Elongation of the Disulfide Bonds of Bovine Pancreatic Ribonuclease and the Effect of the Modification on the Properties of the Enzyme*

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SUMMARY

A method is described for the intramolecular pairing of the sulphydryl groups in fully reduced bovine pancreatic ribonuclease (RNase) by reaction with mercuric ions and formation of S-Hg-S cross-links. The protein derivative obtained contains 4 mercury atoms per protein molecule, sediments in the ultracentrifuge as a single symmetrical peak (s20,w = 1.81), and migrates as a single sharp band on acrylamide gel electrophoresis. The circular dichroism spectrum of the mercury derivative is consistent with the supposition that the tyrosine residue which has been found to be normalized in the RNase-mercury derivative, is tyrosine-92, although other interpretations are not excluded. The RNase-mercury derivative retains about 5% and 25% of the specific hydrolytic activity of the native enzyme towards RNA and cytidine 2',3'-cyclic monophosphate, respectively. Unlike the native protein it is digested by trypsin, although at a lower rate than oxidized RNase, with the consequence of complete loss of the enzymic activity. Both trypsic digestion and the concomitant loss of catalytic activity of the mercury derivative follow first order kinetics (trypsin concentration 10 μg per ml; initial protein concentration 0.1 mg per ml) with similar rate constants. These findings indicate that the RNase-mercury derivative is homogeneous and free of contamination by the native enzyme. It is concluded that the cysteine residues are predominantly paired in the RNase-mercury derivative in the same pattern as the cystines in native RNase, and that elongation of the internal cross-links in RNase by 3 Å each does not abolish, but quantitatively modifies, the activity of the enzyme.

EXPERIMENTAL PROCEDURE

Materials—Bovine pancreatic ribonuclease-A (five times crystallized, type 1A, Lot 95B-0330), cytidine 2',3'-cyclic monophosphate (Lot 94B-0560), and p-hydroxymercuribenzoate sodium salt (Lot 64B-5170) were purchased from Sigma. Trypsin (lyophilized, two times crystallized, Lot TRL 6295) was obtained from Worthington Biochemicals. Water-insoluble polytyrosyl trypsin bound to a synthetic diazotizable resin S-MDA (15) was kindly donated by Dr. L. Goldstein. Ribonucleic acid (yeast nucleic acid Lot 6502) was purchased from Schwarz BioResearch. Mercuric chloride labeled with 203Hg (catalog No. M-109) was obtained from the International Atomic Energy Commission (Bagha, Israel). Analytical reagent grade urea, obtained from BDH, Ltd. (Poole, England), was recrystallized from 95% ethanol; solutions of this compound were prepared immediately before use. All other chemicals were of analytical grade.

Methods—In the preparation of [RNase-4Hg], RNase was reduced as described (16). A solution of the reduced protein (7 to 10 × 10^-6 M in 0.1 M aqueous acetic acid) and a solution of HgCl2 (5.6 to 8 × 10^-4 M) were forced in parallel and in stoichiometric proportions (molar ratio of RNase:HgCl2, 1:4) into

*This research was supported in part by United States Public Health Service Grant GM13637.
Fig. 1. Kinetics of trypsin digestion of [RNase:4Hg] a, first order plot of the digestion of 0.2 mg per ml [RNase:4Hg] by 10 μg per ml of trypsin at pH 7.0, 30°, (M) (see Footnote 1). b, logarithm plot of enzymic activity of [RNase:4Hg] towards cytidine 2',3'-cyclic phosphate versus time (measured spectrophotometrically) during trypsin digestion at pH 7.0; O, 0.1 mg per ml of [RNase:4Hg], 5 μg per ml of trypsin; Δ, 0.07 mg per ml of [RNase:4Hg], 10 μg per ml of trypsin; ●, 0.07 mg per ml of [RNase:4Hg], 10 μg per ml of trypsin.

