Attempts of Chemical Modification of Threonine and Serine Residues in RNase A

We have examined the features of pancreatic bovine ribonuclease (RNase A) after reaction with the oxidizing system formed by dimethylsulfoxide-carbodiimide-phosphoric acid[1-3] to verify the selective oxidation of the alcoholic groups of serine and threonine residues present in the protein.

The experimental conditions for the oxidation of such residues were studied already in model peptides (di- and tri-peptides) and were reported elsewhere[1,2].

RNase A maintained the fully enzymatic activity when treated with DMSO at variable times in agreement with the data of RAMMLER[4]. The activity decreased up to 85% of the initial value after a 78-hours treatment with DMSO.

The retention of full activity was also observed in the presence of both DMSO and phosphoric acid. The presence of the electrophylic agent DCCI in DMSO, on the contrary, caused the biological activity to decrease up to a 50% within 4 h; at the same time this reagent induced an aggregation of the protein as indicated by gel-filtration on the (1.8 × 140) G-75 Sephadex column eluted with 0.1 *M* acetate buffer pH 5. The chromatographic pattern reported in Figure 1 (solid line) illustrates, in fact, the presence of more protein components having different molecular size.

To ascertain whether such an aggregation was induced by DCCI we repeated the same experimental conditions described by SCHERAGA[5]. The dotted line in Figure 1 shows the chromatographic behaviour (on the G-75 Sephadex column) after reacting RNase A with the water-soluble carbodiimide (WSC)[5] in aqueous solution. Only a protein component appeared in a position very close to the one observed for native RNase. This result indicates that the dimethylsulfoxide solution of dicyclohexylcarbodiimide induces the formation of more protein components at different molecular weight. At this point we studied the possible oxidation of RNase A at a concentration of the oxidizing system DCCI-DMSO-H_3PO_4 ranging from 0.35 to 10 μmoles per μmole of RNase A.

The Table summarizes the findings obtained at different conditions. A gradual and consistent decrease in enzymatic activity parallel to a partial disappearance of the threonine and serine residues was noted in any case on increasing this molar ratio. The effective – even if limited – modification of the alcoholic groups present in RNase A is indicated also by the cleavage, with NH_2OH–HCl, of peptide bonds as indicated by positive ninhydrin reaction.

The paper chromatography (No. 1 Whatman paper; Partridge eluent) of oxidized RNase A after NH_2OH–HCl treatment has shown the presence of 3 ninhydrin positive spots with a Rf 0, 0.22 and 0.34 respectively. The NH_2OH·HCl treatment of native RNase A, under the same experimental conditions, indicated no detectable fragmentation.

It was observed that the DCCI/DMSO system causes the aggregation of RNase A either in the presence or in

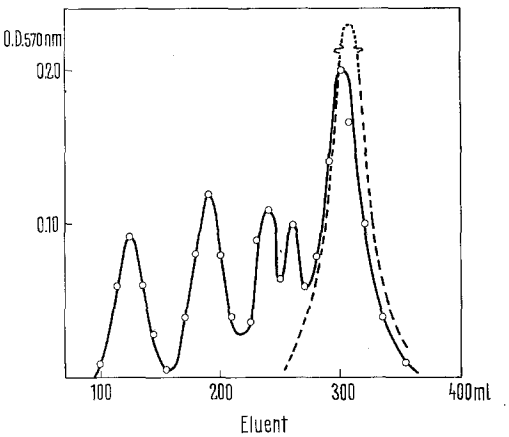

Fig. 1. Gel-filtration on a (1.8 × 140) G-75 Sephadex column eluted with 0.1 *M* acetate buffer pH 5 at 25 °C. Optical density at 570 nm, measured after colorimetric reaction with ninhydrin, is expressed as a function of the elution volume. –⊙–⊙–, chromatographic pattern of RNase A (Worthington product) reacted with DCCI/DMSO system. – – –, chromatographic pattern of RNase A reacted with a water-soluble carbodiimide (WSC)[5] in aqueous solution.

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Oxidative conditions of threonine (Thr) and serine (Ser) residues of RNase A by DCCI/DMSO/H_3PO_4 System

RNase A[a]	DCCI	DMSO	H_2O	H_3PO_4	Time (h)	Yield in oxidized aminoacid[c] (%)		Activity[b] (%)	Paper chromatography (Rf)	
						Thr	Ser		Before	After NH_2·OH treatment
1	–	4900	–	–	4			100	0	0
1	–	4900	–	–	4			100		
1	–	4900	–	–	78			85		
1	–	1535	600	0.8	4			100		
1	2.4	1535	600	–	4	0	0	50	0	0
1	0.35	4000	600	1	4			100		
1	2.0	2550	–	0.8	4	22	38	70		
1	4.3	1237	600	0.8	4	15	34	20	0	0; 0.22; 0.34
1	10.0	2010	–	1.7	7	18	26	15	0	0; 0.22; 0.34

[a] The molar ratio between RNase A and DCCI/DMSO was calculated on the basis of the alcoholic residues content (15 Thr + 10 Ser). [b] Activity percent after elimination of DMSO. Enzymatic activity was measured as initial velocity of RNA hydrolysis[8]. [c] After due correction for the acidic hydrolysis losses[9].

the absence of the protonating agent H_3PO_4. The partial oxidation of the protein takes place, on the contrary, only in the presence of phosphoric acid as indicated in the Table.

