Deisenhofer, J., & Steigemann, W. (1975) *Acta Crystallogr., Sect. B: Struct. Crystallogr. Cryst. Chem. B31*, 238–250.
Dennis, J. E., & Mei, H. H. W. (1975) Technical Report 75-246, Department of Computer Sciences, Cornell University, Ithaca, NY.
Dunfield, L. G., & Scheraga, H. A. (1980) *Macromolecules 13*, 1415–1428.
Gay, D. M. (1983) *ACM Trans. Math. Software 9*, 503–524.
IUPAC–IUB Commission on Biochemical Nomenclature (1970) *Biochemistry 9*, 3471–3479.
Kikuchi, T., Némethy, G., & Scheraga, H. A. (1985) *J. Comput. Chem.* (in press).
Kosen, P. A., Creighton, T. E., & Blout, E. R. (1980) *Biochemistry 19*, 4936–4944.
Kosen, P. A., Creighton, T. E., & Blout, E. R. (1981) *Biochemistry 20*, 5744–5754.
Kosen, P. A., Creighton, T. E., & Blout, E. R. (1983) *Biochemistry 22*, 2433–2440.
Momany, F. A., McGuire, R. F., Burgess, A. W., & Scheraga, H. A. (1975) *J. Phys. Chem. 79*, 2361–2381.
Némethy, G., Pottle, M. S., & Scheraga, H. A. (1983) *J. Phys. Chem. 87*, 1883–1887.
Pottle, C., Pottle, M. S., Tuttle, R. W., Kinch, R. J., & Scheraga, H. A. (1980) *J. Comput. Chem. 1*, 46–58.
Richardson, J. S. (1981) *Adv. Protein Chem. 34*, 167–339.
Salemme, F. R., & Weatherford, D. W. (1981a) *J. Mol. Biol. 146*, 101–117.
Salemme, F. R., & Weatherford, D. W. (1981b) *J. Mol. Biol. 146*, 119–141.
Swenson, M. K., Burgess, A. W., & Scheraga, H. A. (1978) in *Frontiers in Physicochemical Biology* (Pullman, B., Ed.) pp 115–142, Academic Press, New York.

Isolation and Characterization of 101-β-Lysozyme That Possesses the β-Aspartyl Sequence at Aspartic Acid-101

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ABSTRACT: In the reaction of the intramolecular cross-linking between Lys-13 (ε-NH_3^+) and Leu-129 (α-COO^-) in lysozyme using imidazole and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride [Yamada, H., Kuroki, R., Hirata, M., & Imoto, T. (1983) *Biochemistry 22*, 4551–4556], it was found that two-thirds of the protein (both the recovered and cross-linked lysozymes) showed a lower affinity than the rest against chitin-coated Celite, an affinity adsorbent for lysozyme. The protein with the reduced affinity was separated on chitin-coated Celite affinity chromatography and found to be slightly different from native lysozyme in the elution position of the tryptic peptide of Ile-98–Arg-112 on reversed-phase high-performance liquid chromatography. In contrast with native lysozyme, the limited hydrolysis of this abnormal tryptic peptide of Ile-98–Arg-112 in 6 N HCl at 110 °C gave a considerable amount of β-aspartylglycine. Therefore, it was concluded that two-thirds of the protein obtained from this reaction possessed the β-aspartylglycyl sequence at Asp-101–Gly-102. As a result, we obtained four lysozymes from this reaction, the derivative with the β-aspartyl sequence at Asp-101 (101-β-lysozyme), the cross-linked derivative between Lys-13 and Leu-129 (CL-lysozyme), the CL-lysozyme derivative with the β-aspartyl sequence at Asp-101 (101-β-CL-lysozyme), and native lysozyme. In the ethyl esterification of Asp-52 in lysozyme with triethyloxonium fluoroborate [Parsons, S. M., Jao, L., Dahlquist, F. W., Borders, C. L., Jr., Groff, T., Racs, J., & Raftery, M. A. (1969) *Biochemistry 8*, 700–712; Parsons, S. M., & Raftery, M. A. (1969) *Biochemistry 8*, 4199–4205], the same bond rearrangement was detected in the same ratio. Therefore, it is concluded that the Asp-52 ethyl ester lysozyme reported had been a mixture of the derivatives with the α- and β-aspartyl sequences at Asp-101. The mechanism for the formation of 101-β-lysozyme in these reactions is discussed.

Recently, we have reported the preparation of the intramolecularly cross-linked lysozyme between the ε-amino group of Lys-13 and the α-carboxyl group of Leu-129 by the carbodiimide reaction catalyzed by imidazole (Yamada et al., 1983). During the investigation of the nature of the cross-linked lysozyme, we noticed that the thermal stability of the recovered lysozyme from this reaction was much reduced compared with native lysozyme. This observation suggested that some unknown lysozyme derivatives were formed in this reaction. Recently, we have prepared chitin-coated Celite as an affinity adsorbent for lysozyme and found the affinity chromatography on this resin to be sometimes very efficient for separation of the chemically modified lysozyme derivatives (Yamada et al., 1985). On utilizing this chromatography, we found the recovered lysozyme and the cross-linked lysozyme obtained previously were both mixtures of two components, respectively. In both cases, the components with lower affinities were the derivatives with the β-aspartyl sequence at Asp-101. Furthermore, Asp-52 ethyl ester lysozyme produced in the reaction of lysozyme with triethyloxonium fluoroborate (Parsons et al., 1969; Parsons & Raftery, 1969) was also found to be a mixture of two derivatives with the α- and β-aspartyl sequences at Asp-101.

