Modification of Catalytic Groups in Lysozyme with Ethylenimine†

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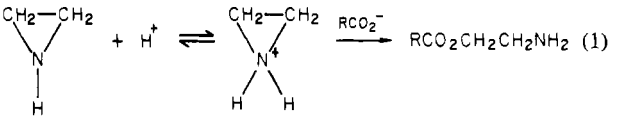
ABSTRACT: The reaction of lysozyme with ethylenimine has been studied at neutral and acidic pH. At least four singly modified lysozyme derivatives have been isolated. From the product analyses, only carboxyl groups in the protein were found to react. One of the derivatives, **1**, was very labile and reverted back to native lysozyme during the isolation procedure. Its formation was markedly inhibited by the presence of tri(*N*-acetyl-D-glucosamine), indicating that the modified carboxyl residue in **1** is at or close to the binding site of lysozyme for the trisaccharide. Two other derivatives were identified as 2-aminoethyl esters of Glu-35 (**2**) and Asp-52 (**3**). At pH 10 and room temperature, these derivatives rearranged to the corresponding 2-hydroxyethylamide derivatives (**5** and **6**). A fourth derivative (**4**) did not contain an ethanolamine moiety; nevertheless, its Glu-35 was found to be modified by sequence analysis. Lysozyme derivatives modified at Glu-35 and Asp-52 were inactive toward glycol chitin but retained high affinity for tri(*N*-acetyl-D-glucosamine). Thus, Glu-35 and Asp-52 are essential for enzyme activity. A mechanism for the selective modification of these carboxyl residues in lysozyme has been proposed and related to the metal binding ability of lysozyme.

Hen egg-white lysozyme is a carbohydrate hydrolase with an acidic pH optimum for certain substrates and is one of the best characterized proteins (Imoto et al., 1972). As a result of X-ray crystallographic studies, three of the ten carboxyl groups (Glu-35, Asp-52, and Asp-101) are found to be located in the active site cleft (Blake et al., 1965, 1967a,b; Phillips, 1967). Based on details of a lysozyme–tri(*N*-acetyl-D-glucosamine) $[(NAG)_3]$[1] complex in the crystalline state and a result of further model building, it has been suggested that two (Glu-35 and Asp-52) of these carboxyls participate in catalysis and a third (Asp-101) is involved in a substrate binding interaction (Blake et al., 1967b; Phillips, 1967). So that more direct proof for these interactions could be obtained, some chemical modifications of carboxyl groups have been developed. Derivatives singly modified at Asp-52 have been prepared by utilizing triethyloxonium fluoroborate (Parsons et al., 1969; Parsons & Raftery, 1969) or the 2,3-epoxypropyl β-glycoside of di(*N*-acetyl-D-glucosamine) (Eshdat et al., 1973, 1974). These derivatives were found to be enzymatically inactive. For Asp-101, we recently developed a method of selective modification of this residue by the carbodiimide–amine nucleophile reaction (Yamada et al., 1981). Derivatives obtained possess reduced binding ability to $(NAG)_3$ by a factor of 10–40 (H. Yamada and T. Imoto, unpublished results) while they are still active against glycol chitin (Yamada et al., 1981). Glu-35 in lysozyme has not yet been selectively modified. Although Imoto et al. (1973) prepared an enzymatically inactive Glu-35–Trp-108 ester derivative in which Glu-35 forms an ester bond with oxindolylalanine-108, Trp-108 is also modified in this derivative. Therefore, it was important to discover a reagent that modified only Glu-35 in lysozyme. In this regard we have tested the reaction of lysozyme with ethylenimine[2] and found it to be this type of reagent.

It is well-known that various metal cations including Mn^{2+} (Gallo et al., 1971; Ikeda & Hamaguchi, 1973), Co^{2+} (McDonald & Phillips, 1969), Ni^{2+} (Ikeda & Hamaguchi, 1973), Cu^{2+} (Teichberg et al., 1974), Gd^{3+} (Secemski & Lienhard, 1974), and Ca^{2+} (Imoto et al., 1981) bind to lysozyme at either Glu-35 or Asp-52 (Kurachi et al., 1975; Perkins et al., 1979). These results suggest that a small reagent having a positive charge might have an affinity to the metal binding sites. Ethylenimine has a pK_a of 8.01 (O'Rourke et al., 1956) and a positive charge by protonation in neutral or acidic solution. Moreover, nucleophilic attack of a carboxylate anion to the protonated ethylenimine occurs to give an aminoethyl ester as shown in eq 1 (Earley et al., 1958).



