Intramolecular Cross-Linkage of Lysozyme. Imidazole Catalysis of the Formation of the Cross-Link between Lysine-13 (ϵ-Amino) and Leucine-129 (α-Carboxyl) by Carbodiimide Reaction†

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ABSTRACT: The salt bridge between Lys-13 (ϵ-NH_3^+) and Leu-129 (α-COO^-) in lysozyme was converted to an amide bond by 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) reaction in the presence of imidazole (0.3–1 M) at pH 5 and room temperature, followed by dialysis at pH 10. Absence of imidazole under a similar condition did not give this intramolecularly cross-linked lysozyme derivative (CL-lysozyme) but resulted in the formation of intermolecularly cross-linked lysozyme oligomers. From the mechanistic studies on the formation of CL-lysozyme, imidazole was suggested to play the following three roles. (1) Some carboxyl groups activated by EDC in lysozyme were converted to acylimidazole groups which protected them from the reaction with amino groups in other lysozyme molecules at pH 5. These could be hydrolyzed at pH 10 to regenerate free carboxyls. (2) High concentrations of imidazole (pH 5) increased the ionic strength of the solution which weakened the salt bridge in lysozyme and facilitated the activation of the α-carboxyl group by EDC. (3) The α-carboxyl group activated by EDC was converted to an acylimidazole group which could react with the ϵ-amino group of Lys-13 in the same molecule to form an amide bond. The last step may involve some conformational change of the backbone of lysozyme and be slower than the hydrolysis reaction of the α-carboxyl group activated by EDC itself. However, acylimidazole groups are stable against hydrolysis at pH 5. This may afford enough time to allow the ϵ-amino group of Lys-13 to attack the acylimidazole group of Leu-129.

Many proteins contain intramolecular covalent cross-links of disulfide groups. The function of cross-links is believed to stabilize the native conformation of protein. Flory (1956) and Poland & Scheraga (1965) have estimated that introducing cross-links will destabilize the unfolded structure due to the entropy loss of random coil. However, this entropy effect has rarely been measured experimentally with a protein in which an artificial cross-link is introduced except in the case of the lysozyme derivative cross-linked between Glu-35 and oxindolylalanine-108 as an ester bond (Johnson et al., 1978). For general evaluation of this entropy effect, it is necessary to compare the experimental results from well-characterized protein derivatives in which artificial cross-links are introduced at various sites, respectively. Hen egg lysozyme is a good protein for this purpose because it is one of the best characterized proteins (Imoto et al., 1972).

Lysozyme contains one clear salt bridge in the crystalline state between groups of opposite charge provided by the ϵ-amino group of Lys-13 and the terminal carboxyl group of Leu-129 (Blake et al., 1967). In this paper, we describe the conversion of this specific salt bridge to an amide bond.

1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC)[1] is known to promote the formation of amide linkages between protein carboxyl and protein amino groups (Sheehan & Hlavka, 1957; Marfey et al., 1965; Combarnous & Henner, 1974; Timkovich, 1977; Weare & Reichert, 1979). However, in the absence of a nucleophile (usually an amine), polymerization of lysozyme was predominant (Timkovich, 1977). Thus, our strategy was activation of the carboxyl group of Leu-129 and simultaneous protection of the other carboxyls with some nucleophiles by EDC reaction. After the activated carboxyl group of Leu-129 is converted to an amide bond with the ϵ-amino group of Lys-13, the other protected carboxyl groups, which are not reactive enough to provide polymerization, are hydrolyzed to regenerate free carboxyl groups. Imidazole and pyridine were found to be useful nucleophiles.

Experimental Procedures

Materials. Five times recrystallized hen egg white lysozyme was donated from Eisai Co. (Tokyo, Japan). The lysozyme derivative, where Asp-101 was modified with ethanolamine, was prepared as described previously (Yamada et al., 1981). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC) was purchased from Protein Research Foundation (Japan). Bio-Rex 70 (100–200 mesh) and Sephadex G-100 were obtained from Bio-Rad Laboratories and Pharmacia, respectively.

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 vaccum, 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. Tryptic digestion of reduced and S-carboxymethylated lysozyme or its derivative and separation of tryptic peptides on reversed-phase high-performance liquid chromatography (TSK-GEL LS-410 ODS SIL, 5 μm, Toyo Soda, Japan) were accomplished as described previously (Yamada et al., 1981; Okazaki et al., 1982).

