Identification of Aspartic Acid 52 as the Point of Attachment of an Affinity Label in Hen Egg White Lysozyme*

(Received for publication, April 11, 1973)

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SUMMARY

Hen egg white lysozyme, fully inactivated by the affinity-labeling reagent, the 2',3'-epoxypropyl β-glycoside of di-(N-acetyl-D-glucosamine), contained 1 mole of the label per mole of protein. Total enzymatic digestion of the reduced and carboxymethylated affinity-labeled enzyme afforded, as a major radioactive product, a compound composed solely of aspartic acid and glucosamine at the molar ratio of 1:2. Peptic digestion afforded a single radioactive peptide corresponding to the sequence Asn-Thr-Gln-Ala-Thr-Asn-Arg-Asn-Thr-Gly-Ser-Thr-Asp-Tyr in the enzyme. Digestion of this peptide with Clostridium histolyticum aminopeptidase gave a radioactive pentapeptide the composition, partial structure, and other properties of which corresponded to the sequence Gly-Ser-Thr-Asp-Tyr in hen egg white lysozyme, and which contained 2 moles of glucosamine per mole of peptide. Our findings show that the affinity label is covalently bound to the enzyme via the β-carboxyl group of Asp 52 and are in accord with our conclusions from immunochemical (MARON, E., ESHDAT, Y., AND SHARON, N. (1972) Biochim. Biophys. Acta 278, 243) and x-ray crystallographic (MOULT, J., ESHDAT, Y., AND SHARON, N. (1973) J. Mol. Biol. 75, 1) studies of the affinity-labeled hen egg white lysozyme.

EXPERIMENTAL PROCEDURE

Materials

Hen egg white lysozyme (salt-free), pepsin (2500 units per mg), L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (200 units per mg), and diisopropyl phosphofluoridate-treated carboxypeptidase A (42 units per mg) were purchased from Worthington Biochemical Corp. Aminopeptidase M was obtained from Rohm and Haas, Darmstadt, Germany. Aminopeptidase of Clostridium histolyticum (11) was kindly supplied by Miss Efrat Kessler and Dr. A. Yaron from the Department of Biophysics, The Weizmann Institute of Science. Subtilisin Carlsberg was obtained from Novo Industries, Copenhagen. Other chemicals, purchased from commercial sources, were of the highest purity available and were used without further purification.

Methods

Enzymatic Assay

Activities of hen egg white lysozyme and of the affinity-labeled enzyme were measured, essentially as described by Shugar (12), in a Beckman DB-G recording spectrophotometer equipped with a cell holder thermostated at 26°C. Reaction...
mixtures consisted of 1 ml of 0.1 M ammonium acetate (pH 6.7) containing 225 µg of suspended cells of Micrococcus lysodeikticus and 3 to 6 µg of the native enzyme or higher amounts (up to 20 µg) of the inactivated enzyme. Absorbance decrease at 700 nm was measured following the addition of the enzyme. Protein concentration was determined spectrophotometrically at 280 nm with an extinction coefficient \( E_{280} = 26.5 \) (13).

Radioactive Counting

Samples (5 to 100 µl of the solution to be tested) were added to vials containing 15 ml of Bray's dioxane scintillation solution (14) and radioactivity was measured with a Packard Tri-Carb scintillation counter. The counting efficiency for \(^3H\) was 56%, and for \(^14C\), 90%.

High Voltage Paper Electrophoresis

High voltage electrophoresis was carried out on Whatman No. 1 or No. 3 paper, at a constant voltage of 50 volts per cm. The buffers and times used were: pH 1.9, formic acid-acetic acid-water (1:4:45, v/v; 3 hours); pH 3.5, pyridine-acetic acid-water (1:10:189, v/v; 60 min); pH 6.5, pyridine acetic acid water (25:1:225, v/v; 45 min). In most cases, a guide strip 0.5 cm wide was cut from the edge of the paper after electrophoresis and stained with the ninhydrin-cadmium reagent (15). Radioactive materials on the paper were located with a Packard radiochromatogram scanner.

Elution of Peptides from Paper

Located areas were cut out and eluted with water or 0.05 M acetic acid according to the method of Eshdat and Mirelman (16). Alternatively, the section of the paper containing the sample was sewn to another Whatman paper of the same number for the next stage of purification.