Experimental Procedures

Materials. Five times recrystallized hen egg-white lysozyme was donated from Eisai Co. (Tokyo, Japan). Ethylenimine[2] was prepared according to the method of Reeves et al. (1951). Bio-Rex 70 (100–200 mesh) and Sephadex G-25 (medium) were obtained from Bio-Rad and Pharmacia, respectively. Glycol chitin, a substrate of lysozyme, was prepared as described elsewhere (Yamada & Imoto, 1981).

Analytical Methods. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer after hydrolysis of protein or peptide samples in 6 N HCl under vacuum, at 110 °C for 20 h. Chromatography of lysozyme and its derivatives was performed on columns of the carboxylic acid cation exchanger Bio-Rex 70 at pH 7 or 10. Protein elution was monitored by absorbance of effluents at 280 nm with a Hitachi 200-10 double-beam spectrophotometer. Hydrolyses of reduced and S-carboxymethylated lysozyme and its derivatives with TPCK[1]-treated trypsin (Worthington) and separations of tryptic peptides on DEAE[1]-cellulose (Whatman, DE-32) ion-exchange chromatography or on high-performance reversed-phase liquid chromatography (LiChrosorb RP-8, 5 μm, Merck) were accomplished as described previously (Yamada et al., 1981). Peptide elution was monitored with a Hitachi

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[1] Abbreviations: $(NAG)_3$, tri(*N*-acetyl-D-glucosamine); PTH, phenylthiohydantoin; DEAE, diethylaminoethyl; TPCK, L-1-(*p*-tosylamido)-2-phenylethyl chloromethyl ketone; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance.

[2] Ethylenimine, also called azirane, is an extremely toxic chemical that must be handled in a good hood. We must also note that this chemical is no longer available from commercial suppliers probably because of its toxicity.

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Table I: Amino Acid Composition of Lysozyme Derivatives[a]

amino acid	native: theory	native: control	III[b]	IV	V	VI	VII	VIII
Asp	21	20.5	20.6	20.6	20.8	20.6	20.7	20.6
Thr	7	6.7	6.6	6.7	6.8	6.6	6.8	6.7
Ser	10	9.1	9.1	9.2	9.2	8.9	9.2	9.0
Glu	5	5.1	5.3	5.2	5.1	5.3	5.4	5.1
Pro	2	1.9	1.8	1.7	1.9	1.9	1.7	1.9
Gly	12	12.2	12.1	12.2	12.1	12.1	12.3	11.9
Ala	12	12	12	12	12	12	12	12
Val	6	5.7	5.9	5.9	5.6	5.9	5.7	5.7
Met	2	1.9	1.8	2.0	2.0	2.0	2.0	1.7
Ile	6	5.6	5.7	5.6	5.5	5.7	5.6	5.6
Leu	8	8.3	8.2	8.1	8.3	8.2	8.1	8.2
Tyr	3	2.9	2.9	3.0	3.0	3.0	3.2	2.9
Phe	3	3.0	3.1	3.1	3.0	3.2	3.3	3.1
Lys	6	5.9	6.2	6.0	6.1	6.0	6.0	5.9
His	1	1.0	1.0	0.9	1.0	1.0	1.0	0.9
Arg	11	11.1	10.9	11.1	11.0	11.0	10.5	10.9
ethanolamine				1.0	0.5	1.0	0.9	

[a] All values are expressed as molar ratios normalized to a value of 12.0 for alanine. [b] See Figure 1.

635 M LC detector. Activities of lysozyme and its derivatives against glycol chitin were determined in 0.1 M acetate buffer (pH 5.5) at 40 °C as described elsewhere (Imoto & Yagishita, 1971). Dissociation constants for the binding of $(NAG)_3$ to lysozyme and lysozyme derivatives in 0.1 M acetate buffer at pH 5.5 and 40 °C were determined by a method of UV difference spectroscopy (Dahlquist et al., 1966) with a Hitachi 557 double-wavelength double-beam spectrophotometer.

Reaction of Lysozyme with Ethylenimine for Product Analysis. Lysozyme (200 mg) and ethylenimine (100 mg) were dissolved in water, and the pH of the solution was adjusted to 5.0 with HCl. After the volume of the solution was adjusted to 5 mL with water, the mixture was stirred for 24 h at room temperature without control of the pH. During the reaction, the pH was changed to 6.1. The reaction mixture was dialyzed against distilled water and lyophilized.

Reaction of Lysozyme with Ethylenimine for Determination of Yields of Products at Various pHs. Ethylenimine was dissolved in a small amount of water, and the pH of the solution was adjusted to an appropriate value (4.0, 5.5, and 7.0) with H_2SO_4. A solution of lysozyme (100 mg/mL in H_2O) was added, and immediately the volume was adjusted to 5.0 mL. The pH was maintained at a desired value by addition of 0.05 N H_2SO_4 with titration system RTS 622 (Radiometer). After 24 h of stirring, the mixture was dialyzed against distilled water at 4 °C and then lyophilized. A similar experiment was carried out in the presence of 1.6×10^{-3} M $(NAG)_3$ at pH 5.5.