Cross-Linking Reaction of Lysozyme or Its Derivative. Lysozyme (100 mg) or its derivative (100 mg) and various additives 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 10 mL, EDC was added to the solution with

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[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; TPCK, L-1-(*p*-tosylamido)-2-phenylethyl chloromethyl ketone; NAG, *N*-acetyl-D-glucosamine; $(NAG)_n$, oligomers of *N*-acetyl-D-glucosamine; Im, imidazole; CL-lysozyme, lysozyme derivative in which the ϵ-amino group of Lys-13 and the α-carboxyl group of Leu-129 are cross-linked in an amide bond.

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

amino acid	native		I[b]	II	III
	theory	control			
Asp	21	20.5	20.7	20.4	20.7
Thr	7	6.7	7.0	6.7	7.0
Ser	10	9.1	9.2	8.9	9.2
Glu	5	5.1	5.4	5.2	5.2
Pro	2	1.9	1.8	2.2	1.8
Gly	12	12.2	12.2	12.1	12.2
Ala	12	12	12	12	12
Val	6	5.7	5.5	5.6	5.7
Met	2	1.9	2.2	1.9	2.1
Ile	6	5.6	5.5	5.5	5.6
Leu	8	8.3	7.2	8.1	8.2
Tyr	3	2.9	3.2	3.0	3.0
Phe	3	3.0	3.2	3.0	3.0
Lys	6	5.9	6.1	6.2	6.3
His	1	1.0	1.0	1.0	1.0
Arg	11	11.1	10.1	11.0	11.3

[a] All values are expressed as molar ratios normalized to a value of 12 for alanine. [b] See Figure 1.

stirring at room temperature. The pH of the solution was maintained at 5.0 for 1 day by adding dilute HCl. Usually, the pH change ceased within 2 h. Then, the reaction mixture was dialyzed against 0.02 M borate buffer at pH 10 and room temperature for several hours and then against distilled water exhaustively. For detection of intermediates, the dialysis was carried out at 4 °C against distilled water directly without exposing the reaction mixture to high pH. Each reaction condition is listed in Table III.

Results

Intramolecular Cross-Linking of Lysozyme in the Presence of Imidazole by EDC Reaction. Lysozyme (100 mg) was stirred with 32 mg of EDC in the presence of 1 M imidazole in 10 mL of water at pH 5 and room temperature for 1 day (run 5 in Table III). The reaction mixture was dialyzed against 0.02 M borate buffer at pH 10 and room temperature for several hours and then against distilled water. Gel filtration of the dialysate through Sephadex G-100 (1.5 × 150 cm column, 0.05 M phosphate buffer at pH 7) showed that a small amount (3.3%) of intermolecularly cross-linked polymer of lysozyme was formed. The monomer fraction was separated and chromatographed on Bio-Rex 70 at pH 7, and the pattern is shown in Figure 1A. The major peak appeared at the elution position of native lysozyme. This peak was rechromatographed on Bio-Rex 70 at pH 10, resulting in the resolution of three peaks as shown in Figure 1B (peaks I–III), where the elution position of native lysozyme is indicated by the arrow. Amino acid compositions of the three fractions are listed in Table I. The derivative in peak I showed a somewhat different amino acid composition from that of native lysozyme and seemed to lack the Arg-Leu carboxy terminus. We have not further investigated this derivative because of its low yield (less than 4%).

The derivative in peak II had the same amino acid composition as that of native lysozyme (Table I) and was eluted at an identical position on Bio-Rex 70 ion-exchange chromatography at pH 7 as well as pH 10. Peptide analysis also indicated that this derivative was identical with native lysozyme (data are not shown). Thus, we conclude that peak II is native lysozyme.

The derivative in peak III showed the same amino acid composition as that of native lysozyme, indicating a small degree of modification. The UV spectrum of this derivative in the 240–300-nm region was identical with that of native

