Further Studies on the Alkylation of the Histidine Residues at the Active Site of Pancreatic Ribonuclease*

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SUMMARY

The chemistry of the active site of bovine pancreatic ribonuclease has been further examined by study of the reaction of iodoacetate with histidine residues 12 and 119 in derivatives of the protein.

In the native enzyme, the stereospecific alkylation of the two imidazole rings is a mutually exclusive, electrostatically oriented reaction leading to a 7:1 ratio of substitution at residues 119 and 12. When inactive des-(121-124)-ribonuclease, prepared by limited peptic hydrolysis according to the method of Anfinsen, is exposed to iodoacetate at pH 5.5, alkylation at histidine-119 is nearly abolished but the reaction at histidine-12 is accelerated. If, as has been assumed for the native enzyme, 1 protonated histidine residue orients the reagent for the alkylation of the unprotonated form of the other, this result with the derivative is interpretable in terms of an increase in the pK of the imidazole ring of histidine-119 accompanying the removal of -Asp-Ala-Ser-Val from the carboxyl end of the chain. In turn, this change in pK would alter the precise interaction between the two imidazole rings which are essential for catalytic action; such a shift could account for the inactivity of the des-(121-124)-derivative.

To support this interpretation of the alkylation of des-(121-124)-ribonuclease, further information on the alkylation reaction itself was sought. The influence of neighboring groups on the course of the alkylation of ribonuclease has been studied with two derivatives in which lysine residues have been modified. When the positive charge on lysine-7 is neutralized by acetylation of the S-peptide, by the procedure of Richards and Vithayathil, the resulting active S-peptide (12) has been acetylated by the procedure of Vithayathil and Richards (8, 7) showed that the ε-NH$_2$ group of lysine-41 is essential for enzymatic activity; from studies with additional alkylating agents, Heinrikson (8) estimated that this group is about 7 to 10 Å from the imidazole ring of histidine-12. These postulates as to the spatial relationships between the 3 residues have proved to be consistent with the results of the x-ray diffraction studies on RNase A by Kartha, Bello, and Harker (9) and on RNase S by Wyckoff and Richards and their associates (10).

The present communication is concerned with further chemical experiments directed at residues which knowledge of the chemistry and the geometry of the enzyme places at or near the active center. The COOH-terminal 4 residues in the chain, -Asp-Ala-Ser-Val, have been implicated in the activity of the enzyme by Anfinsen (11), who showed that, when they are removed by limited peptic hydrolysis, the resulting molecule of 120 residues is inactive. This loss of activity could be a result of the removal of residues that participate directly in the catalytic process or it could be a consequence of secondary changes in the structure of the molecule. To provide information on these alternatives, we have studied the effect of the removal of 4 residues on the alkylation of the two specially oriented imidazole rings.

Experiments have been designed to test further the working hypothesis that in the above case, and in the native enzyme, the protonated form of one imidazole ring serves to attract and orient iodoacetate so that it alkylates the unprotonated form of the other imidazole. This subject has been approached by abolishing the positive charge, in turn, on each of the two other basic groups that the three-dimensional model shows to be not far from 2 histidine residues and studying the effect of this change on the alkylation. For this purpose, the ε-NH$_2$ group of Lys-7 in the S-peptide (12) has been acetylated by the procedure of Vithayathil and Richards (13) and then recombinated with the S-protein to give a modified yet active RNase S which can be submitted to alkylation.

Previous studies (1-5) on the alkylation of histidine residues in bovine pancreatic ribonuclease by iodoacetate at pH 5.5 led to the conclusion that the imidazole rings of histidine-12 and histidine-119 are at the active site of the enzyme and are about 5 Å apart. From experiments with fluorodinitrobenzene at pH 8, Hirs, Halmann, and Kycia (6, 7) showed that the ε-NH$_2$ group of lysine-41 is essential for enzymatic activity; from studies with additional alkylating agents, Heinrikson (8) estimated that this group is about 7 to 10 Å from the imidazole ring of histidine-12.

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1 The abbreviations used are: Cm, carboxymethyl; DNP, 2,4-dinitrophenyl.
has also been studied. The results are discussed in terms of the mechanism of the alkylation originally formulated.