Experimental Procedures

Materials. Five times recrystallized hen egg white lysozyme was donated from Eisai Co. (Tokyo, Japan). 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDC)[1]

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was purchased from Protein Research Foundation (Osaka, Japan). Bio-Rex 70 and Cellulofine GLC-25-m were obtained from Bio-Rad Laboratories and Seikagaku Kogyo Company, Ltd. (Tokyo, Japan), respectively. L-1-(*p*-Tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin (TPCK-trypsin) was the product of Worthington. α- and β-aspartylglycines were synthesized according to the method of Le Quesne & Young (1952). Triethyloxonium fluoroborate was synthesized from boron trifluoride etherate and epichlorohydrin (both from Nakarai Chemicals Ltd., Kyoto, Japan) as described by Meerwein et al. (1937). Chitin-coated Celite, an affinity adsorbent for lysozyme derivatives, was prepared as described elsewhere (Yamada et al., 1985).

Analytical Methods. Amino acid analysis for determination of amino acid composition was performed on a Hitachi 835 amino acid analyzer after hydrolysis of protein or peptide sample in 6 N HCl under vacuum, at 110 °C for 20 h. Analytical affinity chromatography of lysozyme derivatives on a chitin-coated Celite column (4 × 50 mm) using high-performance liquid chromatography (HPLC) was performed at 0 °C with a Hitachi 655 liquid chromatograph equipped with a Hitachi wavelength-tunable effluent monitor as described previously (Yamada et al., 1985). Preparative affinity chromatography of lysozyme derivatives on a 6.5 × 10 cm column of chitin-coated Celite was performed in a cold room (4 °C). The column was preequilibrated with 0.1 M sodium acetate buffer containing 0.5 M NaCl at pH 5.5. After a sample was loaded, the column was eluted with the concave gradient by using two chambers at a flow rate of 1 mL/min. The first chamber contained 1 L of 0.1 M acetate buffer (pH 5.5) containing 0.5 M NaCl, and the second chamber contained 270 mL of 2.5 M acetic acid. The protein elution was monitored by absorbance of effluents at 280 nm with a Hitachi 200-10 double-beam spectrophotometer. The protein thus separated was precipitated with 85% saturation of $(NH_4)_2SO_4$, collected by centrifugation, redissolved in a small amount of water, dialyzed against distilled water, and then lyophilized. Reversed-phase high-performance liquid chromatography (RP-HPLC) of tryptic peptides obtained from lysozymes was done as before (Okazaki et al., 1982). Separation of tryptic peptides with gel chromatography was performed on a 2.5 × 85 cm column of Cellulofine GCL-25-m, which was eluted with 0.1% concentrated HCl at a flow rate of 0.5 mL/min and room temperature. The peptide elution was monitored by absorbance of effluents at 230 nm. For determination of the stability of α- or β-aspartylglycine or asparagine under the condition of the acid hydrolysis, 40 nmol of the sample was dissolved in 200 μL of 6 N HCl in a test tube, and the tube was evacuated and sealed. The sealed tube was heated at 110 °C in a boiling toluene bath for an appropriate period. The hydrolysis was stopped by dipping the sealed tube into ice–water. The content in the tube was evaporated to dryness below 0 °C, and the residue was dissolved in 300 μL of 0.02 N HCl and then analyzed on a Hitachi 835 amino acid analyzer. Similarly, 70 nmol of a tryptic peptide from lysozyme was hydrolyzed in 200 μL of 6 N HCl under vacuum at 110 °C for an appropriate period, and the hydrolysate was analyzed by the amino acid analyzer for detection of α- and/or β-aspartylglycine.

Preparations of Lysozyme Derivatives. The reaction of lysozyme with EDC in the presence of imidazole at pH 5 was carried out under the condition of run 6 of Table III in the previous paper (Yamada et al., 1983). After incubation of the reaction mixture at alkaline pH, the cross-linked lysozyme and the recovered lysozyme were separated as described.

The reaction of lysozyme with triethyloxonium fluoroborate at pH 4.5 was carried out according to the method of Parsons et al. (1969). Isolation of components III and IV designated by them and the conversions from component III to the recovered lysozyme and from component IV to Asp-52 ethyl ester lysozyme were performed as described. The recovered lysozyme and Asp-52 ethyl ester lysozyme were further purified with ion-exchange chromatography on a column of the carboxylic cation exchanger Bio-Rex 70 utilizing the buffer system (pH 7.18) described by them.

Reaction of Lysozyme Derivative with EDC in the Presence of Methylamine. This was done according to the method for native lysozyme described previously (Yamada et al., 1981).

Tryptic Digestion. Fifty milligrams of reduced and S-carboxymethylated lysozyme or its derivative (Crestfield et al., 1963) was suspended in 5 mL of 0.1 M phosphate buffer (pH 8.0) and digested with TPCK-trypsin (1% lysozyme weight) at 40 °C for 4 h. The digestion was stopped by adding phosphoric acid to bring the pH to 2.0, and then the mixture was filtered through a membrane filter (0.45 μm, Toyo Roshi, Japan). The filtrate was kept frozen until used.