Gel Filtration on Sephadex G-25

The affinity-labeled enzyme was isolated on a column of Sephadex G-25 (fine), equilibrated and eluted with 0.1 M ammonium acetate; 2-ml fractions were collected. The optical density of the eluates was continuously monitored at 280 or 220 nm with the aid of a 1-ml flow cell inserted in a Beckman DB-G spectrophotometer between the column and the fraction collector. Radioactivity of aliquots from the fractions was determined as described above.

Gel filtration on Sephadex G-25 (fine) was also employed for the isolation and separation of peptides. In these cases, 0.05 M acetic acid was used for equilibration of the column and for elution, and 1-ml fractions were collected. Radioactivity and absorbance at 220 nm (or at 230 nm) for location of peptides were measured as described above.

Amino Acid and Glucosamine Analyses

Samples were prepared for analysis by hydrolysis in 6 M HCl for 22 hours at 110° in evacuated tubes. After drying of the hydrolysate under reduced pressure in a desiccator, the amino acid composition was determined on an automated amino acid analyzer (17) with loads of 5 to 100 nmoles per amino acid. For the determination of glucosamine on the long column, the buffer change time was set at 75 min instead of 85 min. In some cases, the glucosamine was analyzed on a medium-sized column.

The integration constant for glucosamine (75% of that of aspartic acid) was determined by subjecting N-acetyl-D-glucosamine, in mixture with the amino acids of lysozyme, to the standard conditions of hydrolysis and then to analysis on the amino acid analyzer.

For the determination of glutamine plus asparagine present in enzymatic digests, analysis was performed on the long column at 37° instead of 55°. This procedure permitted separation of the two amines from serine; no such separation could be achieved under the standard conditions at 55°.

End Group Analysis

NH₂-terminal residues of the purified peptides were determined by the 5-dimethylaminonaphthalene-1-sulfonyl chloride method (18). For determination of COOH-terminal residues, dispropyl phosphofluoridate-treated carboxypeptidase A in suspension was washed and dissolved according to the method of Ambler (19). Digestion of peptides was carried out at 37° in 0.1 M NH₄HCO₃ (pH 7.8). A control mixture without the peptides was also prepared. The incubation mixtures were analyzed for free amino acids, either on the amino acid analyzer or by paper electrophoresis at pH 1.9.

Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed in β-alanine-acetic acid at pH 4.5 (20).

Synthesis of Affinity-labeling Reagent

Radioactive 2',3'-epoxypropyl β-glycoside of (GlcNAc)₂ labeled with either \(^14C\) or \(^3H\) at the acetamido group in the sugar residue nearest to the aglycon, was synthesized, according to the method of Thomas (21), from the corresponding labeled octaacetylchitobiose. The label was introduced into the latter compound as described by Horton et al. (22) for the preparation of the analogous deuterated compound 1,3,4,5-tetra-O-acetyl-2-deoxy-2-trideuterio-acetamido-a-d-glucopyranose. The specific activity of the \(^3H\)-labeled (GlcNAc)₂*-Ep was 1.1 \( \times 10^6 \) cpm per mmole and that of the \(^14C\)-labeled (GlcNAc)₂*-Ep was 1.4 \( \times 10^6 \) cpm per mmole.

Preparation of Affinity-labeled Hen Egg White Lysozyme

In a typical experiment, the enzyme (3 ml of 1.25 \( \times 10^{-4} \) M) was incubated with \(^{14C}\)-labeled (GlcNAc)₂*-Ep (1.0 \( \times 10^{-4} \) M) at 37° in water at pH 5.5. At various times, 2-µl aliquots were taken from the incubation mixture for the determination of enzymatic activity. After 24 hours, the affinity-labeled enzyme, (GlcNAc)₂*-Pr-lysozyme, was separated from excess labeling reagent by dialysis against water and isolated by gel filtration on a column (1.5 × 80 cm) of Sephadex G-25 (fine). The fractions containing the radioactive protein were combined, dialyzed against water, and lyophilized to yield (GlcNAc)₂*-Pr-lysozyme.

Reduction and Carboxymethylation of (GlcNAc)₂*-Pr-lysozyme

(GlcNAc)₂*-Pr-lysozyme (90 mg) was dissolved in 1 M Tris buffer at pH 8.0 (10 ml), and 5.76 g of guanidine hydrochloride...
The peptic digest of RCM-(GlcNAc)2*-Pr-lysozyme was lyophilized, dissolved in water, and centrifuged as described above. The supernatant was applied to a column (2 x 145 cm) of Sephadex G-25 (fine) with 0.05 M acetic acid as eluent. Fractions were monitored at 240 nm, and samples were withdrawn for ninhydrin-cadmium reagent. The radioactive fractions were collected and lyophilized. The lyophilized material was applied to a strip of Whatman No. 3 paper and subjected to electrophoresis at pH 6.5. After separation, the paper was dried and put into a desiccator containing three beakers containing, respectively, water, triethylamine, and saturated solution of triethylamine in water. After 7 hours at 37°C, the paper strip was dried at room temperature, sewn onto a fresh sheet of Whatman No. 3 paper, and subjected again to electrophoresis at pH 6.5, perpendicularly to the original direction. The paper was then dried and stained with the ninhydrin-cadmium reagent.

