CHEMISTRY

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1962

SovietRxiv

Abstract

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CHEMISTRY

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STUDY OF THE CATALYTICALLY ACTIVE CENTERS OF LYSOZYME

(Presented by Academician B. A. Kazanskii, 22 I 1962)

According to current concepts of the mechanism of the enzymatic action of esterases and proteases, and also according to data from the study of ribonuclease ([1]), histidine, which is part of the enzyme molecule, is a direct participant in the catalytic act. It seemed highly probable that in lysozyme—an enzyme of purely protein type—the single histidine residue present in its molecule is likewise not indifferent to the manifestation of the enzymatic properties of the protein.

The connection of the histidine present in lysozyme with the enzymatic activity of the latter was noted in Weil' s work ([2]) on the photooxidation of lysozyme. As the protein molecule is oxidized, histidine is destroyed first of all. By the time it is completely destroyed, among the other amino acids only tryptophan is slightly affected, yet the activity of the enzyme falls by 70%. On the other hand, Frenkel-Conrat ([3]) notes that when approximately one residue per molecule of tryptophan is destroyed by ultrasound, the activity of lysozyme is practically not decreased. From this it would seem to follow that histidine must be entirely responsible for the fall in activity. It should be noted that in the cited works the total reaction mixture of proteins was studied without prior isolation of the modified products.

In the present work an attempt was made to clarify—using modern methods for studying proteins—how essential the presence of the imidazole group as such, and consequently also of the histidine residue, is for the performance by lysozyme of its enzymatic function. The active center of lysozyme was studied by selective modification of the enzyme, isolation from the reaction mixture of pure modified protein, and comparative study of the modified and native lysozyme with respect to enzymatic properties and amino-acid composition. The protein was selectively modified by blocking the imino group of histidine with iodoacetic acid. Since it is known that, as a result of the interaction of amino acids with haloacetic acids, compounds are obtained that are stable to prolonged acid hydrolysis ([4]), after complete hydrolysis of the protein it is possible to determine the amino acids blocked in this way and thereby to control the selectivity of the modification.

The work used lysozyme isolated from hen egg white by the method of direct crystallization. After fourfold recrystallization the protein was obtained suffi-

Figure 1: Chromatography of native lysozyme on Amberlite CG-50 resin, column 1 × 33 cm. The elution volume in milliliters is plotted on the abscissa; optical density at 280 mμ is plotted on the ordinate.

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ciently homogeneous, as evidenced by the chromatogram obtained on a column with Amberlite CG-50 resin (Fig. 1). Onto a column measuring 1 × 33 cm, 18 mg of lysozyme was applied. Elution was carried out with 0.2 N sodium phosphate buffer, pH 7.20, at a rate of 3.5 ml/hour, and 1-ml fractions were collected. The fractions were analyzed spectrophotometrically on an SF-4 instrument at $\lambda = 280$ mμ.

In Fig. 1, the peak designated H corresponds to native lysozyme and constitutes 85% of the total protein. The peak designated H_1, emerging from the column somewhat earlier and possessing the same enzymatic activity as peak H, constitutes about 15% of the total amount and represents,

apparently, a protein with a somewhat lower degree of amidation of the free carboxyl groups (6), and, consequently, it cannot be regarded as a foreign impurity. To obtain lysozyme modified at the imidazole ring of histidine, the reaction with iodoacetic acid was carried out at pH 5.5, since under these conditions the hydrogen of the imino group of imidazole is most mobile (7). To an aqueous solution of 40 mg of lysozyme was added an aqueous solution of 40 mg of iodoacetic acid; the pH of the mixture was brought with 0.1 N NaOH to 5.5 and the total volume of the reaction mixture to 10 ml. After heating at 40° for 6 h, the reaction was stopped by one of the two methods described below.

Fig. 1. Chromatography of native lysozyme on Amberlite CG-50 resin, column 1 × 33 cm. On the abscissa is plotted the elution volume in milliliters; on the ordinate, the optical density at 280 mμ.

1. The reaction mixture was passed through a column (0.9 × 10 cm) with carboxymethylcellulose (CMC) in the H^+ form. The protein was sorbed on the CMC, while the iodoacetic acid was removed by washing with water. Desorption of the protein was carried out with 0.2 N sodium acetate-soda solution, pH about 10.5.

As is known, removal of salts from lysozyme solutions is a difficult task. During dialysis the protein readily passes through membranes, and in attempts at desalting on anion-exchange resins it is denatured to a considerable extent. A solution was found in adding strongly cross-linked coarse-grained resin of the KU-2 × 20 type in the H^+ form to the sodium acetate solution of lysozyme. Sodium ions were rapidly bound by the resin, while the protein, as a high-molecular substance, did not penetrate into the resin grains and was practically not sorbed. The desalted protein solution was lyophilically dried in a vacuum

Figure 2: Chromatography of carboxymethylated lysozyme on Amberlite CG-50 resin, column 2.7 × 30 cm. The designations are the same as in Fig. 1.

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Figure 3. Amino acid composition of native lysozyme. Chromatography of the complete hydrolysate on Amberlite CG-120 resin. A —column 0.9 × 150 cm; B —column 0.9 × 20 cm. On the abscissa is plotted the volume of eluate in milliliters; on the ordinate, the optical density of the reaction product with ninhydrin at 570 mμ.