Results and Discussion

Isolations of the Abnormal Lysozyme and the Abnormal Cross-Linked Lysozyme from the Reaction Mixture of Lysozyme with EDC in the Presence of Imidazole. Lysozyme was treated with EDC at pH 5 in the presence of imidazole [run 6 of Table III in the previous paper, Yamada et al. (1983)]. Separation of the recovered lysozyme and the intramolecularly cross-linked lysozyme between Lys-13 and Leu-129 was done as described previously (Yamada et al., 1983). The recovered lysozyme and the cross-linked lysozyme obtained in this way were analyzed by affinity HPLC on a 4 × 50 mm column of chitin-coated Celite. The chromatographic patterns are shown in panels B and C of Figure 1, respectively. For comparison, the pattern of native lysozyme is also shown in Figure 1A. Both the recovered and cross-linked lysozymes gave two peaks of the similar pattern. The ratio of the area of the former peak (peak I or III) to that of the latter peak (peak II or IV) was 2:1 in each lysozyme. The elution positions of the latter peaks were the same as that of native lysozyme (Figure 1A). These results clearly indicate that both the recovered and cross-linked lysozymes obtained previously were mixtures of two components and contained the derivatives with the abnormally reduced saccharide binding ability as major components, respectively. Derivatives I and II or derivatives III and IV could not be separated by Bio-Rex 70 ion-exchange chromatography at pH 7 or 10 (Yamada et al., 1983). Therefore, it is suggested that the abnormal derivatives I and III are the lysozymes modified commonly at the positions around the saccharide binding sites of derivatives II and IV, respectively, and the modification does not alter the net charges under the conditions of ion-exchange chromatography (pH 7 and 10). These four derivatives, abnormal lysozyme (derivative I), normal lysozyme (derivative II), abnormal cross-linked lysozyme (derivative III), and normal cross-linked lysozyme (derivative IV), were isolated respectively by preparative affinity chromatography on a 6 × 10 cm

[1] Abbreviations: EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; TPCK, L-1-(*p*-tosylamido)-2-phenylethyl chloromethyl ketone; RP, reversed phase; HPLC, high-performance liquid chromatography; 101-β-lysozyme, lysozyme derivative with the β-aspartyl sequence at Asp-101; CL-lysozyme, lysozyme derivative in which the ε-amino group of Lys-13 and the α-carboxyl group of Leu-129 are cross-linked intramolecularly as an amide bond; 101-β-CL-lysozyme, CL-lysozyme derivative with the β-aspartyl sequence at Asp-101.

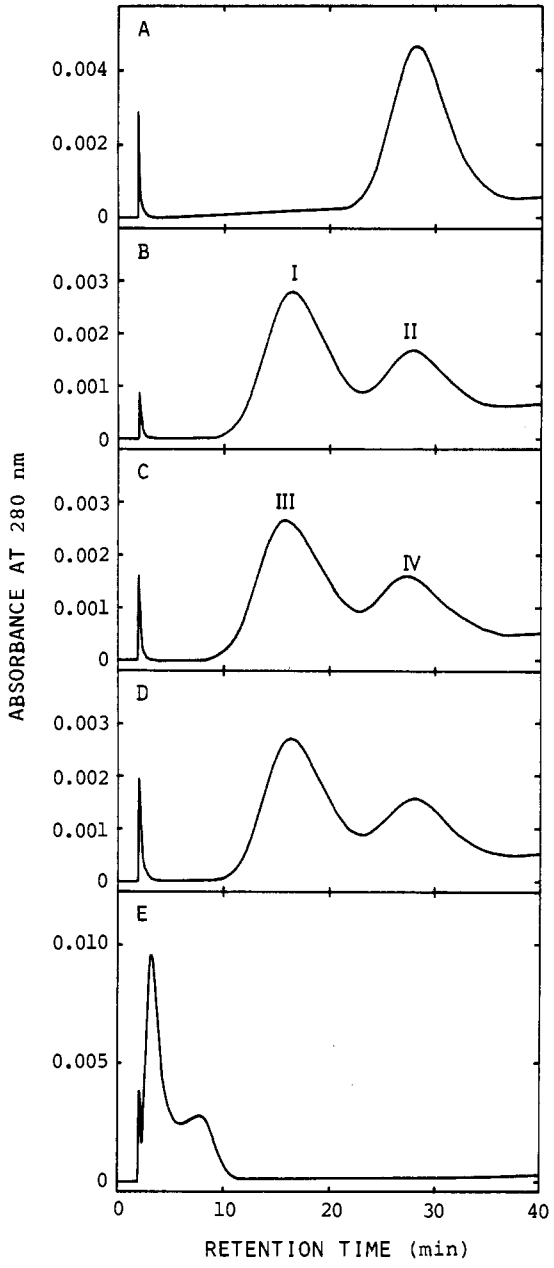


FIGURE 1: Affinity HPLC of lysozyme derivatives on a chitin-coated Celite column (4 × 50 mm). About 10 μg of protein was injected, and the column was eluted with a gradient of 10 mL of 0.1 M acetate buffer (pH 5.5) containing 0.5 M NaCl and 10 mL of 1 M acetic acid at a flow rate of 0.5 mL/min and 0 °C. (A) Native lysozyme; (B) the recovered lysozyme from the reaction of lysozyme with EDC in the presence of imidazole; (C) the cross-linked lysozyme obtained from the reaction of lysozyme with EDC in the presence of imidazole; (D) the recovered lysozyme from the reaction of lysozyme with triethyloxonium fluoroborate; (E) the Asp-52 ethyl ester lysozyme obtained from the reaction of lysozyme with triethyloxonium fluoroborate. Details are given in the text.

column of chitin-coated Celite (data are not shown). Amino acid compositions of these derivatives were all identical with that of native lysozyme (data are not shown).

