Selective Modification of Aspartic Acid-101 in Lysozyme by Carbodiimide Reaction†

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ABSTRACT: A general procedure which selectively introduced a nucleophilic group at a particular location in the active site of lysozyme has been developed. The coupling of hen egg white lysozyme with amine nucleophiles by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) was studied at pH 5 and room temperature. In the presence of an amine nucleophile, such as ethanolamine, ethylenediamine, methylamine, or 4(5)-(aminomethyl)imidazole, the carboxyl side chain of aspartic acid-101 in lysozyme was selectively modified by using a small excess of EDC. The reactivity of Asp-101 is probably due to the specific binding of EDC to the substrate binding site close to Asp-101. With histamine or D-glucosamine, the selectivity of Asp-101 was somewhat decreased. This may be due to the specific binding of these amines to lysozyme in competition with EDC, causing a decrease of the selective activation of Asp-101 by EDC. Depending on the amine employed, the lysozyme derivatives obtained exhibited decreased activity (83–52% of native enzyme), suggesting that the modification of Asp-101 weakened substrate binding.

The alteration of enzyme function by the chemical modification of the substrate binding site is an interesting problem in enzymology. Hen egg white lysozyme is a good model for this purpose because it is stable and plentiful and it is one of the best characterized enzymes.

The carboxyl groups of lysozyme are of particular interest because three (Glu-35, Asp-52, and Asp-101) of the 10 are in the substrate binding site (Blake et al., 1967). Carbodiimides have been frequently used to modify carboxyl groups of proteins (Hoare & Koshland, 1966; Lin & Koshland, 1969; Lin, 1970; Kramer & Rupley, 1973; Atassi et al., 1974). Lin & Koshland (1969) and Lin (1970) have shown that in lysozyme Glu-35 was almost completely unreactive and that Glu-7 and Asp-66 were only half-reacted, while all of the other carboxyl groups were completely modified with 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC)[1]–1 M glycinamide. With 0.25 M aminomethanesulfonic acid, only Glu-35 and Asp-66 were half and one-third unreacted, respectively (Lin & Koshland, 1969). Proteins modified in these ways were reported to be inert toward cell walls of *Micrococcus lysodeikticus* (Lin & Koshland, 1969; Lin, 1970). Kramer & Rupley (1973) have demonstrated that with 0.05 M sulfanilic acid, Glu-35 and Asp-101 were most reactive while Glu-7, Asp-18, and Asp-66 were least reactive. Fifty per cent inactivation was shown at about one group of sulfanilic acid incorporated. They also demonstrated that with 1.2 M sulfanilic acid, the reactivities were increased for Glu-7 and decreased for Glu-35 (ca. 10-fold) and Asp-101 (3-fold). Atassi et al. (1974) have shown that with 1 M glycine methyl ester, only two carboxyls (Asp-119 and carboxyl-terminal Leu-129) were modified, with 73% decrease in lytic activity. Although some selectivity is evident in the carbodiimide modification, no one has studied the selectivity of the reaction with amine by carbodiimide under conditions where less than one carboxyl group in the molecule was reacted. In this paper, we present a general method to modify selectively a single carboxyl side chain of lysozyme, Asp-101, using the carbodiimide–amine nucleophile modification.

Experimental Procedures

Materials. Six times recrystallized hen egg white lysozyme was donated from Eisai Co. (Tokyo, Japan). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) was purchased from Protein Research Foundation (Japan). Bio-Rex 70 (100–200 mesh) and Dowex 50W-X2 were products of Bio-Rad and Dow Chemical Co., respectively. Sephadex G-25 (medium) was obtained from Pharmacia. DEAE-cellulose (DE-32) was obtained from Whatman Ltd. LiChrosorb RP-8 (5 μm) was purchased from Merck. Glycol chitin was synthesized according to the method described by Senzyu & Okimasu (1950) and Yamada & Imoto (1981).

Synthesis of 4(5)-(aminomethyl)imidazole was accomplished by Pyman's procedure (1911) with small modification. Three grams of 4(5)-(chloromethyl)imidazole hydrochloride was dissolved in 50 mL of aqueous ammonia (28%) and kept at 40 °C for 29 h. The mixture was concentrated, and the residue was applied to the column of Dowex 50W-X2 (H^+ form, 2 × 43 cm). The column was eluted with a gradient of 1 L of water and 1 L of 2 N HCl. Ninhydrin-positive fractions were combined and concentrated. The residual solid was recrystallized from ethanol to give 366 mg of 4(5)-(aminomethyl)imidazole dihydrochloride hemihydrate as colorless needles: mp 216–7 °C. Anal. Calcd for $C_4H_7N_3 \cdot 2HCl \cdot 0.5H_2O$: C, 26.83; H, 5.63; N, 23.47. Found: C, 26.89; H, 5.62; N, 23.17. All other chemicals were the highest purity available.