**RESULTS**

Purification and Characterization of Affinity-labeled Enzyme—

Incubation of lysozyme with (GlcNAc)2*-Ep resulted in exponential inactivation of the enzyme with time (Fig. 2). After 24 hours, the specific enzymatic activity of the inactivated lysozyme was less than 2% of that of native lysozyme. Dialysis of the incubation mixture resulted in removal of most of the unreacted affinity-labeling reagent. However, another purification step was required in order to separate completely the affinity-labeled enzyme from the reagent. Results of gel filtration of one preparation of the dialyzed 3C-labeled (GlcNAc)2*-Pr-lysozyme are presented in Fig. 3. Only one radioactive peak, which also absorbed at 280 nm, was obtained. This peak exhibited a constant specific radioactivity, corresponding to 1 mole of (GlcNAc)2*-Ep per mole of enzyme. The protein peak was well separated from the second radioactive peak containing (GlcNAc)2*-Ep. The fractions containing the protein were combined, dialyzed against water, and lyophilized. The product obtained was homogeneous by polyacrylamide gel electrophoresis at pH 4.5, and its enzymatic activity remained about 2% of that of the native enzyme. Amino acid analysis of the inactivated enzyme revealed, in addition to the expected composition, the presence of glucosamine in a ratio of 1.9 moles per mole of enzyme.

Reduction and Carboxymethylation—The radioactivity of (GlcNAc)2*-Pr-lysozyme was quantitatively recovered as the

**Diagonal Electrophoresis of Radioactive Peptic Peptides**

The peptic digest of RCM-(GlcNAc)2*-Pr-lysozyme was lyophilized, dissolved in water, and centrifuged as described above. The supernatant was applied to a column (2 x 145 cm) of Sephadex G-25 (fine) with 0.05 M acetic acid as eluant. Fractions were monitored at 240 nm, and samples were withdrawn for ninhydrin-cadmium reagent. The radioactive fractions were collected and lyophilized. The lyophilized material was applied to a strip of Whatman No. 3 paper and subjected to electrophoresis at pH 6.5. After separation, the paper was dried and put into a desiccator containing three beakers containing, respectively, water, triethylamine, and saturated solution of triethylamine in water. After 7 hours at 37°C, the paper strip was dried at room temperature, sewn onto a fresh sheet of Whatman No. 3 paper, and subjected again to electrophoresis at pH 6.5, perpendicularly to the original direction. The paper was then dried and stained with the ninhydrin-cadmium reagent.
Fraction number

FIG. 3. Separation of ¹⁴C-labeled (GlcNAc)₂-Pr-lysozyme from an excess of (GlcNAc)₄*-Ep by gel filtration on a column (1.5 X 80 cm) of Sephadex G-25 (fine) using 0.1 M ammonium acetate as eluant. Fractions of 2 ml were collected. Absorption at 280 nm and radioactivity were measured as outlined under “Experimental Procedure.”

TABLE I
Amino acid composition of reduced and carboxymethylated affinity-labeled hen egg white lysozyme

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Residues per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found</td>
</tr>
<tr>
<td>S-Carboxymethylcysteine</td>
<td>7.9</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>20.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.8</td>
</tr>
<tr>
<td>Serine</td>
<td>9.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>5.4</td>
</tr>
<tr>
<td>Proline</td>
<td>2.3</td>
</tr>
<tr>
<td>Glycine</td>
<td>12.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.3</td>
</tr>
<tr>
<td>Half cystine</td>
<td>0</td>
</tr>
<tr>
<td>Valine</td>
<td>5.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.0</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>2</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>11.0</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* From Canfield (Ref. 23).