Location of the Abnormal Site in the Tryptic Peptides of Derivative I (or III). In order to clarify what was different between derivatives I and II or between derivatives III and IV, peptide analyses of these derivatives were carried out on reversed-phase high-performance liquid chromatography (RP-HPLC; Okazaki et al., 1982). A reduced and S-carboxymethylated lysozyme (Crestfield et al., 1963) was digested with TPCK-trypsin at pH 8.0, and the digestion was

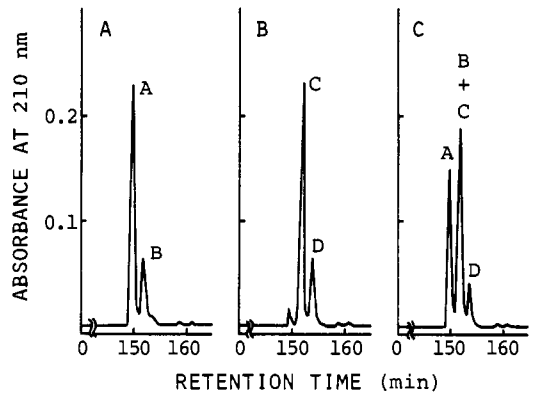


FIGURE 2: RP-HPLC of tryptic peptides derived from reduced and S-carboxymethylated lysozyme derivatives on TSK gel ODS-120A (4 × 300 mm). The column was eluted with a gradient of 40 mL of 1% acetonitrile and 40 mL of 40% acetonitrile, both containing 0.1% concentrated HCl, at a flow rate of 0.4 mL/min. (A) From derivative I; (B) from derivative II; (C) mixture of both.

stopped by lowering the pH to 2.0. The elution pattern of tryptic peptides thus obtained from derivative I seemed to be identical with that obtained from derivative II. However, the coinjection of both peptides revealed that there was a slight but clear difference in the T_{13} peptides (Ile-98–Arg-112). T refers to Canfield's nomenclature (1963). In Figure 2A–C, the elution patterns of the T_{13} peptides from derivative I, derivative II, and the mixture of both are shown, respectively. Previously we have reported the T_{13} peptide from native lysozyme to appear as two peaks and suggested the T_{13} peptide to be present as a monomer–dimer mixture (Yamada et al., 1981). As shown in Figure 2A,B, both the T_{13} peptides from derivative I and derivative II also appeared as two peaks, respectively (peptides A and B from derivative I and peptides C and D from derivative II). However, the coinjection of both tryptic peptides gave three peaks (Figure 2C), and it was found that the latter peak from derivative I (peak B) and the former peak from derivative II (peak C) were completely overlapped under the conditions employed here. The elution positions of all tryptic peptides obtained from derivative II were completely identical with those obtained from native lysozyme. The elution positions of the T_{13} peptide obtained from derivative III were identical with those from derivative I, and also those from IV were identical with those from II (or native lysozyme). Amino acid compositions of peptides A–D were all the same and consistent with that of the T_{13} peptide. These observations and the previous observations (Yamada et al., 1983) indicate that (i) derivative II is native lysozyme and derivative IV is the cross-linked lysozyme with the normal T_{13} peptide (CL-lysozyme) and (ii) derivatives I and III are the lysozyme and the CL-lysozyme with the common abnormality in the T_{13} peptide.

Under the tryptic digestion conditions employed here, the ratio of the peak heights of peptides A and B was almost similar to that of peptides C and D. When the digestion was stopped by heating the mixture in a boiling water bath for 10 min as previously employed (Okazaki et al., 1982; Yamada et al., 1983) instead of lowering the pH to 2.0, not only did the ratio of peak A to peak B or that of peak C to peak D change but also the interconversion between the normal and abnormal T_{13} peptides took place appreciably. This observation suggests that abnormality of derivative I (or III) is not due to an irreversible chemical modification such as deamidation of an asparagine residue and that the normal–abnormal interconversion may be in an equilibrium at high temperatures at least in a peptide stage.

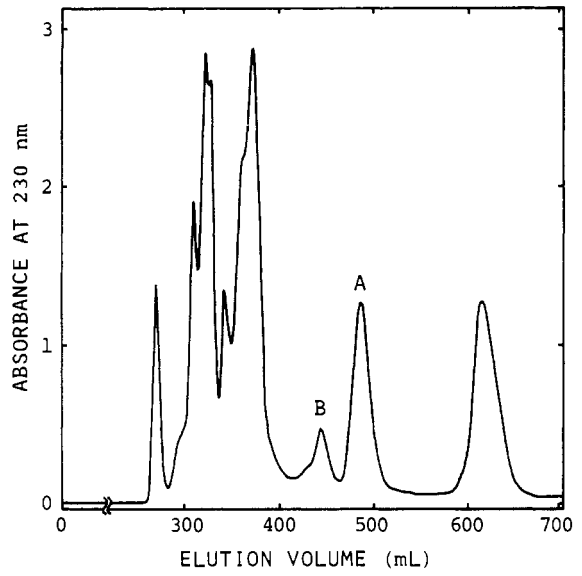


FIGURE 3: Gel chromatography of tryptic peptides derived from derivative I on Cellulofine GCL-25-m (2.6 × 85 cm). Fifty milligrams of peptides was loaded, and the column was eluted with 0.1% concentrated HCl at a flow rate of 0.5 mL/min and room temperature.