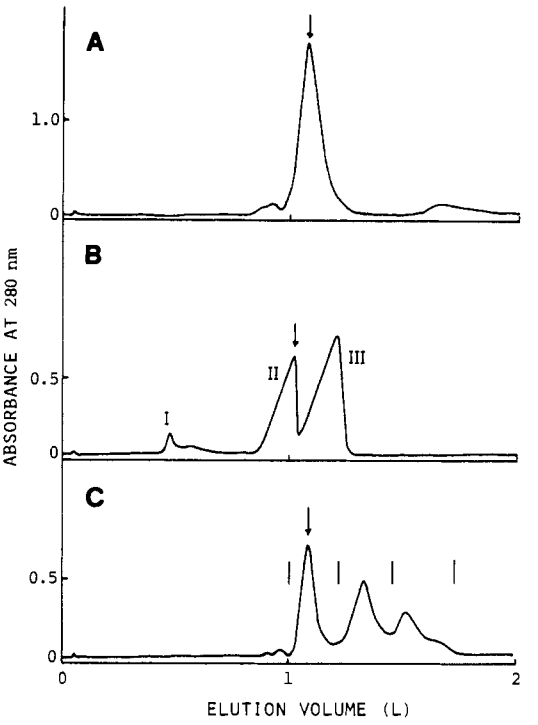


FIGURE 1: Ion-exchange chromatography of derivatives obtained from the reaction of lysozyme with EDC in the presence of imidazole on Bio-Rex 70 (100–200 mesh). (A) Reaction mixture of 100 mg of lysozyme, 1 M imidazole, and 32 mg of EDC in 10 mL of H_2O at pH 5 for 1 day followed by dialysis at pH 10. The column (1.3 × 70 cm) was 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. (B) Rechromatography of the major peak in (A). The column (1 × 200 cm) was eluted with the gradient of 1 L of 0.02 M borate buffer (pH 10) containing 0.03 M NaCl and 1 L of the same buffer containing 0.16 M NaCl. (C) Reaction mixture of 100 mg of lysozyme, 0.3 M imidazole, and 14 mg of EDC in 10 mL of H_2O at pH 5 for 1 day followed by dialysis against distilled water at 4 °C without raising the pH to 10. The elution condition of the column was the same as described for (A).

lysozyme, indicating that reaction did not occur at tryptophan or tyrosine residues, although modification of tyrosine residues of EDC has been reported (Carraway & Koshland, 1968). On Bio-Rex 70 ion-exchange chromatography at pH 7, peak III was eluted at the position of native protein peak (Figure 1A). At pH 10, this peak was eluted just after the native protein peak (Figure 1B). These results suggest that the derivative in peak III has the same net charge as native lysozyme at pH 7 but is more cationic than native lysozyme at pH 10. This ionic behavior of the derivative in peak III is explained if one ϵ-amino group of a lysine residue and one carboxylic group are both modified in this derivative. At pH 7, both ϵ-amino groups and carboxyl groups are fully ionized with opposite charge, so that modification does not change the net charge of the enzyme at this pH. At pH 10, on the other hand, ϵ-amino groups are only partially ionized but carboxyl groups are fully ionized. Thus, such modification reduces one negative charge and less than one positive charge in the modified enzyme at pH 10, leaving the enzyme more cationic than the native.

For location of the modified groups in the derivative in peak III, the derivative was reduced and S-carboxymethylated and then hydrolyzed to peptides with TPCK-treated trypsin. The tryptic hydrolysate was analyzed by reversed-phase high-performance liquid chromatography, and the pattern is shown in Figure 2B. For comparison, the pattern from native lysozyme is shown in Figure 2A, where the assignment of each peak is also shown. In Figure 2B, the peaks corresponding

Table II: Amino Acid Composition of Peptides[a]

amino acid	amino acid ratio in peptide A[b]	B
Glu		1.0 (1)
Gly	1.0 (1)	0.2
Ala		3.0 (3)
Met		0.9 (1)
Leu		2.0 (2)
Lys		1.0 (1)
Arg	1.0 (1)	1.1 (1)
CMC[c]	0.9 (1)	1.0 (1)
assignment	T_{17}	$T_{3+4} + T_{18}$

[a] Normalized to Gly or Ala. Values less than 0.1 are omitted. Numbers in parentheses are theoretical values. [b] See Figure 2. [c] (Carboxymethyl)cysteine.