RESULTS

In order to confirm that the RNase derivative obtained contains stoichiometric amounts of mercury, the above procedure of preparation was repeated with [203Hg]HgCl₂. The product obtained was dialyzed against 0.1 M sodium acetate buffer, pH 4.6, for several hours to remove any free mercuric salt in solution. An aliquot of the protein solution was then removed, its protein content determined spectrophotometrically, and its...
mercury content was determined by radioactive counting. Four mercury atoms were found to be bound per protein molecule.

[RNase-4Hg] was analyzed by acrylamide gel electrophoresis. It moved as a single sharp band; its mobility was however slightly lower than that of native RNase. An artificial mixture of RNase and [RNase-4Hg] could be resolved into two closely separated bands. The slight retardation of [RNase-4Hg] as compared with native RNase is possibly due to some distortion or swelling of the protein molecule upon the introduction of the mercury atoms.

[RNase-4Hg] showed appreciable enzymic activity towards cytidine 2',3'-cyclic monophosphate as substrate. Various preparations of [RNase-4Hg] showed specific activity of 25% ± 5% of the activity of the native enzyme. The enzymic activity towards RNA was much lower, being about 5% ± 1% of that of native RNase. (The activity toward RNA had actually been overlooked in the preliminary studies of [RNase-4Hg] (10).)

It is of interest to note that the specific enzymic activity of [RNase-4Hg] prepared at pH 8.0 was very similar to that of [RNase-4Hg] prepared at pH 4.6.

[RNase-4Hg] is digestible by trypsin at room temperature at pH 7.0 and 8.5. The rate of digestion follows first order kinetics for over three half-life times (see Fig. 1). It is somewhat difficult to compare the rates of trypsinic digestion of [RNase-4Hg] and of oxidized RNase, since the digestion of the latter deviates appreciably from first order kinetics. However, the initial rate of digestion of [RNase-4Hg] is appreciably lower (by about 40%) than that of oxidized RNase. Native RNase is not digested by trypsin under the same conditions.

The course of loss of the enzymic activity of [RNase-4Hg], toward the substrate cytidine 2',3'-cyclic monophosphate, upon digestion of the modified RNase by trypsin is presented in Fig. 1. The activity at any instant was taken as the slope of the curve which described the optical density at 290 mµ as a function of time. The rate of inactivation of [RNase-4Hg] closely follows first order kinetics. When equal amounts of trypsin were used for digestion, the rate constants of inactivation (i.e. the slopes of the plots of the logarithm of the activity versus time) were comparable with the rate constant of the trypsinic digestion of the mercurated protein. When half the concentration of trypsin was used for digestion of [RNase-4Hg], the slopes of inactivation were reduced by about 50% (Fig. 1).

[RNase-4Hg] was also digested by insoluble trypsin with the result of complete loss of enzymic activity of the modified RNase. Under the same conditions, oxidized RNase is digested about twice as fast as [RNase-4Hg], whereas native RNase is not digested at all by the insoluble trypsin.

Fig. 2 presents the circular dichroism spectrum of [RNase-4Hg] in the 250- to 300-mµ region. For comparison, the spectra of native RNase and oxidized RNase are included. As seen from this figure, the circular dichroism spectrum of [RNase-4Hg] in this region is much less intense than that of RNase.

**DISCUSSION**

Reduced RNase reacts quantitatively with 4 mercuric ions. Under selected conditions of reaction, a monomeric product is obtained which retains, within limits, some of the properties of the native enzyme. Thus, 2 of its tyrosine residues titrate abnormally (10) (compared with 3 tyrosines in the native protein (23, 24)), it cross-reacts with anti-RNase (10), and retains an appreciable fraction of the enzymic activity. Contrary to the native enzyme, [RNase-4Hg] is digested by trypsin, although at a lower rate than oxidized RNase.