Scheme I: Amino Acid Sequence of the T_{13} Peptide

98 99 100 101 102 103 104 105 106 107 108 109 110 111 112
Ile-Val-Ser-ASP-GLY-ASN-GLY-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg
α-Asp-Gly or β-Asp-Gly
α-Asp-Gly

Isolation of the T_{13} Peptides with Gel Chromatography. In order to obtain the normal and the abnormal T_{13} peptides in large scales, 50 mg of the tryptic peptides derived from derivative I was chromatographed on a Cellulofine GCL-25-m column (Figure 3). The chromatographic pattern from native lysozyme was identical with that from derivative I. Again, the T_{13} peptides appeared as two peaks in both cases. Each T_{13} peptide was lyophilized and analyzed by RP-HPLC. The former peak in gel chromatography was of peptide B (or D) and the latter was of peptide A (or C), suggesting peptide B to be the dimer of peptide A and peptide D to be that of peptide C. For further investigations, peptide A and peptide C were used.

Detection of β-Aspartylglycine from the Abnormal T_{13} Peptide by Limited Acid Hydrolysis. As shown above, derivative I (or III) possesses a reduced saccharide binding ability compared with native lysozyme (or CL-lysozyme), and the abnormality is in the T_{13} peptide. Aspartic acid-101 has been shown to be involved in saccharide binding (Blake et al., 1967) and to be in the T_{13} peptide (Scheme I; Canfield, 1963; Imoto et al., 1981). Furthermore, the net charge of derivative I (or III) is suggested to be the same as that of native lysozyme (or CL-lysozyme) at pH 7 and 10. Therefore, we tentatively attributed the abnormality in derivative I (or III) to the formation of the β-peptide bond between Asp-101 and Gly-102 residues. As hydrazinolysis or protease digestion (Pronase and/or carboxypeptidases A, B, and Y) of the T_{13} peptide from derivative I (or III) to detect aspartic β-hydrazide or β-aspartylglycine resulted in failure, we decided to detect β-aspartylglycine by limited acid hydrolysis. Under the routine amino acid analysis condition, α- and β-aspartylglycines appeared as well-separate peaks, respectively, as shown in Figure 4A. The ninhydrin color yields for α- and β-aspartylglycines at 570 nm were found to be 164% and 42% of that for glycine, respectively. As can be seen in Scheme I, the T_{13} peptide has

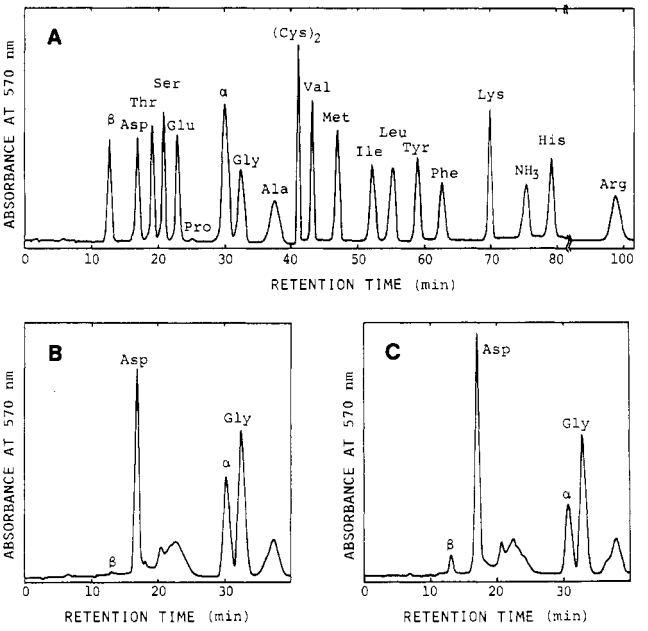


FIGURE 4: Amino acid analyses of the limited hydrolysates of the T_{13} peptides in 6 N HCl at 110 °C for 4 min. (A) Elution positions of α- and β-aspartylglycines under the conditions of amino acid analysis; (B) from 70 nmol of the normal T_{13} peptide (peptide C); (C) from 70 nmol of the abnormal T_{13} peptide (peptide A). The symbols α and β indicate α- and β-aspartylglycines, respectively.