The large activity change concurrent with the partial oxidation of threonine and serine is very likely due to aggregation of RNase A induced by DCCI. Of course a partial denaturation of the protein responsible for the activity loss cannot be under-estimated. Figure 2 reports the chromatographic pattern of oxidized RNase A, which shows a residue 15% enzymatic activity, on the G-75 Sephadex column. Also in this case more proteic components were obtained by gel-filtration.

The presence of an altered conformation of RNase A caused by the above treatment was investigated by circular dichroism measurements in the far ultraviolet (Roussel-Jouan dichrograph II). The dichroic spectra of native RNase A and oxidized RNase A having 15% enzymatic activity were compared. The CD spectrum for native RNase reproduces the dichroic band with a minimum at 222 nm (characteristic of n-π^* peptide transition of α-helix) and the larger band at 208 nm associated with the π-π^* peptide transition of α-helix[6,7]. A slight shift toward lower wavelengths of the dichroic bands relative to oxidized RNase, i.e. to 220 and 207 nm respectively, and a dramatic decrease in their magnitude were observed. The large $[\theta]$ values diminution from 8600 to 1600 and from 10,500 to 2500 at 220 nm and 207 nm respectively is evidently due to a loss of ordered structure.

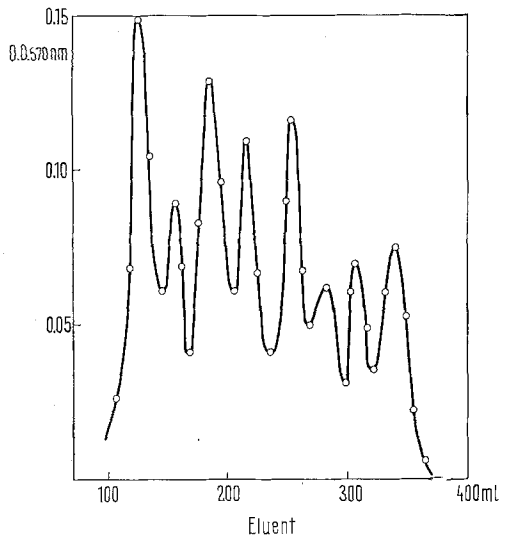

Fig. 2. Gel-filtration of RNase A reacted with the oxydizing system DCCI/DMSO/H_3PO_4. The experimental conditions are the same as in Figure 1.

The IR-spectrum of KBr pellets (Perkin-Elmer Mod. 337 Spectrophotometer) of RNase modified by the oxydizing system shows no appreciable difference from that of native RNase A. In both the cases maxima at 1650, 1530, 1450, 1405, 1240 cm^{-1} were present.

The results reported in this note allow us to conclude that a chemical modification procedure, successfully employed with models, cannot often be apted to protein because it influences dramatically the secondary or tertiary structure.

The present method, however, can be useful in sequence studies together with other chemical or enzymatic methods of cleavage.

Riassunto. È stata studiata la possibilità di modificazione dei residui di Treonina e Serina presenti nella RNase A da parte del sistema ossidante DCCI/DMSO/H_3PO_4 già utilmente impiegata in peptidi modello[1,2].

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The Binding of Some Sodium Substitutes to Chondroitin Sulfate

The need for sodium-free physiologic solutions calls for the use of substitutes to maintain isotonicity. The most commonly used ones are lactose or sucrose, choline chloride and lithium chloride. Such substitution, however, may alter the properties of the tissue incubated or perfused by affecting the state of the protein-polysaccharides which constitute the 'ground substance' and interstitium in general. Whether in the native form of protein-polysaccharides or in partly degraded forms such as chondroitin sulfate, the polysaccharides of the connective tissues are important polyelectrolytes, more precisely, polyanions. These macromolecules will immobilize cations as counter-ions and the kind and concentration of these will determine their domain and their electrical field and, in turn, determine hydration as well. These polyanions are broadly distributed and are present in relatively large amount in the arterial wall[1]. FRIEDMAN and FRIEDMAN[2] have stressed the role that this 'paracellular matrix' can play in altering vascular geometry and affecting peripheral vascular resistance. Equally important physiological effects may be anticipated in other tissues. It seemed thus pertinent to examine the affinities of the commonly used sodium substitutes for such polyanions, of which chondroitin sulfate was chosen as an appropriate representative. It was observed that while lithium and choline substituted efficiently for sodium on the binding sites of this macromolecule, lactose left these sites available to other ions of the medium, that is, in the case of physiological solutions, essentially to polyvalent ions which possess an intrinsically high binding affinity.

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