Results

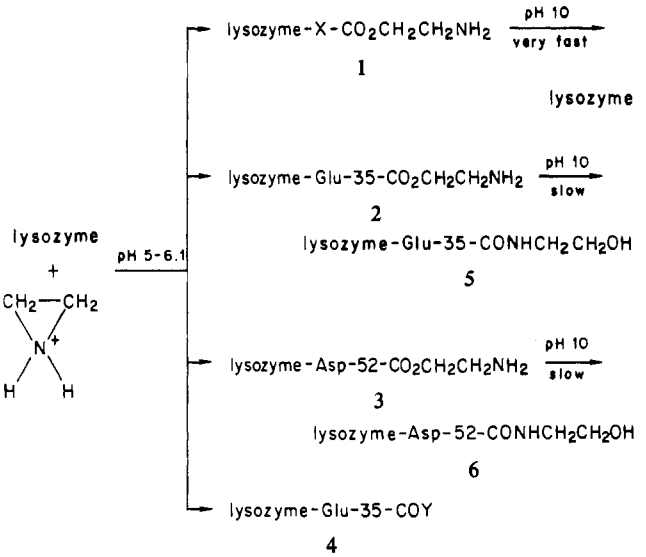
Product Analysis in the Reaction of Lysozyme with Ethylenimine. Lysozyme (200 mg) was allowed to react with ethylenimine (100 mg) in 5 mL of water at acidic pH (5.0–6.1) and room temperature for 24 h. The chromatographic pattern of the reaction mixture of Bio-Rex 70 at pH 7 is shown in Figure 1A. Two peaks (I and II) were eluted after the native protein peak, indicating that the products of the reaction were more cationic than lysozyme. The material in peak II was not analyzed because of its low yield. Peak I was rechromatographed at pH 10 (Figure 1B). Chromatography at this pH was performed at 4 °C to minimize the decomposition of the products (see below) and resulted in the resolution of three peaks (III–V). Amino acid compositions of the three fractions

Table II: Enzymatic Activities of Lysozyme and Lysozyme Derivatives against Glycol Chitin and Dissociation Constants (K_d's) for Binding to $(NAG)_3$ in 0.1 M Acetate Buffer at pH 5.5 and 40 °C

lysozyme	activity (%)	K_d (M)
native	100	2.3×10^{-5}
peak III[a]	101	
Glu-35-$CO_2CH_2CH_2NH_2$ (2)[b]	3	
Glu-35-$CONHCH_2CH_2OH$ (5)	0.6	5.9×10^{-5}
Asp-52-$CONHCH_2CH_2OH$ (6)	1.4	3.5×10^{-5}
Glu-35-$CO_2CH_2CH_2NHCH_2CH_2NH_2$ (4)	3	

[a] See Figure 1. [b] See Scheme I.

Scheme I[a]



[a] Peak I, 1 + 2 + 3 + 4; peak III, lysozyme regenerated from 1; peak IV, 2; peak V, 3 + 4; peak VI, 5; peak VII, 6; peak VIII, 4.

are shown in Table I. There is no significant difference between any of the derivatives and native lysozyme except that fractions IV and V contained ethanolamine, indicating that the reaction did not occur at histidine, lysine, serine, threonine, or tyrosine residues in lysozyme because otherwise the relative ratios of these amino acids should be affected. UV spectra of the fractions of these peaks at a region of 240–300 nm were identical with that of native lysozyme, indicating that the reaction did not occur at tryptophan residues either. These results are consistent with the hypothesis that the reaction of ethylenimine occurs at carboxyl residues and this was confirmed by peptide analyses (see below).

The derivative found in peak III showed the same amino acid composition as that of native lysozyme. Chromatography of a mixture of peak III and native lysozyme on Bio-Rex 70 at pH 7 gave one homogeneous peak. Moreover, the material in peak III was fully active against glycol chitin as shown in Table II. All of these results suggest that the component in peak III is native lysozyme that is regenerated from the original labile derivative during ion-exchange chromatography at pH 10. Although the location of the residue modified in this derivative is unknown, the structure is probably a labile ester, lysozyme–X–$CO_2CH_2CH_2NH_2$ (derivative **1** in Scheme I) from the analogy of the reaction shown in eq 1.