¹ The tryptophan residues were destroyed completely by acid hydrolysis.
² Calculated from affinity label (GlcNAc)₂-Ep

Isolation of Labeled Aspartic Acid after Total Enzymatic Hydrolysis of RCM (GlcNAc)₂*-Pr-lysozyme—¹H Labeled RCM (GlcNAc)₂-Pr-lysozyme (5.35 mg, 40,600 cpm) was subjected to total enzymatic digestion. After 26-hour incubation, the solution was subjected to high voltage electrophoresis at pH 1.9. On staining a guide strip with the ninhydrin-cadmium reagent, many spots were observed, although on strip scanning only two radioactive peaks were detected (Fig. 4). The major radioactive spot was negatively charged and corresponded to a ninhydrin-positive spot, whereas the second radioactive spot was neutral, ninhydrin-negative, and migrated at the same rate as (GlcNAc)₂-Ep. The radioactive materials were eluted from the paper with water, and the eluates were lyophilized. Each of the two materials was dissolved in 0.5 ml of water. Radioactive counting showed that 97% of the radioactivity applied to the paper was eluted, of which 93% is located in the ninhydrin-positive material, and only 7% in the ninhydrin-negative spot.

Gel filtration of the major radioactive products on a column of Sephadex G-25 (fine) gave a single radioactive peak in very high yield, which emerged close to the elution volume of (GlcNAc)₂-Ep. Amino acid analysis of the acid hydrolysate of this material revealed the presence of aspartic acid and glucosamine, in a molar ratio of 1.0:2.1. Also, the specific activity of the product material, based on the results of its amino acid analysis, was 1.05 x 10⁸ cpm per mole (95% of the specific activity of the ³H-labeled (GlcNAc)₂-Ep employed for the labeling of the enzyme used in this experiment).

Isolation and Characterization of Labeled Peptic Peptide (P1)—The electrophoretic pattern at pH 3.5 of the peptides derived from a peptic digest of [¹⁴C]RCM-(GlcNAc)₂-Pr-lysozyme is shown in Fig. 5. Three radioactive peaks were observed, one of them containing about 80% of the total radioactivity. The material under this peak was eluted from the paper, lyophilized, dissolved in 1 ml of 0.05 M acetic acid, and chromatographed on a column (0.9 X 187 cm) of Sephadex G-25 (fine) with 0.05 M acetic acid as eluant (Fig. 6). Only one radioactive peak absorbing at 230 nm was observed. The fractions (70 to 78) comprising this peak were combined and freeze-dried.

Fig. 4. Radioactivity scan of the high voltage electrophoretic pattern of a total enzymatic digest of ³H-labeled RCM-(GlcNAc)₂-Pr-lysozyme. The arrow points to the position of the migration of lysine. For experimental details, see text.

Fig. 5. Electrophoretic pattern of peptides derived from a peptic digest of ¹⁴C-labeled RCM-(GlcNAc)₂-Pr-lysozyme. Visualization of peptides and radioactivity measurements, as well as the conditions used for electrophoresis, are described under “Experimental Procedure.”
The labeled peptic peptide (PI) migrated as a single spot on electrophoresis at pH 1.9 and 6.5. Its amino acid composition was Asp_{2}, Thr_{2}, Ser_{1}, Glu_{1}, Ala_{1}, Tyr_{1}, Arg_{1}, GlcN_{1}. Digestion of PI with carboxypeptidase A released only tyrosine, even after 48-hour incubation, as shown by paper electrophoretic analysis of the digest. In an additional experiment, 100 nmoles of PI were digested with carboxypeptidase A for 3 hours, and the free tyrosine was separated from the residual radioactive peptide by paper electrophoresis at pH 1.9. The peptide was eluted from the paper with 0.1 M acetic acid and found to have the composition Asp_{0}, Thr_{1}, Ser_{1}, Glu_{1}, Ala_{1}, Arg_{1}, GlcN_{1}. With the 5-dimethylaminonaphthalene-1-sulfonyl chloride method, the NH_{2}-terminal amino acid was found to be aspartic acid (or asparagine).

Diagonal electrophoresis of the peptic digest of RCM-(GlcSAc)_{2}*-Pr-lysozyme showed that, in the area of the origin of the electrophoretogram, most of the ninhydrin-positive material lay, as expected, on a diagonal line (Fig. 7). Only one major spot was off this line, in a position corresponding to that of the labeled peptic peptide (PI) in the original direction. This spot was on the anode side of the diagonal, whereas the radioactivity was located on the diagonal in the area of the peptides which are neutral at pH 6.5.

