrier because the amino groups in its adenine and cytosine bases are easily bound to carboxyl groups of enzymes in the presence of water-soluble carbodiimide. Another advantage is that RNA shows no emission peaks near those of the aromatic amino acid residues of trypsin at 330 nm, so fluorescence studies on trypsin bound to RNA can be carried out. This paper reports the enzymic properties of the RNA-trypsin complex and the availability of this type of complex for studies on the conformation of immobilized enzyme.

MATERIALS AND METHODS

Bovine trypsin (type III, 2× crystallized) and α -casein were purchased from Sigma Chemical Co., yeast RNA was from Kohjin Co., Ltd., water-soluble carbodiimide was from the Protein Research Foundation, Osaka, and benzoyl-D,L-arginyl-*p*-nitroanilide (BAPA) from Fluka AG, Buchs SG, Switzerland.

Fluorescence spectra were examined in a Hitachi Fluorescence Spectrophotometer MPF-4.

RNA was deproteinized by the method of Kirby¹⁾ before coupling with trypsin. The RNA-trypsin complex was synthesized as follows; 0.1 g of RNA and 25 mg of trypsin were suspended in 5 ml of 0.02 M

KCl-HCl buffer (pH 2.2). Then 0.05 ml of water-soluble carbodiimide was added with stirring at 0°C for 30 hr, and the mixture was applied to a CM-cellulose column equilibrated with 0.05 M acetate-HCl buffer (pH 3.0) to separate the RNA-trypsin.

The phosphorus content of RNA was determined by the method of Lowry *et al.*²⁾

RESULTS AND DISCUSSION

The elution profile on CM-cellulose column chromatography is shown in Fig. 1. Peak 1 in Fig. 1 is attributable to RNA and water-soluble carbodiimide, and peak 2 to RNA-trypsin, because it has BAPA hydrolyzing activity and contains phosphorus. Free trypsin was obtained in peak 3 by elution with a 0.1~0.3 M NaCl gradient. It was calculated that one molecule trypsin was bound per 31 nucleotides of RNA (Table I).

Table I summarizes some of the properties of trypsin bound to RNA with those of free trypsin for comparison. Free trypsin was prepared at the same time as the trypsin used for synthesis of the RNA-trypsin complex, and hereafter "free trypsin" refers this enzyme: that is, trypsin under the same conditions as the RNA-trypsin complex. The BAPA hydrolyzing activity of RNA bound trypsin was almost the same as that of free trypsin, but the caseinolytic activity and *K_m* value for BAPA at pH 8.1 were different from those of free trypsin. This difference seems reasonable because the microenvironments of the enzymes were different.

FIG. 1. Separation of the RNA-trypsin Complex by CM-cellulose Column Chromatography (column size 2 cm $\times$ 35 cm).

Fractions of 3 ml were collected. An NaCl gradient was applied from fraction No. 47.

—, absorbance at 280 nm; ·····, BAPA hydrolyzing activity, measured as absorbance at 410 nm; - - - -, phosphorus content, measured as absorbance at 700 nm.

TABLE I. COMPARISON OF ENZYMIC PROPERTIES OF RNA-TRYPSIN AND FREE TRYPSIN

BAPA hydrolyzing and caseinolytic activities were measured at pH 8.1 and 37°C on reaction for 10 min and the reactions with BAPA and casein were stopped by adding 10% acetic acid and 7% trichloroacetic acid, respectively.

	Specific activity ^a (substrate: BAPA)	Specific activity ^a (substrate: α -casein)	<i>K_m</i> for BAPA at pH 8.1 (mM)	Phosphorus per trypsin
RNA-trypsin	87	67	1.7	31.2
Free trypsin	100	100	0.67	0.0

^a The values shown were calculated taking the specific activities of free trypsin as 100. Protein concentration was determined by the method of Lowry *et al.*⁽³⁾

The optimum pH for activity of RNA-trypsin toward BAPA was 8.1, which is the same as that of free trypsin.

The heat stability of RNA-trypsin was also apparently the same as that of free trypsin, as shown in Table II, but its resistance to the denaturant SDS was different from that of free

TABLE II. COMPARISON OF THE HEAT STABILITIES OF RNA-TRYPSIN AND FREE TRYPSIN

Samples were preincubated at pH 3.0 for 10 min at each temperature, and then incubated with 2 mM BAPA at pH 8.1 and 37°C for 10 min. The values listed here were determined taking the activities of both enzymes at 37°C as 100. Samples containing 40 μ g protein were used.

	Preincubation temperature	BAPA hydrolyzing activity
RNA-trypsin	37°C	100
	60°C	98
	80°C	70
Free trypsin	37°C	100
	60°C	87
	80°C	70

trypsin as shown in Fig. 2. The fact that trypsin bound to RNA is resistant to 1% SDS, suggests that its conformation does not change on addition of SDS. This possibility was supported by the results of fluorescence spectroscopy shown in Fig. 3: addition of 1% SDS did not change or slightly increased the fluorescence intensity of RNA-trypsin around 330 nm due to tryptophan or tyrosine, whereas it

FIG. 2. Resistance of BAPA Hydrolyzing Activity of RNA-trypsin to SDS.

Experimental conditions were as for Table I.

FIG. 3. Fluorescence Emission Spectra of RNA-trypsin and Free Trypsin.

(a) free trypsin, excited at 289 nm, (b) RNA-trypsin, excited at 291 nm., at pH 8.1; —, at pH 8.1 plus 1% SDS. Fluorescence, calculated for a fixed protein concentration, is shown in arbitrary units.

greatly decreased that of free trypsin. The reasons for these interesting phenomena are unknown.

Katchalsky *et al.*⁽⁴⁾ reported similar results on trypsin bound to Sephadex in the presence of 8 M urea. Further studies are required to elucidate these phenomena.

The transfer RNA-aminoacyl tRNA synthetase complex which was synthesized photochemically by Schimmel *et al.*^(5,6) is useful for obtaining information on the specific nucleotides of tRNA that interact with the specific aminoacyl tRNA synthetase. Our RNA-trypsin complex synthesized in the presence of water-soluble carbodiimide is also useful for investigating the interaction of amino acid residues of the enzyme with carriers. However, we failed to identify the main amino acid residues of trypsin that are covalently bound to RNA: under our conditions the carboxyl groups of trypsin seem to bind amino groups of RNA in a random manner during reaction for 30 hr (data not shown).

It is true RNA is not suitable for studies on the conformation of immobilized enzyme because it has a strong absorption peak around 260 nm, but fluorescence studies should provide information on the structure of the immobilized enzyme and therefore be helpful in improving the method for enzyme-immobilization.

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