The derivative in peak IV contained 1 mol of ethanolamine/mol of protein (Table I). Rechromatography of peak IV on Bio-Rex 70 at pH 7 indicated the presence of small contamination of peak VI (Figure 1C). When the material in peak IV was incubated at pH 10 and room temperature for

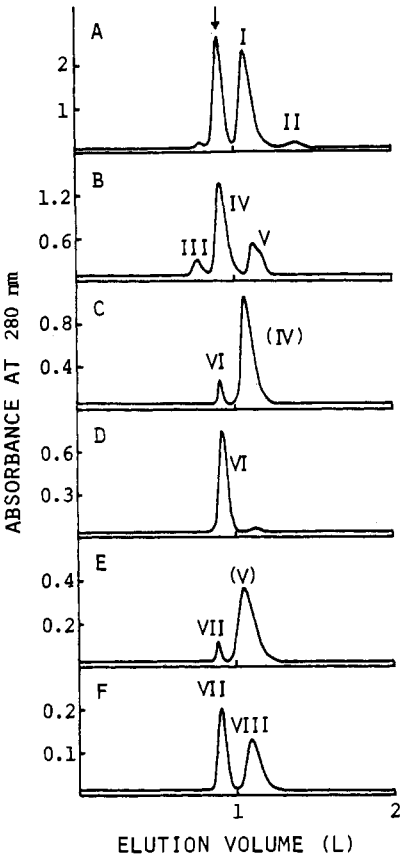
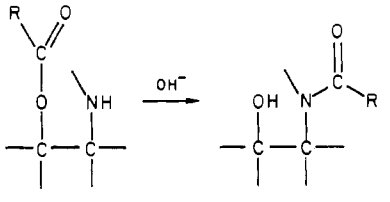


FIGURE 1: Ion-exchange chromatography of derivatives obtained in the reaction of lysozyme with ethylenimine at pH 5.0–6.1 on Bio-Rex 70 (100–200 mesh, 1.3 × 65 cm). All columns except for (B) were eluted with the gradient of 1 L of 0.1 M phosphate buffer and 1 L of 0.4 M phosphate buffer at pH 7 and room temperature. (A) Reaction mixture; arrow indicates the elution position of native lysozyme. (B) Rechromatography of fraction I in (A). The column was eluted with the gradient of 1 L of 0.02 M borate buffer (pH 10) and 1 L of the same buffer containing 0.18 M sodium chloride at 4 °C. (C) Rechromatography of fraction IV in (B). (D) Rechromatography of fraction IV in (B) after incubation at pH 10 and room temperature for 3 days. (E) Rechromatography of fraction V in (B). (F) Rechromatography of fraction V in (B) after incubation at pH 10 and room temperature for 3 days.

3 days and subsequently rechromatographed, peak IV almost disappeared and only peak VI increased (Figure 1D), suggesting that the component in peak IV is also unstable at pH 10 and room temperature and that it is nearly quantitatively converted to the component in peak VI. Amino acid compositions of fractions IV and VI were identical with each other with 1 ethanolamine/molecule (Table I). Since intramolecular oxygen to nitrogen (O → N) acyl migration in *O*-acyl-1,2-amino alcohol in aqueous alkaline solution is well-known (eq 2, Fodor & Kiss, 1950; van Tamelen, 1951), the components



(2)

in peaks IV and VI may be an ester and an amide derivative of ethanolamine, respectively. Incubation of peak IV in 1 M NH_2OH at pH 7 and room temperature for 24 h (Gallop et al., 1960) did not remove an ethanolamine moiety from this derivative but led to the formation of peak VI (ca. 30%), indicating that an O → N acyl migration is faster than hydroxylaminolysis of this ester derivative.