[RNase-4Hg] migrates electrophoretically on acrylamide gel as a single band at a rate that may be distinguished from that of RNase. The ratio of the specific activities of [RNase-4Hg] toward RNA and toward cytidine 2',3'-cyclic phosphate is markedly different from that of the native enzyme. Furthermore, in contradistinction to RNase, [RNase-4Hg] is digested by trypsin with concomitant complete loss of enzymic activity. We may therefore dismiss the possibility that [RNase-4Hg] contains any amount of native RNase.

[RNase-4Hg] is a homogeneous preparation by several criteria. It sediments in the ultracentrifuge as a single symmetrical peak. As mentioned above, it yields a single band on acrylamide gel electrophoresis. It is digested by trypsin according to strict first order kinetics with a single rate constant. Last but not least, the loss in enzymic activity of [RNase-4Hg] proceeds closely in parallel with the extent of its digestion by trypsin (see Fig. 1). The properties of [RNase-4Hg] therefore describe the bulk of the material and are not average properties of a mixture. It may also be concluded that the mercury atoms in [RNase-4Hg] have predominantly paired cysteine residues in the protein according to the pattern of pairing in native RNase.

Enzymically active [RNase-4Hg] can be obtained by reacting reduced RNase with HgCl₂ both at pH 4.6 and at pH 8.0. On the other hand, when reduced RNase is oxidized by atmospheric...
oxygen, one obtains active enzyme in the pH range of 6.2 to 8.5, but no active material is obtained at pH 4.6 (25). It should be noted that at room temperature the native conformation of RNase is still stable at pH 4.6 and starts to melt out only below pH 3 (20); one may therefore expect proper pairing of the cysteine residues of reduced RNase also at pH 4.6, as has actually been found in this work when the pairing is brought about by mercuric ions in the presence of p-mercuribenzoate. The failure to do so when the pairing is brought about by oxidation is therefore probably due to inhibition of the oxidation reaction of sulfhydryl groups at acid pH (27).

The 275-μm circular dichroism band of RNase has been studied by various authors and some controversy has arisen as to its origin. The band was attributed to the normal tyrosines of the protein (28), the abnormal tyrosines (29, 30), especially to tyrosine-92 (29), or to electronic transitions involving both cystines and tyrosines (31, 32). The 275-μm circular dichroism band of [RNase-4Hg] is much less intense than the corresponding band of the native enzyme. Simpson and Vallee (29) have found by optical rotary dispersion measurements marked diminution of the intensity of the Cotton effect at 275 μm upon normalization of the abnormal tyrosine-92. In this connection it may be recalled that one of the abnormal tyrosines of RNase has been normalized in [RNase-4Hg] (10). Our circular dichroism data may thus be rationalized if we assumed that the tyrosine residue which has been normalized in [RNase-4Hg] is tyrosine-92. Indeed, inspection of the model of RNase (33) shows that this tyrosine residue, which is hydrogen bonded to aspartate-88 (34), is a good candidate for normalization upon conversion of the S–S bond of cystine II-VII into an S-Hg-S bond, which forces Residues 38 and 92 slightly apart. On the other hand, one cannot exclude the possibility of a contribution of the S-Hg-S bonds in [RNase-4Hg] to the 275 μm circular dichroism band of this protein derivative, as evidenced from circular dichroism studies of low molecular weight model compounds.

The enzymic activity of [RNase-4Hg] is about 5% and 25% (compared with native RNase) when tested on RNA and cytidine 2',3'-cyclic monophosphate, respectively. It is pertinent to note that a change in the relative reactivity toward high and low molecular weight substrates has also been reported before for an RNase derivative in which the two disulfide bonds, IV-V and I-III, have been split by phosphorothioic acid (3). On the other hand, no change in enzymic activity had been detected when only one crosslink, the disulfide bond IV-V, had been lengthened by the introduction of a mercury atom into the disulfide bond IV-V (2). It may thus be concluded that lengthening the cystine crosslinks I–VI, II–VII, and III–VIII in RNase by about 3 Å each, does not abolish the enzymic activity of the enzyme, although it changes it quantitatively.

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J. Biol. Chem. 1971, 246:715-718.

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