Tryptic Digestion of Labeled Peptic Peptide—From the tryptic digest of the purified labeled peptic peptide, a negatively charged radioactive peptide was isolated by electrophoresis at pH 6.5 (Fig. 8). It was further purified by gel filtration on a column (0.9 x 187 cm) of Sephadex G-25 (fine) with 0.05 M acetic acid as eluent. The fractions containing the only radioactive peptide peak (absorbance at 220 and 280 nm) were collected and lyophilized. The amino acid composition of the purified labeled tryptic peptide was found to be Asp_{1}, Thr_{1}, Ser_{1}, Glu_{1}, Tyr_{1}, GlcN_{1}.

Isolation and Characterization of Labeled Peptide (P2) Obtained by Digestion of P1 with Aminopeptidase—Amino acid analysis of the radioactive peptic peptide (P1) after it had been digested by the aminopeptidase of C. histolyticum revealed the presence of the following free amino acids (the amounts are expressed relative to aspartic acid = 1.0): aspartic acid_{1.0}, threonine_{2.0}, asparagine + glutamine_{2.0}, alanine_{1.0}, and arginine_{1.0}. Gel filtration of the bulk of the digest on a column (0.9 x 214 cm) of Sephadex G-25 (fine), with 0.05 M acetic acid as eluant, gave only one radioactive peak with absorbance at both 220 and 280 nm. The labeled peptide located under this peak (P2) was homogeneous on paper electrophoresis at pH 6.5 with a specific activity of 1.1 x 10^6 cpm per mmole, and had the composition Asp_{1}, Thr_{1}, Ser_{1}, Glu_{1}, Tyr_{1}, GlcN_{1}. Carboxypeptidase A digestion of 20 nmoles of P2 for 30 min released tyrosine (17 nmoles) as the sole amino acid. Glycine was found to be NH_{2}-terminal by the 5-dimethylaminonaphthalene-1-sulfonyl chloride method.

In order to obtain additional evidence on the nature of the chemical bond between the affinity label and the peptide, 10 nmoles of P2 were applied to each of two Whatman No. 1 paper strips (S1 and S2), and 20 nmoles were applied to a third strip (S3). S2 and S3 were treated with triethylamine in the same way as was done in the analysis of the peptic digest by the diagonal electrophoresis. The three paper strips were subjected to high voltage electrophoresis at pH 6.5. Two of the strips (S1 and S2) were then stained with the ninhydrin cadmium reagent. On S1, not treated with triethylamine, a ninhydrin-positive radioactive spot (C1) was located in the area of neutral peptides at pH 6.5 (Fig. 9). On S2, which was treated with triethylamine, a ninhydrin-positive, nonradioactive spot (C2) was observed on the anode region, whereas the radioactivity was...
located in the area of neutral materials (C3). The areas corresponding to C2 and C3 on S3 were cut out and eluted with water. Amino acid analysis of an acid hydrolysate of C2 gave the composition Asp, Thr, Ser, Gly, Tyr. The radioactive material eluted from C3 migrated on Silica Gel G plates (acetone-methanol, 2:1) as the 2,3-propanediol α-glycoside of (GlcNAc)₂ (obtained by treatment of (GlcNAc)₂-Ep with 0.1 M HCl at 90° for 40 min). Its analysis on an amino acid analyzer after acid hydrolysis revealed the presence of glucosamine only.

**DISCUSSION**

Hen egg white lysozyme, irreversibly inactivated by the affinity label (GlcNAc)₂-Ep, was homogeneous by gel filtration on Sephadex G-25 and gel electrophoresis at pH 4.5 and had virtually no enzymatic activity when assayed with M. lysodeikticus cells. Preliminary experiments have shown that the affinity-labeled enzyme does not digest low molecular substrates of lysozyme. This inactivated enzyme was subjected to a variety of chemical and enzymatic reactions in order to identify the site of attachment of the affinity label to the protein.

To digest the affinity-labeled lysozyme with proteolytic enzymes, it was necessary to cleave irreversibly its S-S bridges. The reduced carboxymethylated protein was found to contain 8 carboxymethylcysteines per molecule, and it preserved all of the radioactivity and GlcNAc incorporated into the enzyme in the inactivation process. Total enzymatic hydrolysis of RCM-(GlcNAc)₂-Pr-lysozyme with subtilisin and aminopeptidase M afforded one major radioactive product (93% of the total radioactivity) composed solely of glucosamine and aspartic acid. The specific activity of the product was identical with that of the affinity-labeling reagent employed in this experiment. A second minor radioactive product was also obtained; its acid hydrolysate contained only glucosamine. This glucosamine is most probably derived from the 2,3-propanediol β-glycoside of (GlcNAc)₂ which had been released from the affinity-labeled enzyme during the proteolytic digestion. These findings show that the affinity label is bound solely to aspartic acid in the inactivated enzyme.