Analytical Methods. Amino acid analyses were performed on a Hitachi 835 amino acid analyzer. For analysis, lysozyme and its derivatives were hydrolyzed in 4 N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole, under vacuum, for 24 h at 118 °C, and tryptic peptides were hydrolyzed in 6 N HCl, under vacuum, for 20 h at 110 °C. Chromatography of lysozyme and its derivatives was performed on columns (1 × 65 cm) of the carboxylic cation exchanger Bio-Rex 70, utilizing two pH buffer systems. The first system was a gradient of 1 L of 0.1 M phosphate buffer and 1 L of 0.4 M phosphate buffer at pH 7.0. The second system was a gradient of 1 L of 0.02 M borate buffer (pH 10.0) and 1 L of the same buffer containing 0.15 M sodium chloride. Protein elution was monitored by absorbance of effluents at 280 nm with a Hitachi 200-10 double-beam spectrophotometer. Activities of lysozyme and its derivatives using glycol chitin as substrate were measured in 0.1 M acetate buffer (pH 5.5) at 40 °C as described

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[1] Abbreviation used: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride.

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

amino acid	native theory	native control	$EtOHNH_2$ (1C)[b]	H_2NCH_2Im (1E)	H_2NCH_2-CH_2NH_2 (1F)	CH_3NH_2 (1G)	histamine (2B)	$GlcNH_2$ (2C)
Asp	21	21.1	20.5	20.7	21.2	20.4	20.6	20.4
Thr	7	6.8	6.5	6.4	6.0	5.9	6.3	6.1
Ser	10	8.8	8.6	8.7	8.5	8.1	8.7	8.0
Glu	5	5.3	5.0	5.0	5.1	5.1	4.9	5.1
Pro	2	1.8	2.0	1.8	1.4	1.7	1.9	1.9
Gly	12	12.1	12.0	12.3	12.5	12.2	12.3	12.1
Ala	12	12	12	12	12	12	12	12
Val	6	5.8	5.8	5.8	5.7	5.6	5.6	5.9
Met	2	1.8	2.0	1.8	1.9	2.0	1.8	2.0
Ile	6	5.5	5.5	5.5	5.5	5.6	5.3	5.7
Leu	8	7.8	7.9	8.0	8.0	8.0	7.9	8.0
Tyr	3	3.0	2.9	3.0	3.4	3.1	3.0	3.0
Phe	3	3.1	3.1	3.1	2.9	3.0	3.0	3.0
Lys	6	6.2	6.0	6.3	5.8	6.3	6.2	6.0
His	1	1.0	1.0	1.0	1.1	1.0	1.0	1.0
Arg	11	11.2	11.0	11.2	11.0	11.2	11.1	11.2
Trp	6	6.3	6.2	6.1	5.8	5.8	6.4	5.7
ethanolamine			1.1					
methylamine						0.7		

[a] All values are expressed as molar ratios normalized to a value of 12.0 for alanine. [b] See Figures 1 and 2.

elsewhere (Imoto & Yagishita, 1971).

Tryptic Hydrolysis of Protein. Five to twenty milligrams of reduced, S-carboxymethylated protein (Crestfield et al., 1963) in 10 mL of 5 mM NH_4HCO_3 solution (pH 8.2) was hydrolyzed by TPCK-treated trypsin (Worthington, 1% lysozyme weight) for 2 h at 40 °C. The pH of the solution was maintained at 8 during the reaction by addition of aqueous NaOH. The mixture was heated in a boiling water bath for 10 min and centrifuged, and the supernatant was lyophilized.

Peptide Separation—DEAE-cellulose Ion-Exchange Chromatography. The washed DEAE-cellulose (DE-32) was suspended in degassed distilled water and packed in a 1 × 55 cm column, and the column was equilibrated with 0.02 M NH_4HCO_3. A lyophilized tryptic hydrolysate of protein (5–20 mg) was dissolved in a small amount of water and applied to the column. The column was eluted with increasing salt concentration of NH_4HCO_3 by using three chambers. The first (450 mL) and 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. The peptide elution was monitored by absorbance(s) of effluent at 220 and/or 280 nm with a Hitachi 635M LC detector. The chromatographic procedure was performed below 25 °C. Peptide fractions were lyophilized and further purified on a Sephadex G-25 column (1 × 200 cm) which was eluted with 0.02 M NH_4HCO_3.

High-Pressure Reversed-Phase Liquid Chromatography. LiChrosorb RP-8 (5 μm) was packed in a 0.4 × 25 cm stainless column and attached to a Hitachi 635A liquid chromatograph equipped with a Hitachi 635M LC detector. The column was equilibrated with 1% EtOH containing 0.1% concentrated HCl. About 0.5 mg of tryptic hydrolysate in 20 μL of water was injected to the column, and the column was eluted with a gradient of 40 mL of 1% EtOH–0.1% concentrated HCl and 40 mL of 50% EtOH–0.1% concentrated HCl. The peptide elution was monitored by absorbance(s) of effluent at 210 and/or 280 nm. Effluents containing peptides were collected manually, concentrated by evaporation at 40 °C, and used for amino acid analyses. The column was regenerated by washing with 80% EtOH–0.1% concentrated HCl and then with starting solvent.