Figure 3: Figure 3. Amino acid composition of native lysozyme. Chromatography of the complete hydrolysate on Amberlite CG-120 resin. A —column 0.9 × 150 cm; B —column 0.9 × 20 cm. On the abscissa is plotted the volume of eluate in milliliters; on the ordinate, the optical density of the reaction product with ninhydrin at 570 mμ.

desiccator over alkali; the protein yield was 95–98%. After this the mixture of proteins was chromatographed on a column under the conditions used for chromatography of native lysozyme.

Fig. 2. Chromatography of carboxymethylated lysozyme on Amberlite CG-50 resin, column 2.7 × 30 cm. The designations are the same as in Fig. 1.

2. The reaction mixture was chromatographed directly on a column with Amberlite CG-50 resin. Because of the large initial volume of solution, this method was used only when working on preparative columns. Simultaneously with removal of the iodoacetic acid, chromatographic separation of the reaction products also took place. A typical chromatogram of the separation of the products of lysozyme carboxymethylation under the indicated conditions is shown in Fig. 2. Peak H corresponds to native lysozyme, and peaks I, II, III, IV to the modification products.

Fig. 3. Amino acid composition of native lysozyme. Chromatography of the complete hydrolysate on Amberlite CG-120 resin. **A** —column 0.9 × 150 cm; **B** —column 0.9 × 20 cm. On the abscissa is plotted the volume of eluate in milliliters; on the ordinate, the optical density of the reaction product with ninhydrin at 570 mμ.

Figure 4. Amino acid composition of carboxymethylated lysozyme. Method of analysis and designations as indicated in Fig. 3.

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Upon carboxymethylation the protein becomes more acidic. The lower the degree of carboxymethylation, the closer the acidity of the modified protein is to that of the native protein. In chromatography, however, the more acidic the protein, the more rapidly it is eluted. Consequently, peak *I* among the other modified proteins (*II*, *III*, *IV*) corresponded to the lowest degree of carboxymethylation and therefore was of the greatest interest for detailed study. This peak, as is seen from the curve, because of the optimality of the carboxymethylation conditions, also contained the bulk of the modified protein. A test of enzymatic activity, carried out nephelometrically with *Micrococcus lysodeikticus*, showed that the activity of the modified lysozyme corresponding to peak *I* not only did not disappear or decrease, but even increased somewhat, amounting to 110–112% as compared with the native protein.

Information on the degree and character of carboxymethylation of lysozyme was provided by a study of its amino-acid composition in comparison with the native protein. The analysis was carried out chromatographically by the method of Moore and Stein^(5) on the ion-exchange resin Amberlite CG-120. For analysis, the protein was hydrolyzed in evacuated ampoules with 6 *N* HCl (a 200-fold excess of acid) at 105–110° for 24 h. The results of the amino-acid analyses for native lysozyme are given in the curve in Fig. 3, and for the modified protein (peak *I*) in the curve in Fig. 4.

As is seen in Fig. 4, in the modified protein the histidine peak is completely absent, but an additional peak has appeared in the cystine region, which according to literature data^(8) corresponds to the position of 3-(N-carboxymethyl)histidine. No other changes occurred in the amino-acid composition of lysozyme; i.e., carboxymethylation proceeded selectively only at the imidazole of histidine.

It is interesting to note that when a similar reaction is carried out with ribonuclease under the same conditions, 40 min are required for modification of half of the protein at histidine, and the carboxymethylated ribonuclease proves to be completely inactive. In the case of lysozyme, however, 6 h were required for the same degree of modification, which indicates the considerably greater inertness of histidine in the lysozyme molecule. The absence of any decrease in the enzymatic activity of lysozyme carboxymethylated at histidine, as well as the relative inertness of histidine in lysozyme, make it possible to state with a high degree of probability that histidine is not a constituent of the catalytically active center of lysozyme.

The data obtained also make it possible, with some justification, to suggest that the mechanism of action of β-glucosidases, to which lysozyme belongs, differs substantially from the mechanism of action of ribonuclease and of enzymes of the protease and esterase type.

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Received
20 I 1962

REFERENCES CITED

1. H. G. Gundlach, W. H. Stein, S. Moore, *J. Biol. Chem.*, **234**, 1754 (1959).
2. L. Weil, A. R. Buchert, J. Maher, *Arch. Biochem. and Biophys.*, **40**, 245 (1952).
3. H. Fraenkel-Conrat, *Arch. Biochem. and Biophys.*, **27**, 109 (1950).
4. S. Korman, H. T. Clarke, *J. Biol. Chem.*, **221**, 113 (1956).
5. S. Moore, W. H. Stein, *J. Biol. Chem.*, **211**, 893 (1954).
6. H. N. Tallan, W. H. Stein, *J. Biol. Chem.*, **200**, 507 (1953).
7. V. V. Mosolov, *Advances in Contemporary Biology*, **50**, 2773 (1960).
8. V. H. Stein, S. Moore, V International Biochemical Congress, IV Symposium, No. 1, Moscow, 1961.

Note: Figure translations are in progress. See original paper for figures.