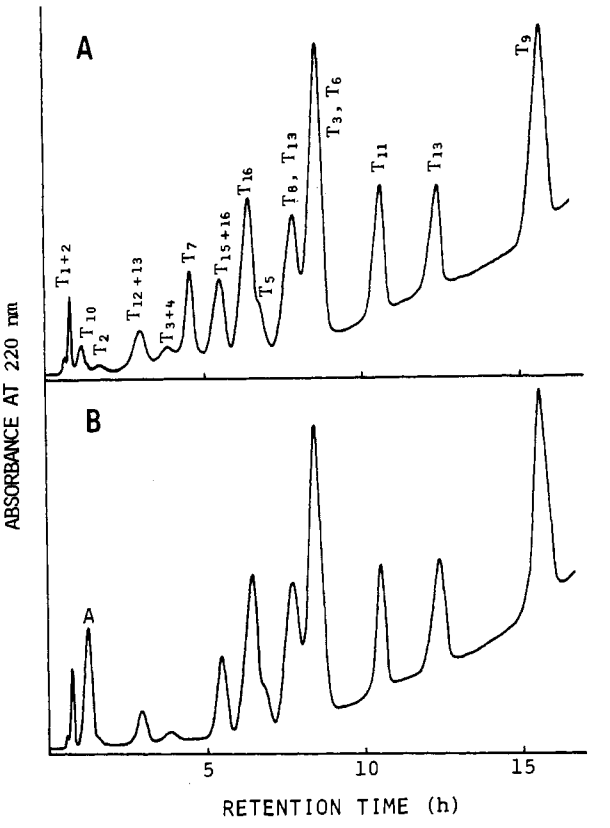


FIGURE 2: Ion-exchange chromatography of tryptic peptides from reduced and S-carboxymethylated lysozyme on a 1 × 55 cm column of DEAE-cellulose (DE-32, Whatman). The column was eluted with increasing salt concentration of NH_4HCO_3 by using three chambers. The first (450 mL) and the second (110 mL) chambers contained 0.02 M NH_4HCO_3, and the third chamber (110 mL) contained 1.5 M NH_4HCO_3. The flow rate was 0.7 mL/min. (A) From native lysozyme; (B) from fraction VI in Figure 1D.

For location of the modified carboxyl group in the component in peak VI (or IV), the derivative in peak VI was reduced and S-carboxymethylated and then hydrolyzed to peptides with TPCK-treated trypsin. The tryptic hydrolysate was chromatographed on DEAE-cellulose. The pattern of peptide elution is shown in Figure 2B. For comparison, the pattern from native lysozyme is shown in Figure 2A where the assignment of each peak (Yamada et al., 1981) is also shown. In Figure 2B, the peak corresponding to native peptide T_7 (Phe-34–Arg-45) has disappeared, and a new peak (peptide A) has appeared at the position of peptide T_{10}. T refers to the Canfield's nomenclature of tryptic peptides (Canfield, 1963). All other peptides were eluted in the same elution volumes as those of native peptides. Peptide A was separated from peptide T_{10} through a column of Sephadex G-25 (1 × 200 cm, medium, eluted with 0.02 M NH_4HCO_3) and subjected to amino acid analysis (Table III). Peptide A was assigned to the peptide T_7 containing one ethanolamine. Since peptide T_7 contains only one carboxyl residue, Glu-35, it is evident that the carboxyl side chain of Glu-35 is modified in the component in peak VI. The peptide analysis results using the derivative in peak IV were completely identical with those using the derivative in peak VI, indicating that the ester bond between Glu-35 and ethanolamine in the derivative in peak IV may have been converted to the corresponding amide bond at the stage of the peptide separation.

The shape of peak V in Figure 1B suggests that this peak is due to a mixture of derivatives. This was supported by the amino acid composition of fraction V, where only 0.5 mol of ethanolamine/mol of protein was detected (Table I). Re-

Table III: Amino Acid Composition of Peptides[a]

amino acid	amino acid ratio in peptide		
	A[b]	B	C
Asp	2.8 (3)	3.9 (4)	3.0 (3)
Thr	1.7 (2)	1.8 (2)	1.9 (2)
Ser	0.9 (1)	2.0 (2)	1.1 (1)
Glu	1.9 (2)	1.0 (1)	2.0 (2)
Gly		2.0 (2)[a]	
Ala	1.0 (1)[a]		1.0 (1)[a]
Ile		1.8 (2)	
Leu		1.0 (1)	
Tyr		1.0 (1)	
Phe	1.8 (2)		1.7 (2)
Arg	1.0 (1)	0.9 (1)	1.0 (1)
ethanolamine	0.8	0.9	
assignment	T_7	T_8	T_7

[a] Normalized to Ala or Gly. Values less than 0.1 are omitted. Numbers in parentheses are theoretical values. [b] See Figures 2 and 3.

chromatography of peak V on Bio-Rex 70 at pH 7 indicated the presence of a small peak (peak VII) before the large peak (Figure 1E). When fraction V was incubated at pH 10 and room temperature for 3 days, peak VII increased up to about half and the rest (called peak VIII) remained at (or near) the original position (Figure 1F). Further incubation of this fraction at pH 10 did not affect the ratio of peaks VII and VIII.

The derivative in peak VII contained 1 ethanolamine/molecule (Table I) and was formed on incubation at pH 10, indicating this peak to be due to an amide derivative of ethanolamine. Tryptic hydrolysate prepared from the derivative in peak VII was analyzed by high-performance reversed-phase liquid chromatography, and the pattern is shown in Figure 3B. For comparison, the pattern from native lysozyme is shown in Figure 3A, where the assignment of each peak (Yamada et al., 1981) is also shown. Obviously, a new peak (peptide B) has appeared with the disappearance of native peptide T_8 (Asn-46–Arg-61). Amino acid composition of peptide B showed that this peptide was the peptide T_8 containing one ethanolamine (Table III). Because peptide T_8 contains two carboxyl residues, Asp-48 and Asp-52, one of them is probably modified in the derivative in peak VII. The activity of fraction VII against glycol chitin was found to be only 1.4% that of native lysozyme (Table II, derivative **6**). Lin & Koshland (1969) have demonstrated that the lysozyme derivative in which all of the carboxyl residues except Glu-35 and Asp-52 are modified with aminoethanesulfonic acid shows over 50% of the activity of the native enzyme against *Micrococcus lysodeikticus* cells. Eshdat et al. (1973, 1974) and Parsons et al. (1969) have shown that derivatives singly modified at Asp-52 are enzymatically inactive. It would appear that the modified carboxyl residue in our derivative is Asp-52 rather than Asp-48.