Table I: Stabilities of α- and β-Aspartylglycines in 6 N HCl at 110

heating period (min)	α-aspartylglycine[a]		β-aspartylglycine[a]	
	α-peptide[b] (%)	β-peptide[b] (%)	α-peptide[b] (%)	β-peptide[b] (%)
2	77.2	0.6	0.1	72.1
3	66.2	1.0	0.3	56.3
4	56.7	1.7	0.4	45.9
5	49.0	1.0	0.4	38.2

[a] Starting aspartylglycines. [b] Yields of the recovered aspartylglycines.

one aspartylglycyl sequence (Asp-101–Gly-102) and one asparaginylglycyl sequence (Asn-103–Gly-104). From the former sequence, α- or β-aspartylglycine could be obtained by limited acid hydrolysis of the T_{13} peptide depending on the structure of this bond. From the latter sequence, α-aspartylglycine could be obtained if the deamidation of Asn-103 residue is fast under the acid hydrolysis condition. Furthermore, α- and β-aspartylglycines have been reported to be interconversible (Buchanan et al., 1962). Therefore, the stabilities of asparagine and α- and β-aspartylglycines were examined in 6 N HCl under vacuum at 110 °C followed by amino acid analysis in order to determine the conditions of the limited acid hydrolysis of the T_{13} peptide. In the case of asparagine, 3-min heating resulted in complete hydrolysis to give aspartic acid. In the cases of α- and β-aspartylglycines, their peptide bonds were somewhat stabler than the β-amide bond of asparagine against hydrolysis. As shown in Table I, on heating for 3–5 min, considerable amounts of α- and β-aspartylglycines were recovered and the interconversion between them was minor. Thus, we could elucidate their original structures by the limited acid hydrolysis followed by amino acid analysis. The hydrolysis of β-aspartylglycine was somewhat faster than that of the α-isomer, but the difference was not so large. These results suggest that the 3–5 min heating may be suitable for the limited acid hydrolysis of the T_{13} peptide.

The normal T_{13} peptide (peptide C) and the abnormal T_{13} peptide (peptide A) were hydrolyzed in 6 N HCl under vac-

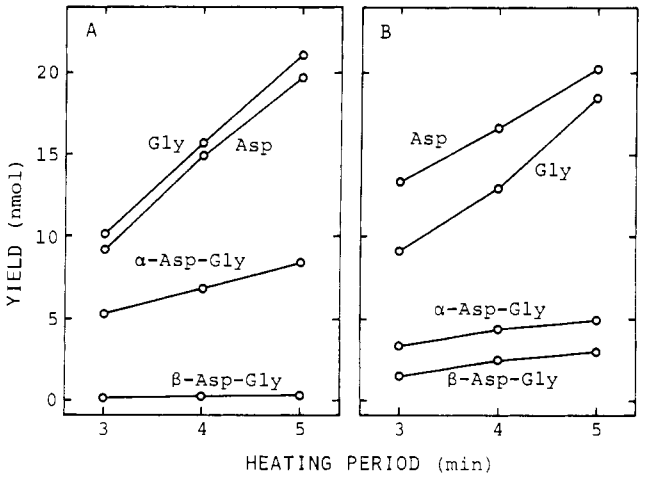
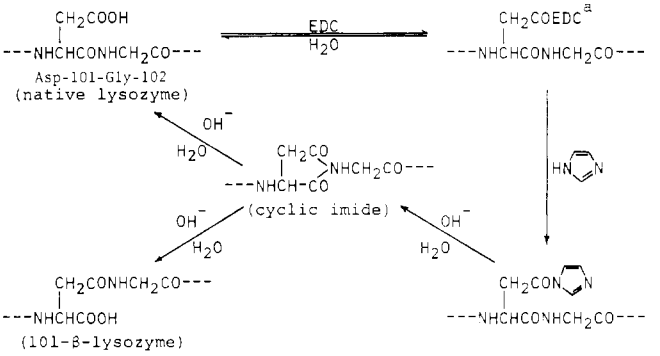


FIGURE 5: Yields of aspartic acid, glycine, and α- and β-aspartylglycines in the limited hydrolysis of 70 nmol of the T_{13} peptide in 6 N HCl at 110 °C: (A) from the normal T_{13} peptide; (B) from the abnormal T_{13} peptide.

uum at 110 °C for 3, 4 and 5 min, respectively, and then each hydrolysate was analyzed by the amino acid analyzer. In Figure 4B,C, the chromatographic patterns around the elution positions of α- and β-aspartylglycines obtained from the 4-min hydrolyses of the normal and abnormal T_{13} peptides are shown as representatives, respectively. From the normal T_{13} peptide, much α-aspartylglycine and only a trace amount of β-aspartylglycine were obtained (Figure 4B). To the contrary, from the abnormal T_{13} peptide, a considerable amount of β-aspartylglycine was obtained with the reduced amount of α-aspartylglycine (Figure 4C). Since β-aspartylglycine, aspartic acid, α-aspartylglycine, and glycine were obtained as well-separate peaks, their yields were determined in both cases. In Figure 5A,B, their yields thus determined were plotted against the hydrolysis period for the normal and abnormal T_{13} peptides, respectively. The yield of β-aspartylglycine from the abnormal T_{13} peptide was about 10 times higher than that from the normal T_{13} peptide. As for α-aspartylglycine, the yield from the abnormal T_{13} peptide was considerably lower than that from the normal T_{13} peptide. However, the total yields of α- and β-aspartylglycines were almost equal in both cases when compared at respective hydrolysis periods. As indicated above, the asparagine residue should be completely hydrolyzed to an aspartic acid residue under the conditions employed (in 6 N HCl, for 3 min or more at 110 °C). Therefore, these results clearly indicate that α- and β-aspartylglycines obtained from the abnormal T_{13} peptide were due to the sequences of Asn-103–Gly-104 and Asp-101–Gly-102, respectively. From the normal T_{13} peptide, both of these two sequences would give α-aspartylglycine, and this was the reason for the higher yield of α-aspartylglycine from the normal T_{13} peptide than from the abnormal one. The small amount of β-aspartylglycine from the normal T_{13} peptide was considered to be formed by the rearrangement of the α-isomer during the hydrolysis. Therefore, we can conclude that both derivatives I and III possess the β-peptide linkage between aspartic acid-101 and glycine-102. Hereafter we call derivatives I and III the 101-β-lysozyme and 101-β-CL-lysozyme, respectively. The structure of 101-β-lysozyme (or 101-β-CL-lysozyme) can explain the fact that it could not be separated from native lysozyme (or CL-lysozyme) by Bio-Rex 70 ion-exchange chromatography at pH 7 and 10, because the carboxylic acid groups at Asp-101 in native lysozyme (or CL-lysozyme) and 101-β-lysozyme (or 101-β-CL-lysozyme) may both fully dissociate at pH ≥7, and the difference in net charges of these