Coupling Reaction of Lysozyme with Amine by EDC. Lysozyme and amine were dissolved in water, and the pH of the solution was adjusted to 5.0 with HCl. EDC was added to the solution with stirring at room temperature to initiate the reaction. The pH was maintained at 5.0 with HCl during the reaction. After the pH change ceased (2–3 h), the solution was dialyzed exhaustively against distilled water and then analyzed by ion-exchange chromatography with Bio-Rex 70. Conditions employed for the couplings were as follows: ethanolamine (0.01, 0.1, or 1 M), lysozyme (0.7 mM), and EDC (2.6 mM), 10 mL, for 3 h; ethylenediamine (0.05 M), lysozyme (0.35 mM), and EDC (3.5 mM), 30 mL, for 2 h; 4-(5)-(aminomethyl)imidazole (0.019 M), lysozyme (1.4 mM), and EDC (5.2 mM), 5 mL, for 2.5 h; histamine (0.04 M), lysozyme (1.4 mM), and EDC (10.4 mM), 5 mL, for 3 h; methylamine (0.05 M), lysozyme (0.35 mM), and EDC (1.7 mM), 30 mL, for 2 h; D-glucosamine (0.1 or 1.0 M), lysozyme (0.7 mM), and EDC (5.2 mM), 10 mL, for 3 h.

Results

Modification of Lysozyme with Amine by EDC. Coupling reactions of lysozyme with various amine nucleophiles by EDC were carried out at pH 5 and room temperature. In the case of ethanolamine, the chromatographic pattern of the reaction mixture on Bio-Rex 70 at pH 10 is shown in Figure 1C. For comparison, the pattern for the native enzyme is shown in Figure 1A. The major derivative (Figure 1C, shadowed peak) was rechromatographed on Bio-Rex 70 at pH 7 and found to be homogeneous. Reaction of lysozyme with other nucleophiles including 4(5)-(aminomethyl)imidazole (Figure 1E), ethylenediamine (Figure 1F), and methylamine (Figure 1G) yielded similar chromatographic patterns, indicating that one major product was obtained in each case. On the other hand, the reaction of lysozyme with histamine (Figure 2B) or D-glucosamine (Figure 2C) gave a rather complicated chromatographic pattern, although one major product was still present (shadowed peak in Figure 2). The effect of the concentration of amine on the reaction was investigated in cases of ethanolamine and D-glucosamine. In the case of ethanolamine, the yield of the major derivative increased (11%, 50%, and 60% at 0.01 M, 0.1 M, and 1 M ethanolamine, respectively) as the concentration of ethanolamine was increased (Figure 1B–D). On the other hand, in the case of D-glucosamine, no detectable effect of the concentration of D-glucosamine (0.1 M and 1 M) was observed (Figure 2C,D).

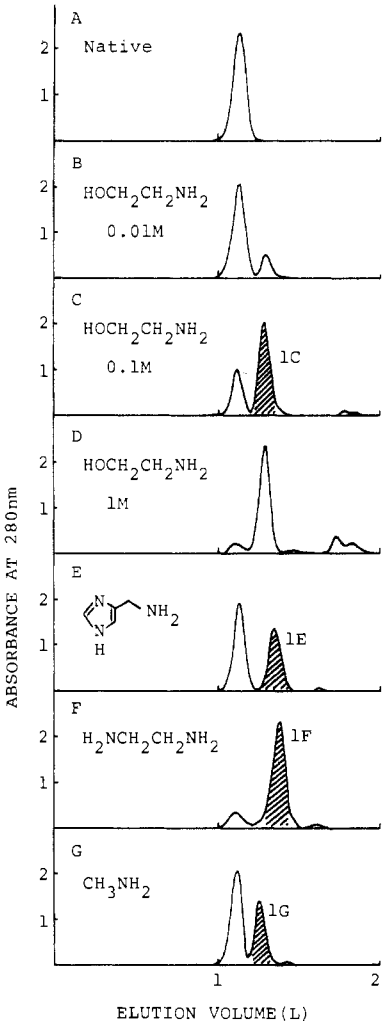

FIGURE 1: Ion-exchange chromatography of the reaction mixture of lysozyme with amine catalyzed by EDC on Bio-Rex 70 at pH 10. (A) Native lysozyme, (B) reaction with 0.01 M ethanolamine, (C) reaction with 0.1 M ethanolamine, (D) reaction with 1 M ethanolamine, (E) reaction with 4(5)-(aminomethyl)imidazole, (F) reaction with ethylenediamine, and (G) reaction with methylamine. Details are given in the text.