Peak VIII showed the same amino acid composition as that of the native enzyme, having no ethanolamine (Table I), but exhibited a different elution position from that of native lysozyme on ion-exchange chromatography. The elution pattern of tryptic peptides obtained from fraction VIII through high-performance reversed-phase liquid chromatography is shown in Figure 3C. Peptide C appeared as a new peak with lack of the peak corresponding to native peptide T_7. Peptide C showed the amino acid composition of peptide T_7, suggesting that something other than ethanolamine is bound to Glu-35 as an ester or amide and is undetectable with the amino acid analyzer under the usual conditions. To confirm this, we

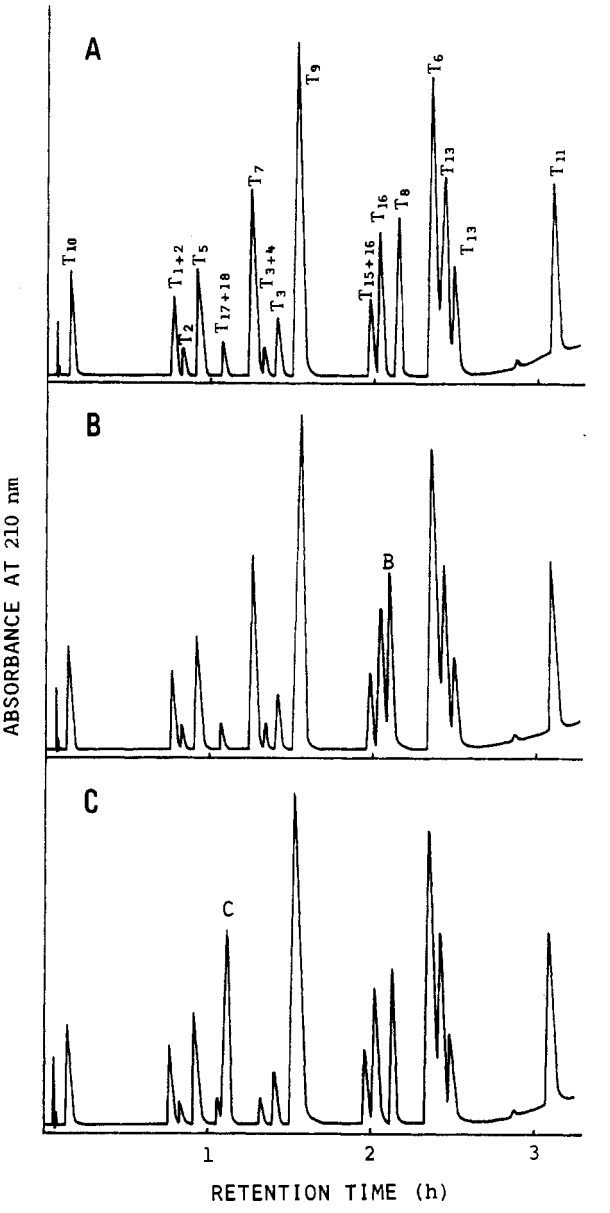


FIGURE 3: High-performance reversed-phase liquid chromatography of tryptic peptides from reduced and S-carboxymethylated lysozymes on a 0.4 × 25 cm column of LiChrosorb RP-8 (5 μm, Merck). The column was eluted with the gradient of 40 mL of 1% EtOH–0.1% concentrated HCl and 40 mL of 50% EtOH–0.1% concentrated HCl. (A) From native lysozyme; (B) from fraction VII in Figure 1F; (C) from fraction VIII in Figure 1F.