to native peptides T_{17+18} (Gly-126–Leu-129), T_3 (Cys-6–Lys-13), and T_{3+4} (Cys-6–Arg-14) have disappeared, and two new peaks (peptides A and B) have appeared. T refers to the Canfield's nomenclature of tryptic peptides (Canfield, 1963). All other peptides were eluted at the same elution volumes as those of native peptides. Peptides A and B were isolated and subjected to amino acid analyses, and the results are shown in Table II. Amino acid composition data showed peptide A to be the peptide T_{17} (Gly-126–Arg-128) and peptide B to be the peptide T_{3+4} plus T_{18} (Cys-6–Arg-14 plus Leu-129). All of these results clearly indicate that the derivative in peak III is the cross-linked lysozyme (CL-lysozyme) with the ϵ-amino group of Lys-13 and the C-terminal carboxyl group of Leu-129 intramolecularly bound as an amide. This derivative is consistent with its ionic behavior on Bio-Rex 70 ion-exchange chromatography. The terminal peptide bond of Arg-128–Leu-129 in lysozyme was hardly cleaved by trypsin (Figure 2A; Okazaki et al., 1982), but the same bond in CL-lysozyme was completely hydrolyzed. These results also suggest that this bond is not a terminal one in CL-lysozyme.

Effects of Imidazole and Other Additives on the Formation of CL-Lysozyme. In order to test the effects of imidazole and other additives [NaCl, NAG, $(NAG)_n$, phenol, *N*-hydroxysuccinimide, and pyridine] on the formation of CL-lysozyme, reactions of lysozyme and EDC at pH 5 and room temperature under various conditions were carried out. The conditions

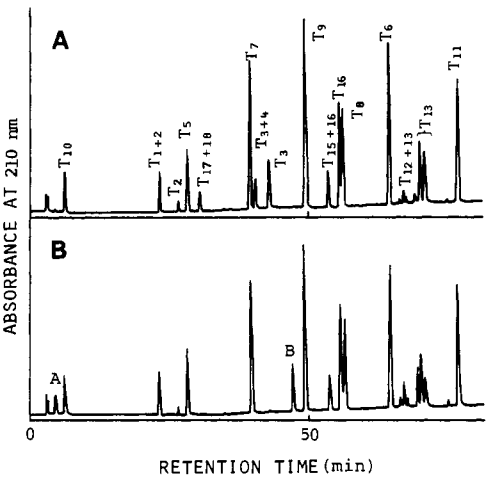


FIGURE 2: Reversed-phase high-performance liquid chromatography of tryptic peptides derived from reduced and S-carboxymethylated lysozyme derivatives on TSK-GEL LS-410 ODS SIL (4 × 300 mm). The column was eluted with the gradient of 40 mL of 1% acetonitrile and 40 mL of 40% acetonitrile, both containing 0.1% concentrated HCl at a flow rate of 0.8 mL/min. (A) From native lysozyme; (B) from fraction III in Figure 1B.

employed and the yields of CL-lysozyme and oligomer formed are listed in Table III. In the absence of any additive, CL-lysozyme was not formed, but intermolecularly cross-linked oligomer was obtained (11.4%, run 1). In the presence of NAG (run 3), polymerization of lysozyme was reduced (4.6%) but still CL-lysozyme was not obtained. In marked contrast, the presence of imidazole (runs 5–8) resulted in good yields of CL-lysozyme. At 0.3 M imidazole, lysozyme oligomer was not detected, but CL-lysozyme was formed, and the amount increased with EDC concentration (runs 6–8). The highest concentration of imidazole (1 M, run 5) increased the yield of CL-lysozyme (49%), compared with the case of 0.3 M imidazole (27–29%, runs 6 and 7), although some lysozyme oligomer was also formed. Addition of 0.5 M NaCl to the reaction mixture in the presence of 0.3 M imidazole also increased the yield of CL-lysozyme (45%, run 10) with some formation of lysozyme oligomer, but addition of 10 mg of a mixture of *N*-acetyl-D-glucosamine oligomers [$(NAG)_n$, n = 1–7] decreased the yield of CL-lysozyme (15%, run 9).

Table III: Yields of CL-Lysozyme and Lysozyme Oligomer under Various Conditions in the Reaction of Lysozyme with EDC in 10 mL of Aqueous Solution at pH 5.0 and Room Temperature for 1 Day[a]

run	lysozyme (100 mg)	additive, concn	amount of EDC used (mg)	yield (%) CL-lysozyme	yield (%) oligomer
1	native		30	0	11.4
2	native	NaCl, 0.5 M	30	0	72
3	native	NAG, 100 mg	30	0	4.6
4	native	NAG, 100 mg; NaCl, 0.5 M	30	0	49
5	native	Im,[c] 1 M	32	49	3.3
6	native	Im, 0.3 M	30	27	0
7	native	Im, 0.3 M	30	29	0
8	native	Im, 0.3 M	14	13	0
9	native	Im, 0.3 M; $(NAG)_n$,[d] 10 mg	30	15	0
10	native	Im, 0.3 M; NaCl, 0.5 M	30	45	5.3
11	native	pyridine, 1 M	30	15	0
12	native	phenol, 0.1 M	30	3	0
13	native	*N*-hydroxysuccinimide, 1 M	30	5	0
14	Asp-101-$CONHCH_2CH_2OH$[b]	Im, 0.3 M	30	22	0