**EXPERIMENTAL PROCEDURE**

*Materials—* Bovine pancreatic RNase A (RAF grade) and trypsin (twice crystallized) were obtained from Worthington, pepsin (three times crystallized) from Pentex, and subtilisin BN (Nagarse) from Enzyme Development Corporation, New York, New York. Iodoacetate-1-14C acid and acetic-1-14C anhydride were obtained from New England Nuclear. e-Lys(DNP)-41-RNase (6, 7) was a gift from Dr. C. H. W. Hirs.

**Assay of RNase—** The activity of the enzyme with cyclic cytidylylate (Schwarz Bio-Research) as substrate was determined by the column procedure of Fruhct and Crestfield (14). Activity toward ribonucleic acid (Worthington) was measured by the method of Egami, Takahashi, and Uchida (15).

**Amino Acid Analysis—** Analyses of 22-hour hydrolysates (16) were performed with the equipment of Spackman, Stein, and Moore (17) by an accelerated system (18) on Beckman/Spinco AA-15 and AA-27 resins.

**Preparation of des-(121-124)-RNase—** The procedure of Anfinsen (11) for limited peptic digestion was followed, with the use of sulfoethyl Sephadex instead of IRC-50 for the chromatography and of gel filtration for the desalting steps. To 50 mg of RNase in 5 ml of H2O adjusted to pH 1.8 with HCl, 10 μg of pepsin (in 10 μl of H2O) were added. After 10 min at 37°C, digestion was stopped by the addition of 0.5 ml of 1 m phosphate buffer, pH 6.45. The mixture was applied to a column (0.9 × 60 cm) of sulfoethyl Sephadex C-25 equilibrated with 0.13 m phosphate buffer, pH 6.45, at 4°C (10). The fractionation was followed by taking 50 μl samples from the effluent fractions for alkaline hydrolysis and ninhydrin reaction (14, 20). The tetrapeptide was eluted at 15 ml, RNase A at 35 ml, and des-(121-124)-RNase at 100 ml. The fractions containing the modified protein were pooled and the derivative was desalted on a column (2 × 35 cm) of Sephadex G-25 equilibrated with 5% acetic acid; the yield of lyophilized des-(121-124)-RNase was about 30%. The tetrapeptide was desalted on Sephadex G-10.

**Preparation of S-Peptide and S-Protein—** Proteolysis was performed as described by Richards and Vithayathil (12) and gel filtration (21, 22) was used for separation of the S-peptide. RNase A, 100 mg, was dissolved in 5 ml of 0.1 M KCl, chilled to 0°C and adjusted to pH 8.0. Subtilisin, 200 μg in 50 μl of H2O, was added and the digestion was allowed to proceed at 0°C for 3 hours; the mixture was maintained at pH 8.0 with 0.2 N NaOH in a pH-stat. At the end of the digestion, 5 ml of glacial acetic acid were added and the mixture was applied to a column (2 × 100 cm) of Sephadex G-75 equilibrated with 50% acetic acid. The protein fraction contained about 90% S-protein and 10% RNase A, well separated from the zone containing the S-peptide. Acetic acid was removed from the pooled fractions by lyophilization. The S-peptide was sufficiently pure for the current purpose without the further fractionation described by Doscher and Hirs (22), the present preparation gave an amino acid composition very close to the theoretical and a single detectable zone on high voltage paper electrophoresis at pH 6.5 with pyridine acetate buffer.