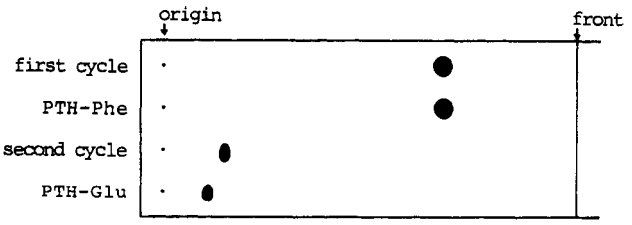


FIGURE 4: TLC pattern of the products of the first and the second cycles of Edman degradation of peptide C on silica gel (DC-Alufolien Kieselgel 60 F_{254}, Merck) developed with chloroform–methanol (9:1).

subjected peptide C to Edman degradation. As is seen in Figure 4, phenylthiohydantoin-Phe (PTH-Phe,[1] Phe-34) was identified on TLC[1] as the product of the first cycle of degradation, but the product of the second cycle was clearly different from PTH-Glu (Glu-35). Thus, the structure of the component in peak VIII is concluded to be lysozyme–Glu-35–COY (derivative **4** in Scheme I).

Table IV: Yields of Products in the Reaction of Lysozyme with Ethylenimine at Various pHs

pH	additive	yield of product (%) native lysozyme	1[a]	2 (5)	3 (6)	4
4.0		80.0	7.5	1.5	1.0	nd[b]
5.5		54.5	7.5	16.4	3.7	3.1
7.0		37.4	2.6	21.7	5.8	7.6
5.5	$(NAG)_3$[c]	58.4	3.7	20.0	3.0	3.4

[a] See Scheme I. [b] Not detected. [c] 1.6×10^{-3} M.

In Scheme I, the products identified in the reaction of lysozyme with ethylenimine and the components in the peaks resolved by ion-exchange chromatography are summarized.

Effect of pH and the Presence of $(NAG)_3$ on the Yields of Products in the Reaction of Lysozyme with Ethylenimine. To determine the effect of the pH of the reaction on the yield of each product, we allowed 100 mg of lysozyme to react with 50 mg of ethylenimine in 5 mL of water for 24 h at various pHs. The mixture was analyzed by ion-exchange chromatography with Bio-Rex 70 at pH 7 (room temperature) and then at pH 10 (4 °C) as described above. The materials in peak V were reduced, S-carboxymethylated, digested with TPCK-treated trypsin, and then analyzed by high-performance reversed-phase liquid chromatography. The yield of each product was calculated from the relative peak area in ion-exchange chromatography and reversed-phase liquid chromatography. The results are shown in Table IV. The result in the presence of 1.6×10^{-3} M $(NAG)_3$ at pH 5.5 is also shown in Table IV.

As can be seen in Table IV, the yield of each derivative was affected by the pH of the reaction. The yields of derivatives modified at Glu-35 (**2**) and at Asp-52 (**3**) increased as the pH was increased in a range of pH 4–7. This result is consistent with the mechanism of nucleophilic attack of carboxylate anion rather than corresponding carboxylic acid to the protonated ethylenimine as shown in eq 1. As for derivative **1**, however, the yield decreased at pH 7 compared with those at pH 4.0 and 5.5. The reason for this is unknown but probably is related to the instability of **1** at higher pH.

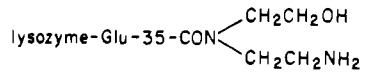
Addition of $(NAG)_3$ in the solution at pH 5.5 markedly reduced the formation of **1** while the yields of other derivatives were not much affected. Under these conditions, more than 90% of lysozyme is present as the $(NAG)_3$ complex. Thus, the carboxyl residue modified in derivative **1** is concluded to be at or close to the binding site of lysozyme for the trisaccharide.

Enzymatic Activities of Lysozyme Derivatives and Their Binding Ability to $(NAG)_3$. The enzymatic activities of lysozyme derivatives isolated here were determined with glycol chitin as a substrate in 0.1 M acetate buffer at pH 5.5 and 40 °C (Table II). Activities of derivatives in peak III and peak VII (derivative **6**) have been mentioned above. Every derivative modified at Glu-35 (**2**, **5**, and **4**) showed less than 3% of the activity of native lysozyme. Table II also shows the dissociation constants (K_d's) for the interactions of native lysozyme, Glu-35–amide derivative **5**, and Asp-52–amide derivative **6** to $(NAG)_3$ in 0.1 M acetate buffer (pH 5.5) at 40 °C. Derivatives **5** and **6** had values for K_d of 5.9×10^{-5} M and 3.5×10^{-5} M, respectively, compared with $K_d = 2.3 \times 10^{-5}$ M for native lysozyme.

Discussion

Ethylenimine was found to react specifically with carboxyl residues in lysozyme at neutral or acidic pH to give at least four derivatives (**1**, **2**, **3**, and **4**). To the best of our knowledge, this is the first example that ethylenimine is used to modify carboxyl residues in protein.