[a] The pH of the solution was adjusted to 5 with HCl. [b] Lysozyme derivative where Asp-101 was modified with ethanolamine (Yamada et al., 1981). [c] Imidazole. [d] A mixture of *N*-acetyl-D-glucosamine oligomers: n = 1, 22%; n = 2, 22%; n = 3, 21%; n = 4, 18%; n = 5, 11%; n = 6, 5%; n = 7, 1% (w/w).

All other nucleophiles tested in place of imidazole protected lysozyme from the formation of oligomer, but the yield of CL-lysozyme was very dependent on the kind of nucleophile present (runs 11–14). In the case of phenol (run 12) or *N*-hydroxysuccinimide (run 13), the yield of CL-lysozyme was very low (less than 5%), but in the case of pyridine, CL-lysozyme was obtained in relatively good yield (15%, run 11). In every case, no other prominent products were obtained.

Detection of Intermediates in the Reaction of Lysozyme in the Presence of Imidazole. As EDC has been used for modification of the carboxyl groups of lysozyme with nucleophiles (Hoare & Koshland, 1966; Lin & Koshland, 1969; Lin, 1970; Kramer & Rupley, 1973; Yamada et at., 1981; Okazaki et al., 1982), some of the carboxylic groups of lysozyme must be modified with the nucleophiles employed here also. However, all products are expected to be unstable under the hydrolysis condition (pH 10), since the modified carboxyl groups expected here are acylimidazole, phenyl ester, *N*-hydroxysuccinimide ester, and acylpyridinium salt. In order to detect intermediates in the case of imidazole, the reaction mixture of 100 mg of lysozyme, 14 mg of EDC, and 0.3 M imidazole in 10 mL of solution at pH 5 and room temperature for 1 day (run 6 in Table III) was directly dialyzed against distilled water at 4 °C without raising the pH of the solution to 10 and then analyzed by Bio-Rex 70 ion-exchange chromatography at pH 7. As shown in Figure 1C, the pattern was different from that shown in Figure 1A, and a considerable amount of protein was eluted after the native protein peak, indicating that some of the carboxyl groups were modified with imidazole. Eluent containing protein was fractionated to three portions as shown with vertical bars in Figure 1C, and each fraction was incubated at pH 10 for 1 day and dialyzed against distilled water. Rechromatography of each fraction on Bio-Rex 70 at pH 7 gave a pattern similar to that shown in Figure 1A, indicating that the modified carboxyl groups with imidazole were hydrolyzed to free carboxyl groups during the incubation at pH 10. Furthermore, analysis of each fraction by ion-exchange chromatography on Bio-Rex 70 at pH 10 indicated that all of the fractions contained CL-lysozyme and native lysozyme in a similar ratio (total yield of CL-lysozyme was 13%).

Effects of Modification at Asp-101 in Lysozyme on the Formation of CL-Lysozyme. As mentioned above, substrate analogues, $(NAG)_n$, which bind the active site of lysozyme far from the site of cross-linking, affected the formation of CL-lysozyme. As Asp-101 is located at the edge of the active site cleft, it is interesting to study whether modification of Asp-101 in lysozyme affects the formation of CL-lysozyme. Thus, the reaction of the lysozyme derivative modified at Asp-101 with ethanolamine was tested (run 14). The yield of the cross-linked product (22%) was somewhat lower than that obtained from native lysozyme (27–29%, runs 6 and 7) under a similar condition.