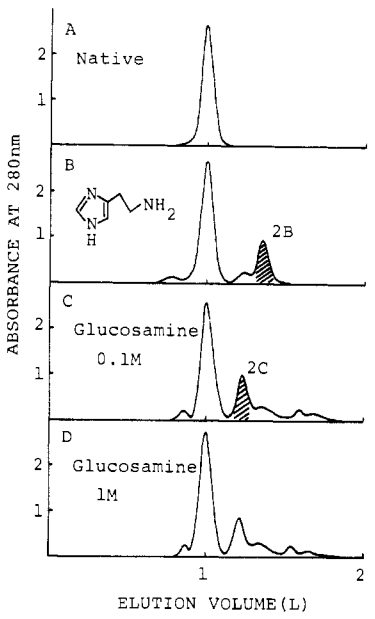

FIGURE 2: Ion-exchange chromatography of the reaction mixture of lysozyme with amine catalyzed by EDC on Bio-Rex 70 at pH 7. (A) Native lysozyme, (B) reaction with histamine, (C) reaction with 0.1 M D-glucosamine, and (D) reaction with 1 M D-glucosamine. Details are given in the text.

In Table I, amino acid compositions of the products (shadowed peaks in Figures 1 and 2) are listed. There is no significant difference in amino acid composition between any of the products and native lysozyme, indicating a small degree of modification. The presence of one amine per molecule is clearly shown in the products from ethanolamine (Figure 1C) and methylamine (Figure 1G), respectively. As for D-glucosamine, the peak area of glucosamine could not be determined because of interference of the peak of Leu in amino acid analysis. As for other amines, the number of amines incorporated into the product was not determined because these amines did not elute from the amino acid analyzer under the usual condition. However, only one amine was considered to be incorporated into lysozyme in every case, as judged from the behavior during ion-exchange chromatography (Figures 1 and 2), and this was confirmed by the peptide analysis described below.

Location of the Carboxyl Group Modified in Lysozyme. For determination of the carboxyl group modified in lysozyme, tryptic hydrolysates of derivatives were analyzed by DEAE-cellulose ion-exchange chromatography and/or by high-pressure reversed-phase liquid chromatography using Li-Chrosorb RP-8. Figure 3A shows the chromatographic pattern of peptides from native lysozyme by ion-exchange chromatography. Each peak was collected respectively, further purified by gel filtration through Sephadex G-25, and then identified by amino acid analysis after acid hydrolysis. The results are shown in Figure 3A. All peptides were identified in a straightforward manner except T_{13}. T refers to the Canfield's nomenclature of tryptic peptides (1963a). T_{13} (Ile-98–Arg-112) appeared as two peaks (3A2 and 3A3 in Figure 3A). We do not know why multiple forms of peptide T_{13} were generated from unmodified enzyme. Originally residue 103 was reported to be Asp (Canfield, 1963b; Jollès et al., 1963), but this residue was correctly determined to be Asn [J. R. Brown cited by Imoto et al. (1972)]. Deamidation of lysozyme recrystallized in the presence of 5% $NaHCO_3$ at pH 9.5 was shown by Tallan & Stein (1953). Also, a rapid deamidation of Asn-103 in T_{13} peptide, particularly during desalting in an acidic medium, has been discussed recently (Jollès et al., 1979). If Asn-103 was unstable in peptide T_{13}, the peak having a shorter retention time (peak 3A2) may be T_{13} with Asn-103, and the later peak (peak 3A3) may be T_{13} with Asp-103, considering the nature of the anion-exchange chromatography. But we confirmed that Asn-103 was never deamidated under the condition employed (T. Imoto et al., unpublished results). Both peptides, 3A2 and 3A3, showed the identical UV spectra, indicating that tryptophans were not changed in these peptides under conditions employed. Gel filtration of these peptides through Sephadex G-25 showed that peptide 3A2 had a higher hydrodynamic volume than peptide 3A3, indicating that there was heterogeneity in size of these peptides. So we tentatively conclude that the T_{13} peptide is present as a monomer–dimer mixture that separates during the ion-exchange chromatography. The same situation may occur when the T_{13} peptide is modified (see below). Figure 4A shows the elution pattern of tryptic hydrolysate of lysozyme using reversed-phase chromatography. The assignment of each peak is also shown in Figure 4A. T_{13} again appeared as two peaks.

In every lysozyme derivative obtained in this study, the peaks of native T_{13} and T_{12+13} (Lys-97–Arg-112) disappeared and new peaks appeared (Figures 3 and 4). All other peptides appeared at the same positions as those of native peptides. The