Most of the derivatives intially formed were not stable at alkaline pH. Two of the derivatives were the esters of Glu-35 (**2**) and Asp-52 (**3**) with ethanolamine, and they probably rearranged at pH 10 and room temperature to the corresponding amide derivatives **5** and **6**, respectively. The ester derivative of Asp-52 (**3**) was not separated from derivative **4**, but derivatives **2**, **5**, and **6** were isolated by repetition of ion-exchange chromatography. Derivative **2** or **5** is the first example in which only Glu-35 in lysozyme is singly modified. Derivative **1** was estimated to be an ester between unknown carboxyl residue X and ethanolamine, but this derivative was not isolated from other derivatives because it was highly labile and reverted to native lysozyme under the conditions of ion-exchange chromatography at pH 10. In the presence of $(NAG)_3$, the formation was markedly inhibited (Table IV). Since only Asp-101 is known to be a carboxyl residue involved in a saccharide binding as shown from X-ray crystallographic study of the lysozyme–$(NAG)_3$ complex (Blake et al., 1967b), the carboxyl residue modified in **1** is probably Asp-101. Parsons et al. (1969) have also obtained a very labile ethyl ester derivative in the reaction of lysozyme with triethyloxonium fluoroborate. They have not determined the modified carboxyl residue because of its lability. It is possible that the carboxyl residue modified with both reagents is the same since the products are so unstable. Derivative **4** was not separated from **3** directly by ion-exchange chromatography, but it was separated after **3** was converted to **6**. As shown under Results, it is evident that Glu-35 is modified in **4** but it does not contain an ethanolamine moiety, suggesting that some unusual reaction has occurred. As ethylenimine has a tendency to dimerize to give *N*-(2-aminoethyl)ethylenimine (Schatz & Clapp, 1955; Schmitt & Maris, 1971), we tentatively assign the structure of **4** to be an ester (lysozyme–Glu-35–CO_2CH_2-$CH_2NHCH_2CH_2NH_2$) derived from the ethylenimine dimer or an O → N acyl-migrated amide



as **4** was isolated after incubation at pH 10.

Under the conditions employed, only Glu-35, Asp-52, and probably Asp-101 in lysozyme were modified with ethylenimine, and other carboxyl residues were not modified appreciably. This observation strongly suggests that there are specific interactions between these carboxyl groups and ethylenimine due to the microenvironment of the protein.

As ethylenimine is cationic by protonation under the conditions employed, it is interesting to compare our results with the results from metal binding studies with lysozyme. X-ray crystallographic results have indicated that lysozyme has two strong binding sites for metal cation, site 1 at about 2.5 Å from the closest carboxyl oxygen of Glu-35 and site 2 at about 3 Å from that of Asp-52 (Kurachi et al., 1975; Perkins et al., 1979). Dill & Allerhand (1977) have shown that there is a third weak binding site for metal cation close to Asp-101 from the results of ^{13}C NMR[1] studies. From the comparison of our work with these results, we postulate the following mechanism to explain the specificity in the reaction of lysozyme with ethylenimine. At neutral or acidic pH, ethylenimine is protonated and this cation binds to lysozyme close to Glu-35, Asp-52, or Asp-101, preferentially as metal cations do. Under the conditions where these carboxyl groups are present as the carboxylate anion form, these anions attack nucleophilically

to the bound ethylenimine to give ester derivative **1**, **2**, or **3** selectively.

Since Glu-35 as well as Asp-52 has been postulated as a catalytic group in lysozyme (Blake et al., 1967b), it is interesting to study the enzymatic activities of the lysozyme derivatives (**2**, **4**, **5**, and **6**) isolated here. As can be seen in Table II, these derivatives showed less than 3% of the activity of native lysozyme. In order to know whether the remaining small activities are inherent in these derivatives or due to the contamination of a nativelike enzyme, we measured pH dependencies of activities of **5**, **6**, and native lysozyme throughout the pH range 3–11, respectively, because optimal pH should be affected by modification of Glu-35 or Asp-52. Both derivatives and native lysozyme showed a similar pH–activity profile with pH optimum near 5 and almost no activity at pH 9–11 (data are not shown). These results indicate that the remaining small activities in the derivatives are due to the contamination of the nativelike enzyme. Table II also shows the dissociation constants (K_d's) for the interaction of native lysozyme, Glu-35–amide derivative **5**, and Asp-52–amide derivative **6** to $(NAG)_3$ under the same conditions as their activities were measured. Derivative **5** and derivative **6** possessed about 2.6 and 1.5 times less binding strength against $(NAG)_3$ than native lysozyme, respectively, indicating that the ethanolamine moiety linked to Glu-35 or Asp-52 is not so bulky for binding of $(NAG)_3$ and more importantly that the active site structure in these derivatives is not destroyed by the modification. All of these results clearly indicate that Glu-35 as well as Asp-52 is essential for the activity of lysozyme as postulated by Blake et al. (1967b).

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