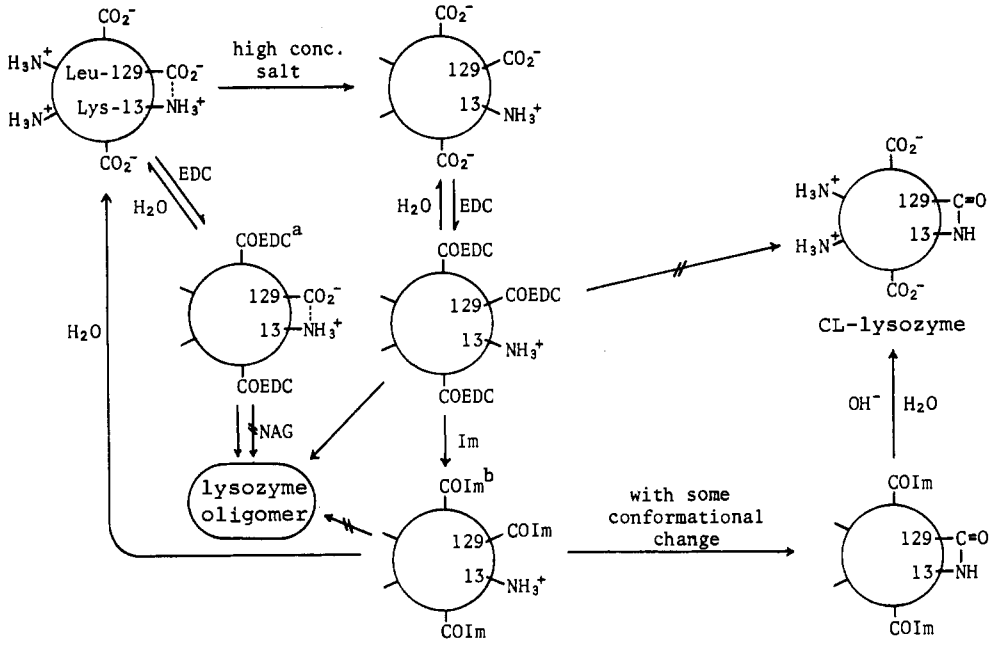
Discussion

X-ray crystallographic studies of hen egg white lysozyme indicated that Lys-13 (ϵ-amino) forms a salt bridge with the carboxyl terminus, Leu-129 (α-COO^-) (Blake et al., 1967). Recently, it has been shown that this salt bridge is also present in solution (Gerken et al., 1982). Although EDC is frequently used for amide bond formation in proteins (Sheehan & Hlavka, 1957; Marfey et al., 1965; Combarnous & Henner, 1974; Timkovich, 1977; Weare & Reichert, 1979), direct use of EDC for conversion of this specific salt bridge in lysozyme to an amide bond was not successful because it gave only lysozyme oligomer (Table III, run 1; Timkovich, 1977). However, in the presence of 0.3–1 M imidazole, reaction of lysozyme by EDC at pH 5 and subsequent dialysis at pH 10 gave the desired cross-linked lysozyme (CL-lysozyme) with much reduced formation of lysozyme oligomer (Table III, runs 4–7). Thus, we conclude that imidazole not only catalyzes the formation of CL-lysozyme but also protects lysozyme from polymerization by EDC reaction.

Mechanism of Formation of CL-Lysozyme. Polymerization of lysozyme by EDC results from attack of an amino group in one lysozyme molecule with the EDC-activated carboxyl in the other molecule. In the presence of an amine nucleophile, the nucleophilic attack of the amine molecule to the activated carboxyl groups in lysozyme may become exclusive as was found in many studies (Hoare & Koshland, 1966; Lin & Koshland, 1969; Lin, 1970; Kramer & Rupley, 1973; Yamada et al., 1981; Okazaki et al., 1982). When imidazole is employed as a nucleophile, some of the carboxyl groups of lysozyme are converted to acylimidazole groups. This can be seen in Figure 1C where ion-exchange chromatography on Bio-Rex 70 at pH 7 was performed without prior dialysis of the reaction mixture at pH 10 (Table III, run 7). This result indicates that the acylimidazole groups are stable and prevent the polymerization of lysozyme at least under the reaction conditions employed (pH 5). On incubation of the reaction mixture at pH 10, the acylimidazole groups are hydrolyzed to regenerate free carboxyl groups (Figure 1A). Thus, imidazole works as a protecting reagent of the carboxyl groups of lysozyme from the undesired polymerization reaction.