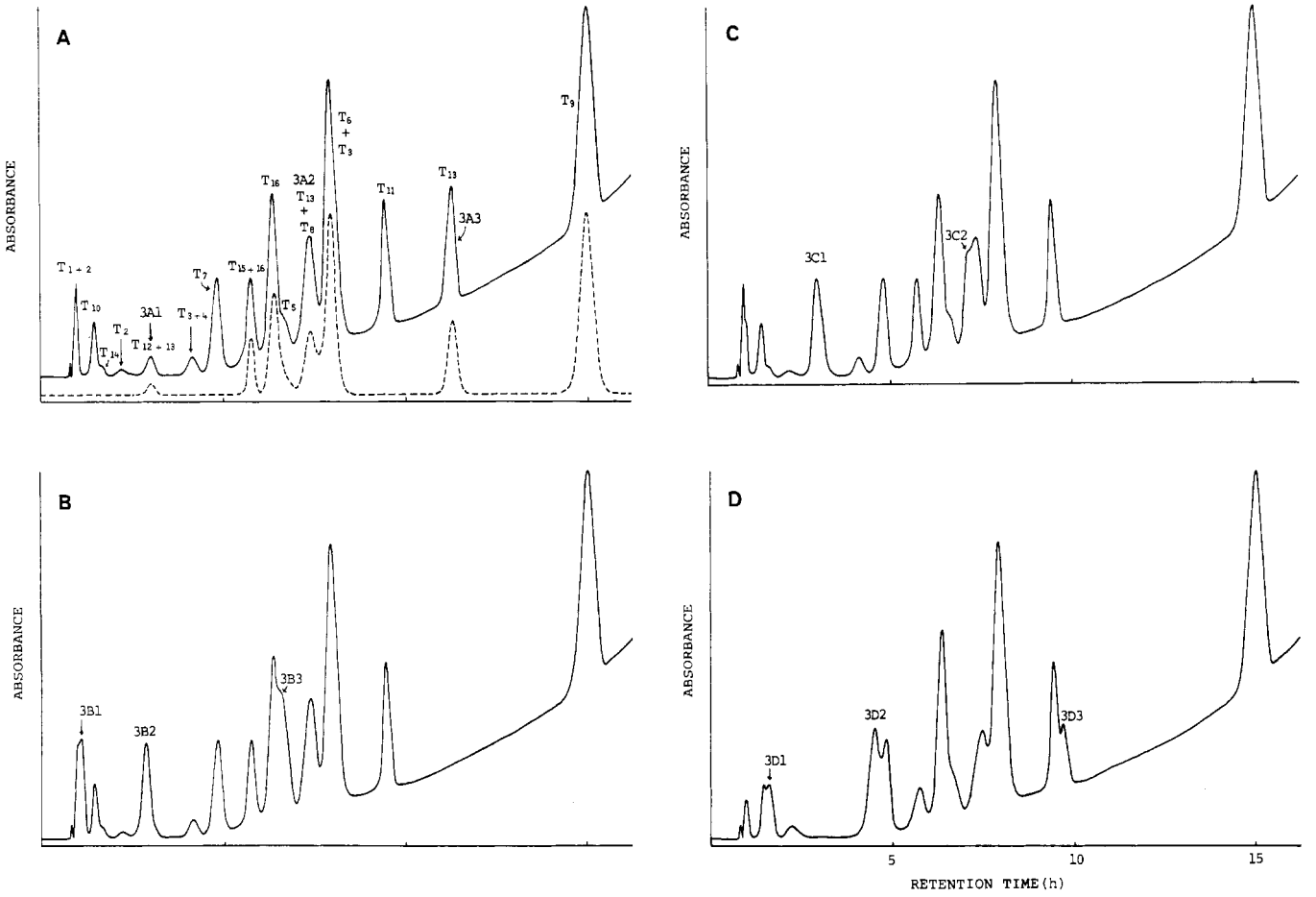

FIGURE 3: Ion-exchange chromatography of tryptic peptides from reduced, S-carboxymethylated lysozyme derivatives on DEAE-cellulose. (A) From native lysozyme, (B) from the product in the reaction with ethanolamine, (C) from the product in the reaction with D-glucosamine, and (D) from the product in the reaction with ethylenediamine. (—) A_{220}; (---) A_{280}. Details are given in the text.

new peptides obtained were characterized by amino acid analyses. These results as well as those of native T_{13} and T_{12+13} are shown in Table II. All new peaks except those obtained from the reaction of lysozyme with ethylenediamine were assigned to peptide T_{13} or T_{12+13}. One amine per molecule was also detected in the peptides obtained from modification with ethanolamine, methylamine, and D-glucosamine, respectively. Since T_{13} (or T_{12+13}) contains only one carboxyl residue, Asp-101, it is evident that the carboxyl side chain of Asp-101 is modified by the carbodiimide–amine reaction. As for the case of ethylenediamine, one of the new peaks (3D1 in Figure 3D and Table II) was assigned to the peptide Ile-98–Asp-101. The remaining two peaks (3D2 and 3D3) had the identical amino acid composition which was consistent with that of peptide Gly-102–Arg-112. These observations indicate that the peptide bond between Asp-101 and Gly-102 is cleaved by trypsin. Bertrand et al. (1976) have reported that trypsin cleaves peptides at the (aminoethyl)asparagine residue (amide of β-carboxyl of aspartic acid with ethylenediamine) because of its structural similarity to lysine.

Discussion

Reactivity of Carboxyl Groups. Modification of carboxyl groups in lysozyme by the carbodiimide–nucleophile procedure has been developed recently (Hoare & Koshland, 1966; Lin & Koshland, 1969; Lin, 1970; Kramer & Rupley, 1973; Atassi et al., 1974). This procedure can be used to introduce a new function into lysozyme because various amines can be used as nucleophiles. Unlike the previous studies, we have determined the selectivity of the reaction of the carboxyl groups in lysozyme under conditions where less than one carboxyl group on the average is modified.