Scheme II: Mechanism for Formation of 101-β-Lysozyme in the Carbodiimide Reaction Catalyzed by Imidazole



[a] Activated β-carboxyl group of Asp-101 by EDC.

two lysozymes cannot be expected.

Mechanism of the Inversion of the Peptide Bond from α to β in the Aspartylglycyl Sequence (Asp-101–Gly-102) of Lysozyme. We elucidated that the α-peptide bond between Asp-101 and Gly-102 in lysozyme was preferentially inverted to the β one during the reaction with EDC in the presence of imidazole. To clarify the role of imidazole, lysozyme was treated with EDC at pH 5 in the absence of imidazole [run 1 in the previous paper, Yamada et al. (1983)]. After removal of the minor polymerized lysozyme, the monomer portion was analyzed by chitin-coated Celite affinity chromatography to afford only the native lysozyme peak, indicating imidazole to be a catalyst for the inversion of this peptide bond. Previously we have shown that, in the reaction of lysozyme with EDC in the presence of imidazole, some of the carboxyl groups are converted to acylimidazole groups, which are stable at pH 5.5 but hydrolyzed at alkaline pH to regenerate free carboxyl groups (Yamada et al., 1983). We have also shown that in the reaction of lysozyme with EDC in the presence of an amine nucleophile the β-carboxyl group of Asp-101 is selectively modified with the amine (Yamada et al., 1981). Therefore, in the presence of imidazole, the β-carboxyl group of Asp-101 must be modified first to form acylimidazole. Battersby & Robinson (1955) have demonstrated that alkaline hydrolysis of ethyl ester of either *N*-benzoyl-α- or *N*-benzoyl-β-aspartylglycine *n*-hexylamide produces a mixture of *N*-benzoyl-α- and *N*-benzoyl-β-aspartylglycine *n*-hexylamides in a ratio of 1:3. In order to explain these results, they have proposed the formation of the common five-membered ring intermediate, the cyclic imide, during the hydrolyses of two esters. The formation of this kind of cyclic imide or anhydroaspartic acid residue has been also proposed for the selective cleavage of the asparaginylglycyl bond in collagen with basic hydroxylamine by Bornstein (1970), who has demonstrated that the anhydroaspartic acid residue is preferentially formed when the following residue is glycine because of its lower steric hindrance. Thus, the sequence of Asp-101 and Gly-102 in lysozyme might be preferable to the formation of anhydroaspartic acid. Therefore, we also propose the formation of this cyclic imide at Asp-101 in lysozyme as an intermediate during alkaline hydrolysis of the acylimidazole group at Asp-101. In Scheme II, the proposed mechanism for the formation of 101-β-lysozyme catalyzed by imidazole in the reaction with EDC is shown. The opening ratio of the cyclic imide to α- and β-aspartyl sequences may be 1:2 in this case, which is different from the ratio 1:3 observed in the hydrolysis of the ethyl ester of *N*-benzoyl-α- or *N*-benzoyl-β-aspartylglycine *n*-hexylamide (Battersby & Robinson, 1955). This contradiction may be due to the difference between the microenvi-

ronments in the folded lysozyme molecule and in the small dipeptide. As for the carboxyl group activated by EDC at Asp-101, a nucleophilic attack by water or imidazole may be very fast. Thus, in the absence of imidazole, the carboxyl group activated by EDC at Asp-101 may be just hydrolyzed without forming the cyclic imide intermediate, and therefore, 101-β-lysozyme is not produced.

Formations of 101-β-Lysozyme and Its Asp-52 Ethyl Ester Lysozyme Derivative in the Reaction of Lysozyme with Triethyloxonium Fluoroborate. Parsons et al. (1969) have reported that in the reaction of lysozyme with triethyloxonium fluoroborate at acidic pH one of the carboxyl groups is ethyl esterified initially and then the second carboxyl group is esterified. The second carboxyl group has been identified as Asp-52 (Parsons & Raftery, 1969), but the first one has not been determined because this ethyl ester is easily hydrolyzed at pH 7.2 and room temperature. On hydrolyzing this labile ester, they demonstrated that native lysozyme is recovered from the singly esterified derivative (component III in their papers) and that Asp-52 ethyl ester lysozyme (component II) is obtained from the doubly esterified derivative (component IV). Previously, we have suggested this labile ester to be the β-ethyl ester of the Asp-101 residue (Yamada et al., 1981, 1982). If our suggestion were true, both the recovered lysozyme and the Asp-52 ethyl ester lysozyme obtained by them sould be mixtures of the derivatives with the α- and β-aspartyl sequences at Asp-101 as discussed above. Therefore, the reaction of lysozyme with triethyloxonium fluoroborate was reexamined.