In the presence of imidazole at pH 5, the ionic strength of the reaction medium will be high. On the other hand, in the absence of imidazole, the ionic strength of the reaction medium is very low (run 1). The yield of CL-lysozyme increased markedly when the concentration of imidazole was increased (runs 5–7), although the amounts of EDC used were not precisely the same. Addition of 0.5 M NaCl to 0.3 M imidazole solution also increased the formation of CL-lysozyme. These results indicate that the salt bridge between Lys-13 (ϵ-NH_3^+) and Leu-129 (α-COO^-) in lysozyme is weakened in a high ionic strength medium, thus facilitating activation of the α-carboxyl group by EDC. It is possible that low ionic strength is the reason why no CL-lysozyme was formed in the absence of imidazole (run 1). In order to test this possibility, the experiments in the presence of 0.5 M NaCl without imidazole were carried out (run 2). No Cl-lysozyme was formed, but polymerization of lysozyme became extensive (72%). The increased polymerization of lysozyme under the condition used may be explained by the increased self-association of lysozyme by salts (Hampe, 1972). We found that NAG, a substrate analogue, reduced the polymerization of lysozyme even in the absence of nucleophile (run 3). This may also be explained by the self-association of lysozyme which is inhibited by NAG (Sophianopoulos, 1969). In the presence of both NAG and NaCl, more lysozyme was recovered as monomer than in the presence of NaCl alone, but no CL-lysozyme was formed (run 4). In these cases, the α-carboxyl group should be activated by EDC because ionic strength of the reaction medium was high enough (0.5 M NaCl). Thus, it is concluded that not only high ionic strength but also the presence of imidazole are necessary for the formation of CL-lysozyme, indicating that the α-carboxyl group of lysozyme is converted to acylimidazole. One possible explanation for this follows. In the crystalline state of lysozyme, the distance between the nitrogen of the ϵ-amino group of Lys-13 and the α-carboxyl carbon of Leu-129 is 3.15 Å (Imoto et al., 1972). For amide bond formation, the distance between them must be shortened by about 1.8 Å.

Scheme I



[a] Activated carboxyl group by EDC. [b] Acylimidazole.

This may require some conformational change of the backbone in lysozyme and may be a slow process. If the α-carboxyl group activated by EDC is not stable, nucleophilic attack by water or imidazole may be faster than amide bond formation. Then, in the absence of imidazole, the α-carboxyl group activated by EDC is just hydrolyzed, and no cross-linking occurs. On the other hand, in the presence of imidazole, the α-carboxyl group activated by EDC is converted to the acylimidazole group which is stable against hydrolysis under the reaction condition (pH 5) as mentioned before. This may afford enough time to allow the ε-amino group of Lys-13 to attack the acylimidazole group of Leu-129, resulting in the formation of CL-lysozyme. Another explanation is that some conformational change of the backbone in lysozyme is not required for amide bond formation but that the ε-amino group of Lys-13 and the α-carboxyl group activated by EDC are not properly oriented to form the amide bond. When the α-carboxyl group is converted to acylimidazole, the orientation improves for the formation of CL-lysozyme. It is known that oligosaccharides of *N*-acetyl-D-glucosamine stabilize the native conformation of lysozyme (Imoto et al., 1976; Nakanishi et al., 1973; Pace & McGrath, 1980), suggesting that the conformational change mentioned above may be also restricted by them. Thus, in the former mechanism, the presence of oligosaccharides of *N*-acetyl-D-glucosamine is expected to reduce the formation of CL-lysozyme, but in the latter mechanism, it may cause a minor effect on the formation of CL-lysozyme. The fact that the presence of a mixture of oligomers of *N*-acetyl-D-glucosamine reduced the yield of CL-lysozyme (run 9) indicates that the former mechanism is more probable. It is interesting to note that the lysozyme derivative modified with ethanolamine at Asp-101 gave a somewhat lower yield of the cross-linked derivative than native lysozyme. Asp-101 is located in the active site cleft of lysozyme and far from the site of cross-linking. Therefore, it is possible that the ethanolamine residue at Asp-101 tightens the native conformation of the enzyme as substrate analogues do.

In Scheme I, the most probable mechanism for the formation of CL-lysozyme catalyzed by imidazole in the EDC reaction is presented.

Effects of Nucleophiles as a Catalyst on the Formation of CL-Lysozyme. It is evident from Table III that a nitrogen nucleophile (imidazole or pyridine) is more effective than an oxygen nucleophile (phenol or *N*-hydroxysuccinimide) as a catalyst for the formation of CL-lysozyme. The reason for this is not clear but may be related to the difference of nucleophilicity between nitrogen and oxygen. As for nitrogen nucleophiles, imidazole seems more effective than pyridine. Since the pK_a of imidazole [7.14 (Paiva et al., 1976)] is higher than that of pyridine [5.25 (Linnell, 1960)], the nucleophile with a higher pK_a may be more effective. Thus, imidazole was found to be the best catalyst among the nucleophiles tested here for the formation of CL-lysozyme.