With most amines except D-glucosamine and probably histamine, the reaction of lysozyme by EDC at pH 5 and room temperature was very selective, and only Asp-101 was modified. Although the yield of modified lysozyme depended on the kind of amine, reaction was significant even at low concentration of EDC. In the case of ethanolamine, the amount of EDC used was only 3.7 times the amount of lysozyme (actually 0.37 times the amount of carboxyl groups), and the drivative was obtained in more than 50% yield (Figure 1C,D). As expected, the yield of the derivative increased as the concentration of ethanolamine was increased. At high concentration of ethanolamine (1 M), the formation of other derivatives become evident (totally 19% yield, Figure 1D), and only 10% of native enzyme was recovered. Amino acid analyses showed that these derivatives contained two ethanolamines per molecule. These observations suggest that Asp-101 is modified first, followed by modifications of other carboxyls.

In cases of histamine and D-glucosamine, the reaction was less selective as judged from the chromatographic pattern, while the major product was a derivative modified at Asp-101 (Figure 2B,C). Therefore it is evident that there are two types of amines. One type of amine (type A) causes selective modification of Asp-101 in lysozyme by EDC reaction, and the other type of amine (type B) causes somewhat random reaction.

The high reactivity of Asp-101 may be explained partially by the fact that this residue has the most exposed carboxyl side chain (Imoto et al., 1972). However, the difference in selectivity of the reaction between two types of amines mentioned above is not understood only from this fact. We pos-

Table II: Amino Acid Composition of Tryptic Peptides of Lysozyme Derivatives[a]

amino acid	T_{12+13} theory	T_{13} theory	native 3A1[b]	native 3A2	native 3A3	EtOHNH$_2$ 3B1	EtOHNH$_2$ 3B2	EtOHNH$_2$ 3B3	GlcNH$_2$ 3C1	GlcNH$_2$ 3C2	$H_2NCH_2CH_2NH_2$ 3D1	$H_2NCH_2CH_2NH_2$ 3D2	$H_2NCH_2CH_2NH_2$ 3D3	EtOHNH$_2$ 4B1	EtOHNH$_2$ 4B2	EtOHNH$_2$ 4B3	H_2NCH_2Im 4C1	H_2NCH_2Im 4C2	histamine 4D1	histamine 4D2	histamine 4D3	CH_3NH_2 4E1	CH_3NH_2 4E2
Asp	3	3	3.0	3.2	2.9	2.7	2.9	3.2	3.3	3.2	1	1.9	1.9	3.0	3.0	3.0	3.5	2.9	2.7	2.9	3.0	2.8	2.9
Ser	1	1	0.9	1.1	0.9	1.0	0.9	1.0	1.1	1.0	0.9	0	0	1.0	0.9	1.1	1.1	0.9	1.1	1.0	1.0	1.0	1.1
Gly	2	2	2.1	2.4	1.9	2.2	2.1	2.3	2.5	2.3	0	1.9	2.0	2.0	2.0	2.2	2.2	1.9	1.9	2.1	2.1	2.1	2.1
Ala	2	2	2	2	2	2	2	2	2	2	0	2	2	2	2	2	2	2	2	2	2	2	2
Val	2	2	1.7	1.5	1.5	1.6	1.5	1.6	1.9	1.5	0.7	0.9	0.9	1.7	1.8	1.6	2.1	1.6	1.6	1.5	1.5	1.6	1.5
Met	1	1	0.9	1.0	0.9	0.8	0.7	0.7	1.0	0.8	0	0.9	0.9	1.1	0.9	1.2	0.6	0.8	0.9	0.7	0.7	0.8	0.5
Ile	1	1	0.7	0.6	0.6	0.5	0.6	0.7	0.7	0.6	0.7	0	0	1.0	0.8	0.6	1.1	0.6	0.7	0.7	0.6	0.6	0.7
Lys	1		1.0	0	0	1.1	0.1	0.1	0.4	0	0	0	0	1.0	0	0.3	1.1	0.3	1.0	0	0.1	0	0
Arg	1	1	1.0	1.4	1.0	0.6	0.9	1.0	1.0	1.0	0	0.9	0.9	1.0	1.0	0.7	1.1	1.0	0.8	1.0	1.0	0.9	0.9
EtOHNH$_2$						0.7	0.6	0.6						0.7	0.9	0.6							
GlcNH$_2$									0.6	0.4												0.8	0.8
CH_3NH_2																							
assignment			T_{12+13}	T_{13}		T_{12+13}	T_{13}		T_{13}		Ile-98–Asp-101	Gly-102–Arg-112		T_{12+13}	T_{13}		T_{12+13}	T_{13}	T_{12+13}	T_{13}		T_{13}	

[a] Residues per molecule. All values except in 3D1 are expressed as molar ratios normalized to a value of 2.00 for alanine. The values in 3D1 are expressed as molar ratios normalized to a value of 1.00 for aspartic acid. [b] See Figures 3 and 4.