In order to identify to which of the eight aspartic acid side chains present in lysozyme the affinity label is bound, RCM-(GlcNAc)₂-Pr-lysozyme was subjected to peptic digestion. A labeled peptic peptide (P1) was isolated in high yield (about 80%). Amino acid analysis and end group analysis suggested that this peptide corresponds to that located between Asn 39 and Tyr 53 in lysozyme (23). This conclusion was substantiated by the finding that P1, with a single arginine residue (Arg 45), was cleaved by trypsin to give two peptides. Both the radioactivity and the glucosamine were associated with the peptide which had a composition corresponding to that of residues 46 to 53 (Table II).

Diagonal electrophoresis of the peptic digest provided evidence concerning the nature of the chemical bond formed between the enzyme and the affinity label. Treatment of the labeled peptic peptide with triethylamine resulted in loss of radioactivity and conversion of the peptide to a negatively charged one at pH 6.5. This implies that (GlcNAc)₂-Ep is bound to the labeled peptide via an ester bond.

Since the labeled peptic and tryptic peptides contain only 2

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**Fig. 9.** Electrophoresis pattern of the radioactive peptide, P2, before (S1) and after (S8) treatment with triethylamine. Visualization of the peptides and radioactivity measurements, as well as the conditions used for electrophoresis at pH 6.5, are described under "Experimental Procedure."

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**Table II**

Labeled peptides derived from proteolytic digests of RCM-(GlcNAc)₂-Pr-lysozyme

<table>
<thead>
<tr>
<th>Peptide*</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>P1</td>
<td>H₂N-Asn-Thr-Gln-Ala-Thr-Asn-Arg-Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-COOH</td>
</tr>
<tr>
<td>CP1</td>
<td>H₂N-Asn-Thr-Gln-Ala-Thr-Asn-Arg-Asn-Thr-Asp-Gly-Ser-Thr-Asp-COOH</td>
</tr>
<tr>
<td>T1</td>
<td>H₂N-Asn-Thr-Asp-Gly-Ser-Thr-Asp-Tyr-COOH</td>
</tr>
<tr>
<td>P2</td>
<td>H₂N-Gly-Ser-Thr-Asp-Tyr-COOH</td>
</tr>
</tbody>
</table>

* P1, labeled peptide derived from peptic digestion of RCM-(GlcNAc)₂-Pr-lysozyme; CP1, labeled peptide derived from carboxypeptidase A digestion of P1; T1, labeled peptide derived from tryptic digestion of P1; P2, labeled peptide derived from Clostridium histolyticum aminopeptidase digestion of P1.
aspartic acid residues, it could be concluded at this stage that the affinity label is bound to lysozyme through the \(\beta\)-carboxyl group of either Asp 48 or Asp 52.

The fact that carboxypeptidase A cleaves only tyrosine from the peptide peptide even after 48-hour digestion suggested that the identification of the aspartic acid to which the \((\text{GlcNAc})_2\)*-Ep is linked could be carried out only by degradation of the labeled peptide from its amino end. Preliminary experiments, including digestion of the labeled peptide by pronase and by aminopeptidase M, led to the isolation of labeled peptides, the smallest of which corresponded to the peptide located between Asp 48 and Asp 52. However, only digestion of the peptide peptide by the aminopeptidase of \(C.\ histrionicum\) enabled us to identify unequivocally the site of attachment. Analysis of the amino acids liberated from the peptide peptide by digestion with this aminopeptidase identified the aspartic acid residues, it could be concluded at this stage that the affinity label is bound to lysozyme through the \(\beta\)-carboxyl group of either Asp 48 or Asp 52.

The methods employed in this work can be used in mapping and sequence studies of the active site region of lysozymes, derived from other organisms, which are also irreversibly inhibited by the 
\(\text{epoxy glycosides}\) (9).

Our findings clearly show that Asp 52 forms part of the active site of lysozyme. However, they do not give evidence on the proposed catalytic role of this residue (5). Such evidence might be obtained from studies which include chemical reactions with the \(\beta\)-aspartyl ester, or the glycosidic bond between \((\text{GlcNAc})_2\) and the aglycon, which are unique in the affinity-labeled lysozyme.

Acknowledgments—We thank Dr. J. Hildesheim for his assistance in the preparation of the radioactive affinity label. Thanks are also due to Miss Efrat Kessler and Dr. A. Yaron for the aminopeptidase of \(C.\ histrionicum\).

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