**Acetylation of S-Peptide and Preparation of N'-diAc-Lys-Ala-Lys-Ala-Lys-(N'-Ac-Lys)-7-RNase S—** Acetylation was carried out according to the procedure of Vithayathil and Richards (13) with minor changes. The S-peptide (12 mg) was dissolved in 3 ml of 0.2 N acetic acid, chilled to 0°C, and adjusted to pH 6.0. Five 20-μl portions of acetic-1-14C anhydride (specific activity 36 μCi per mmole) were added at 30-min intervals. The reaction mixture was maintained at pH 6.0 with 1 N NaOH in a pH-stat. One hour after the last addition of acetic anhydride, the reaction mixture was lyophilized. The sample of the S-protein, prepared as described above and used for the recombination, contained about 10% RNase A, which was separated in the subsequent chromatographic step; 40 mg of the protein in 3 ml of H2O were added to 12 mg of acetylated S-peptide (in 3 ml of 0.2 N acetic acid) and the mixture was made to 0.1 m in phosphate and brought to pH 6. After 2 hours at room temperature, the solution was applied to a column (2 × 35 cm) of sulfoethyl Sephadex C-25 equilibrated with 0.1 m phosphate buffer, pH 6.54, at 4°C. The order of elution of the fully separated components was triacetyl S-peptide, the acetylated RNase S, and RNase A; the excess S-protein was retained on the column.

The triacetyl RNase S prepared as described contained 29 acetyl groups per molecule of enzyme, based upon 14C content. Thus it is assumed that all three amino groups in the S-peptide, namely the α- and ϵ-amino groups of lysine-I and the ϵ-amino group of lysine-7 were acetylated. There are no other amino acids in the S-peptide known to be acetylated under the conditions used. The triacetyl derivative of RNase S had full activity against cyclic cytidylylate.

**Alkylation of Derivatives of RNase—** The conditions for reaction with iodoacetate acid were similar to those described by Crestfield, Stein, and Moore (3). For the alkylation, 16 μmoles (3.0 mg) of iodoacetate-1-14C acid (specific activity 0.8 C per mole) in 1.4 ml of water, adjusted to pH 5.5 with 0.1 N NaOH, were added to about 2 μmoles (27 mg) of RNase A or its derivatives. The reaction mixture was maintained at pH 5.5 with a pH-stat. After 5 hours at 25°C, excess iodoacetate was removed on a column of Sephadex G-25 equilibrated with 5% acetic acid. The radioactivity was measured with a Nuclear-Chicago liquid scintillation counter, model 720. Scintillation counting was also used in conjunction with the amino acid analyzer (cf. Reference 23); a 1-ml flow cell was used with Nuclear-Chicago Chroma/Cell, model 6351-8713.

**Cleavage by Cyanogen Bromide—** In the application of the procedure of Gross and Witkop (24), CNBr (analytical grade, Eastman, about 160 kmoles) dissolved in 3 ml of 0.2 M HCl was added to 1 μmole (13 mg) of alkylated des-(121-124)-RNase. After the reaction had proceeded for 20 hours at 25°C, 3 volumes of water were added and the mixture was lyophilized. The C-peptide, residues 1 to 13, was separated on a column (0.9 × 160 cm) of Sephadex G-25 equilibrated with 0.2 N acetic acid.

**Tryptic Digestion—** To compare the susceptibility of des-(121-124)-RNase and reduced and carboxymethylated RNase (25) to tryptic action, the proteins were dissolved (0.2φ% solution) in 0.05 m phosphate buffer, pH 7.5. Hydrolysis proceeded at 25°C after the addition of trypsin (1% of protein by weight). Samples (100 μl) were taken from each solution at zero time and at 1 hour for estimation of amino groups by the ninhydrin method (20).

**RESULTS**

**Properties of des-(121-124)-RNase—** Several additional criteria of homogeneity have been applied to the products of the limited peptic digestion of RNase and the results confirm fully the conclusions of Anfinsen (11) in 1956 on the course of the cleavage. Quantitative amino acid analyses of the chromatographically purified protein moiety and the tetrapeptide are given in Table.
I. To test for internal points of hydrolysis, the —S—S— bonds in the modified RNase were oxidized with performic acid under the conditions of Hirs (26) and the product was submitted to gel filtration on Sephadex G-25. The result gave no evidence of any secondary cleavages.

Anfinsen (11) showed that the modified molecule was inactive toward yeast ribonuclease. In the present experiments, we find that the enzymatic activity toward cyclic cytidylate cannot be recovered by the addition at pH 2 of a 500-fold excess of tetrapeptide prior to assay at pH 7.5.