Table III: Enzymatic Activity of Lysozyme Derivatives

lysozyme	activity (%)
native	100
Asp-101–CONHCH$_2$CH$_2$OH (1C)[a]	83
Asp-101–CONHCH$_2$Im (1E)	73
Asp-101–CONHCH$_2$CH$_2$NH$_2$ (1F)	53
Asp-101–CONHCH$_3$ (1G)	67
Asp-101–CONHCH$_2$CH$_2$Im (2B)	52
Asp-101–CONHGlc (2C)	81

[a] See Figures 1 and 2.

tulate the following mechanism to explain this difference. Asp-101 is located at the edge of the substrate binding site. EDC binds specifically to this site with proximity to Asp-101 and activates the carboxyl side chain of Asp-101 to cause selective modification of this residue with the amine, unless the amine has no specific interaction with enzyme (amines of type A). Some amines (type B) may also bind to the substrate binding site in competition with EDC. With this type of amine, enzyme–nucleophile interactions predominate, and the selective activation of Asp-101 by EDC is reduced to lead a more random reaction. It is not clear at present why there was no effect of the concentration of D-glucosamine on the reaction.

Enzymatic Activity of Lysozyme Derivative. Since Asp-101 has been postulated to be involved in a hydrogen-bond contact with the saccharide in the lysozyme–tri-*N*-acetylglucosamine complex (Blake et al., 1967), the effect of modification of this residue on the enzymatic activity should be consequential. The activity of lysozyme has been mainly determined by lysis of bacterial cell walls from *Micrococcus lysodeikticus* (Imoto et al., 1972). Recently, an alternative method that uses glycol chitin as the substrate has been developed (Hamaguchi & Funatsu, 1959; Hamaguchi et al., 1960; Hayashi et al., 1964; Imoto & Yagishita, 1971). There appears to be considerable difference between the activity of lysozyme toward glycol chitin and its activity toward cell walls. This observation is based on the facts that lytic activity shows unusual ionic strength effects (Sela & Steiner, 1963; Davies et al., 1969; Saint-Blancard et al., 1970), that acetylation of amino groups in lysozyme affects lytic but not glycol chitin activity (Yamasaki et al., 1968), and that lytic activity and glycol chitin activity show different pH–activity profiles (Davies et al., 1969; Imoto et al., 1974). These differences for the action of lysozyme are considered to be due to the complex nature of the cell wall substrate (Imoto et al., 1972). Glycol chitin resembles low molecular weight substrates such as oligosaccharides of *N*-acetylglucosamine with pH optimums near 5 and without curious ionic strength effects (Banejee et al., 1973). In these respects, we judged that glycol chitin reflected the true enzymatic activity more than cell walls. Thus, we employed here the glycol chitin method for determination of enzymatic activities of lysozyme derivatives obtained in this study. As shown in Table III, enzymatic activity was decreased by the modification of Asp-101. But the degree of inactivation depended on the amine employed. This suggested that enzymatic activity was affected not only by the loss of hydrogen bonding ability of Asp-101 but also by other factors. The bulkiness of the nucleophile may be one of the factors as seen in the cases of 4(5)-(aminomethyl)imidazole (73% activity of native lysozyme) and histamine (52%), but there is no obvious correlation here when all modifications are considered. Another factor may be the nature of amine introduced which can be classified into three groups. The first group includes amines with a hydrophobic side chain (methylamine) which have no