The detailed properties of CL-lysozyme will be published elsewhere.

Conclusions

We found that the salt bridge between Lys-13 (ϵ-NH_3^+) and Leu-129 (α-COO^-) in lysozyme can be converted to an amide bond, catalyzed by imidazole in the EDC reaction. Imidazole was found to act not only as a catalyst for this bond formation but also as a protecting group of other carboxylic acids that cause the polymerization of lysozyme. This method may be useful in studying intramolecular salt bridges in other protein molecules or intermolecular salt bridges in contact areas of oligomeric proteins. Amide bonds may be formed without modifying any other residues.

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Registry No. Lysozyme, 9001-63-2; Lys, 56-87-1; Leu, 61-90-5; EDC, 25952-53-8; Im, 288-32-4; phenol, 108-95-2; *N*-hydroxysuccinimide, 6066-82-6; pyridine, 110-86-1; Asp, 56-84-8.

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Inhibition of Collagenase from *Clostridium histolyticum* by Phosphoric and Phosphonic Amides†

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ABSTRACT: Di- and tripeptides with sequences present in collagen that are known to occupy the S_1' through S_3' subsites at the active site of the collagenase from *Clostridium histolyticum* do not themselves inhibit this zinc protease. Thus glycylproline, glycylprolylalanine, and their C-terminal amides are not inhibitors. N^{α}-Phosphorylglycylproline, N^{α}-phosphorylglycyl-L-prolyl-L-alanine, and their C-terminal amides are weak inhibitors with IC_{50}'s (concentration causing half-maximal inhibition) of 4.6, 0.8, 3, and 1.5 mM, respectively. Extension of glycyl-L-prolyl-L-alanine to L-leucyl-glycyl-L-prolyl-L-alanine gives a tetrapeptide known to occupy the S_1, S_1', S_2', and S_3' subsites of collagenase when present in collagen but that still does not itself inhibit the enzyme. (Isoamylphosphonyl)glycyl-L-prolyl-L-alanine, a peptide containing a tetrahedral phosphorus atom at the position of the amide carbonyl carbon of the L-leucylglycyl amide bond of the parent tetrapeptide, inhibits collagenase with an IC_{50} of 16 μM, at least 1000-fold more potent than the parent peptide. Substitution of the two-carbon ethyl chain of alanine for the five-carbon isoamyl chain of leucine increases the IC_{50} to 46 μM. Substitution of the *n*-decyl chain for the isoamyl chain does not change the IC_{50}. (Isoamylphosphonyl)glycyl-glycyl-L-proline contains a tripeptide that does not occupy the S_1' through S_3' subsites of collagenase when this peptide is present in collagen and thus has an IC_{50} of 4.4 mM. (Isoamylphosphonyl)glycyl-L-prolyl-L-alanine may be an analogue of the tetrahedral transition state for the hydrolysis of the natural collagen substrate. However, the IC_{50} of this inhibitor is 3-4 orders of magnitude higher than those of the best phosphorus-containing transition-state analogues of other zinc proteases. In addition, this inhibitor lacks specificity for its target, having a K_i for angiotensin converting enzyme of 11 μM, about equal to its IC_{50} for collagenase.

The collagenase from *Clostridium histolyticum* (EC 3.4.24.3) is a zinc metalloprotease also known as clostridiopeptidase A and collagenase A (Seifter & Harper, 1971). This collagenase makes a large number of cleavages in native triple-helical collagen, usually at the X-glycine bond in the sequence X-glycine-proline-Y where X is any amino acid and Y is frequently alanine or hydroxyproline. Synthetic oligopeptides are cleaved with similar specificity. Exceptions to this specificity that have been observed could be due to heterogeneity of the enzyme preparations (four homogeneous collagenases from *C. histolyticum* have been described; Lwebuga-Mukasa et al., 1976) or to the natural activity of pure collagenase (Seifter & Harper, 1971). The triple-helical region of native soluble collagen is highly resistant to nearly every protease except the collagenases (Burleigh, 1977). Compounds that are nonspecific inhibitors of all zinc metalloproteases such as cysteine inhibit collagenase (Seifter &

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