Native RNase is highly resistant to tryptic digestion (27). The susceptibility of des-(121-124)-RNase toward trypsin can serve as an indication of whether the molecule has undergone appreciable conformational change. After 1 hour of exposure to trypsin, the increase in the ninhydrin color was 10% for RNase A and 75% for the molecule in which the —S—S— bonds have been reduced to trypsin, the increase in the ninhydrin color represents roughly the hydrolysis of one to two bonds per molecule. Although the derivative is not as resistant to trypsin as the parent enzyme, the majority of the trypsin-sensitive bonds remain inaccessible.

Alkylations by Iodoacetate—The inactivation of RNase A by iodoacetate has been studied extensively (1-5). At pH 5.5 and 25°, alkylation occurs primarily at the N-terminal of histidine-119 and the 3-nitrogen of histidine-12 with a ratio of about 1:7.1. The current results on the alkylation of RNase A, des-(121-124)-RNase, e-Lys(DNP)-41-RNase, and the triacetyl RNase S by iodoacetate is shown in Table II. The derivatized amino acids were characterized by their positions of elution on the amino acid analyzer. Differentiation of histidine-12 in two of the derivatives from the native RNase were oxidized with performic acid under the conditions of Hirs (26), and the product was submitted to gel filtration on Sephadex G-25. The result gave no evidence of any secondary cleavages.

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**Discussion**

Crestfield et al. (3) suggested, as a working hypothesis to explain the mutually exclusive carboxymethylation of histidine-119 or histidine-12, that the negatively charged iodoacetate ion may be attracted to the positive charge on one imidazole ring and thus be oriented for alkylation of the unprotonated form of the other, and vice versa. This hypothesis is considerably strengthened by experiments which show that the orientation is not supplied by several other positive charges which might be close enough to serve such a function. When the ε-NH₂ group of lysine-41 is dinitrophenylated (Table II) or carboxymethylated (8), the result of the alkylation is essentially unchanged. The x-ray crystallographic studies of RNase S (10) indicate that the next closest positive charges are those on lysine-7 and arginine-39, although both would appear to be sufficiently far away from the histidine residues to have no direct role in the iodoacetate reaction. To obtain chemical evidence on this point, in the present experiments the ε-NH₂ group of lysine-7 has been acetylated (along with the more distant NH₂-groups of lysine-4) and Takahashi (32) has modified arginine-39 with pheylglycine to form a disubstituted guanidino group. Upon reaction of these derivatives with iodoacetate, the ratio of alkylation at histidine-119:histidine-12 is in each case similar to that in the native enzyme.

The working hypothesis for the general course of the alkylation of native RNase by iodoacetate gains support from the recent experiments of Meadows et al. (33), who have been successful in assigning pK values to the individual histidine residues through study of the nuclear magnetic resonance spectra of the enzyme. Their results give pK 5.8 for histidine-119 and pK 6.2 for histidine-12. At pH 5.5 histidine-12 is the more ionized of the two, and would tend to attract the COO⁻ group of iodoacetate, while histidine-119 exists to a greater extent in the unprotonated form and would be the more susceptible of the two to alkylation. The observed ratio of 7:1 for carboxymethylation at histidine-119:histidine-12 (3) is consistent with the pK data and with the mechanism already proposed for the alkylation of the imidazole rings at the active site of RNase.

When the last 4 residues of the chain (121 to 124) are removed by the action of pepsin, however, the situation is changed. Alkylation at histidine-119 is almost completely eliminated; on the other hand, reaction at histidine-12 is more rapid than in the native enzyme. The persistence of a facilitated alkylation reaction at histidine-12 suggests that the 2 histidine residues are still about 5 A apart. The simplest explanation of these findings would be that the removal of the 4 carboxyl-terminal residues has resulted in an increase in the pK of the imidazole ring of histidine-119.³ The effect of increasing the imidazolium form would be to decrease the susceptibility of histidine-119 to alkylation and to increase its tendency to orient iodoacetate for alkylation at histidine-12. In turn, a change in the pK of 1 of the 2 histidine residues would alter the precisely poised interaction between them and could thus account for the enzymatic inactivity of the des(121-124)-derivative.

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**References**