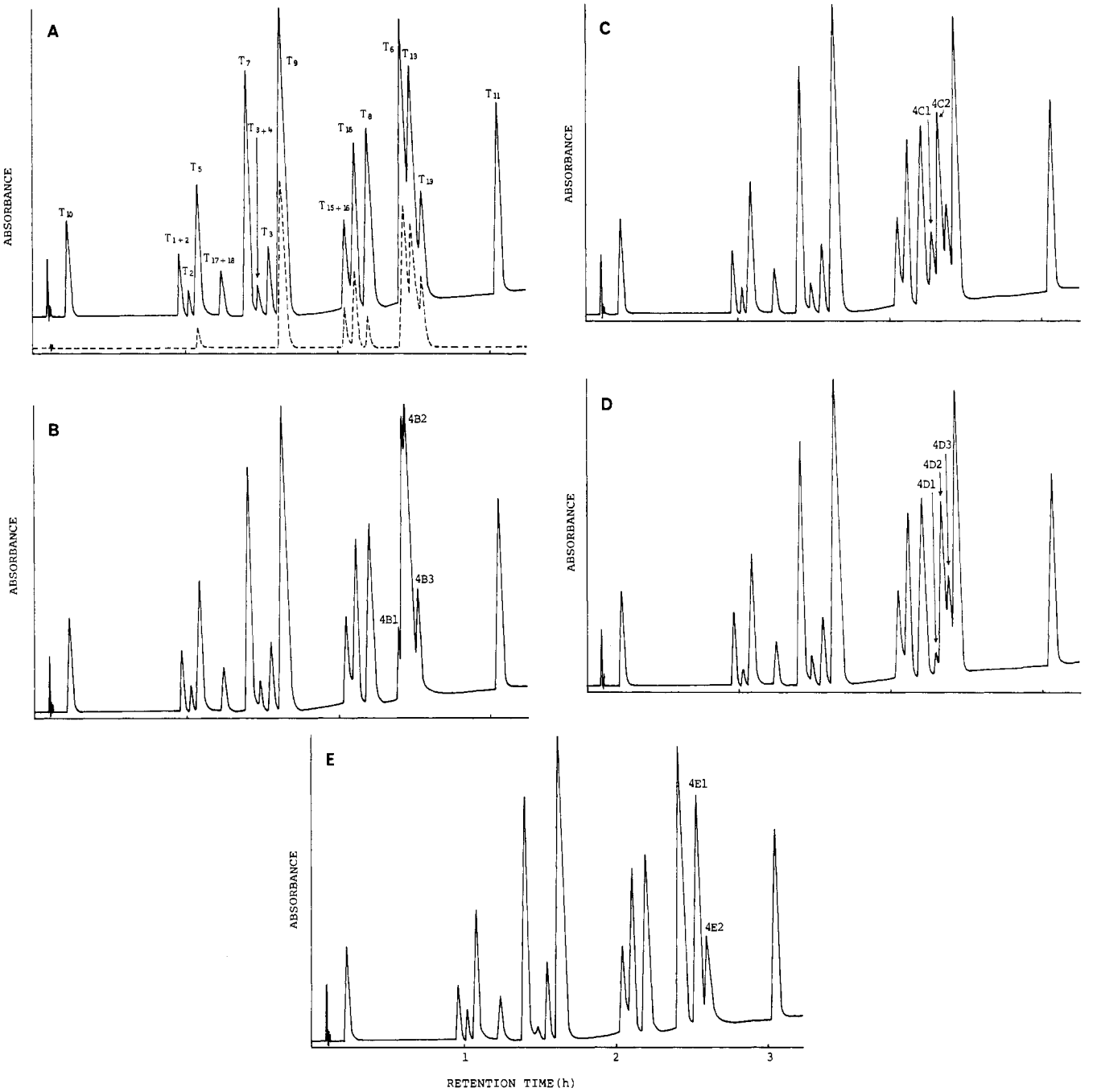

FIGURE 4: Reversed-phase liquid chromatography of tryptic peptides from reduced, S-carboxymethylated lysozyme derivatives on LiChrosorb RP-8. (A) From native lysozyme, (B) from the product in the reaction with ethanolamine, (C) from the product in the reaction with 4(5)-(aminomethyl)imidazole, (D) from the product in the reaction with histamine, and (E) from the product in the reaction with methylamine. (—) A_{210}; (- - -) A_{280}. Details are given in the text.

hydrogen-bonding ability. The second includes amines with amino or imidazole nitrogens on the side chain (ethylenediamine, 4(5)-(aminomethyl)imidazole, or histamine). The third are amines with a hydroxyl group on the side chain. Amino and imidazole nitrogens do not act as proton acceptors in hydrogen bonding at the pH studied (pH 5.5) while a hydroxyl group does. Modifications of Asp-101 by nucleophiles in groups 1 and 2 decreased the activity of lysozyme more than nucleophiles in group 3. In order to determine the correlation between the activity and the type of amine introduced more precisely, we need to do more experiments which are in progress.

Lin & Koshland (1969) have demonstrated that the lysozyme derivative in which all of the carboxyl groups except Glu-35 and Asp-52 are modified with aminomethanesulfonic acid shows over 50% activity of the native enzyme against cell walls. This value is comparable to those of derivatives obtained here. Therefore the decrease in activity of aminomethanesulfonic acid modified lysozyme is probably due to the modification of Asp-101.

Parsons et al. (1969) have reported that modification of carboxyl groups in lysozyme with triethyloxonium fluoroborate gives a singly esterified lysozyme derivative possessing 57% activity of native enzyme against cell walls. This ester is very labile, and they have not determined the modified carboxyl group. But the comparison of the activity of this derivative with those of lysozyme derivatives obtained by us suggests that the modified carboxyl group here is also Asp-101.

Peptide Separation. In this study, the separation of tryptic peptides of reduced, S-carboxymethylated lysozyme derivatives

was performed on DEAE-cellulose ion-exchange chromatography and/or high-pressure reversed-phase liquid chromatography. In the former system, the solvent (aqueous NH_4HCO_3) is volatile and transparent enough in the ultraviolet region to allow detection of peptides by monitoring at 220 nm. This system is very sensitive to changes in the charge of a peptide and most effective in analyzing lysozyme derivatives where carboxyl groups have been modified. The latter system has been discussed elsewhere (Imoto & Okazaki, 1981) when used for the separation of tryptic peptides of lysozyme. In the present study, the high-pressure liquid chromatographic method was also effective for a rapid analysis of tryptic peptides of lysozyme derivatives.

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