According to the method described by Parsons et al. (1969), lysozyme was esterified with triethyloxonium fluoroborate at pH 4.5, and components III and IV were isolated, respectively. The recovered lysozyme and the Asp-52 ethyl ester lysozyme were obtained on incubations of components III and IV at pH 7.2 and room temperature for 48 h, followed by ion-exchange chromatography on Bio-Rex 70, respectively. The recovered lysozyme and the Asp-52 ethyl ester derivative thus obtained were analyzed by affinity chromatography on chitin-coated Celite. The results are shown in Figure 1D,E, respectively. The recovered lysozyme obtained from this reaction was separated into two peaks of 101-β-lysozyme and native lysozyme in a ratio of 2:1 (Figure 1D). This result is completely the same as that observed in the previous case (Figure 1B). These observations clearly indicate that the carboxyl group initially esterified with triethyloxonium fluoroborate is Asp-101 and that the hydrolysis of the β-ethyl ester of Asp-101 occurs through the same cyclic imide intermediate as shown in Scheme II. This might explain the fact that this ester was abnomally labile. These findings strongly suggest that the Asp-52 ethyl ester lysozyme is also a mixture of the derivatives with the α- and β-aspartyl sequences at Asp-101 in a ratio of 1:2. As shown in Figure 1E, the presences of two components in the Asp-52 ethyl ester derivative are obvious, although the ratio of them is not clear because of the low resolution of two peaks. Thus, it is concluded that the Asp-52 ethyl ester lysozyme obtained by the method of Persons & Raftery (1969) has been a mixture of two derivatives with α- and β-aspartyl sequences at Asp-101. When the peptide bond between Asp-101 and Gly-102 is inverted from the α-peptide bond linkage to the β one, the ionization behavior of the resulting α-carboxyl group at Asp-101 may be slightly different from the original β-carboxyl group. Since the ionization behavior of the catalytic carboxyl groups of lysozyme has been mostly investigated by comparing native lysozyme and this Asp-52 ethyl ester lysozyme mixture (Parsons & Raftery, 1972a–c; Kuramitsu et al., 1974), the previous data must be reexamined with the pure Asp-52 ethyl ester lysozyme.

Reaction of 101-β-Lysozyme with EDC in the Presence of Methylamine. We have reported the selective modification of the β-carboxyl group of the Asp-101 residue in lysozyme with an amine nucleophile by the carbodiimide reaction (Yamada et al., 1981). As mentioned above, in the absence of imidazole, the reaction of lysozyme with EDC does not produce 101-β-lysozyme. Therefore, the Asp-101 modified lysozyme derivatives reported should have only the α-aspartyl sequence at Asp-101 when the usual primary amine was used as a nucleophile. However, we used histamine or 4(5)-(aminomethyl)imidazole also as a nucleophile (Yamada et al., 1981). As this kind of amine contains both an imidazole group and a primary amino group in a molecule, the imidazole group could also react with Asp-101 activated by EDC to give an acylimidazole group. Although the acylimidazole group is considered to be stable during the reaction at pH 5 (Yamada et al., 1983), it could be possible that some of the acylimidazole group is hydrolyzed even at pH 5 to give 101-β-lysozyme, which is again activated by EDC to produce the Asp-101-modified lysozyme possessing the β-aspartyl sequence. However, in contrast with native lysozyme (Yamada et al., 1981), 101-β-lysozyme did not give any product in the reaction with EDC in the presence of methylamine when the mixture was analyzed by Bio-Rex 70 ion-exchange chromatography (data are not shown). Thus, it is concluded that all of the Asp-101-modified lysozymes reported (Yamada et al., 1981) possess the only α-aspartyl sequence at Asp-101.

Conclusions

In the cross-linking reaction of the salt bridge between Lys-13 (ϵ-NH_3^+) and Leu-129 (α-COO^-) in lysozyme using imidazole and EDC, it was found that the two-thirds of the reacted protein possessed the β-aspartyl sequence at Asp-101. Therefore, it is concluded that the peptide bond inversion from the α-aspartyl sequence to the β one also occurs in this reaction procedure besides the cross-linking. Although we have documented that the imidazole–EDC reaction may be useful to cross-link bridges between ammonium and carboxylate groups in proteins to give amide bonds without modifying any other residues (Yamada et al., 1983), we must correct this document to that this method sometimes leads the peptide bond inversion from the α-aspartyl sequence to the β one in proteins especially in the protein with the aspartylglycyl sequence. It should be also noted that the Asp-52 ethyl ester lysozyme, obtained from the reaction of lysozyme with triethyloxonium fluoroborate, also contained the derivative with the β-aspartyl sequence at Asp-101 as a major component. Therefore, much attention should be paid to the chemical modification of a protein, since it could be accompanied by hardly detectable side reactions as observed here. Sometimes these lead to serious misinterpretations of the properties of the derivatives. Present results may serve a good example for this, since 101-β-lysozyme showed quite different properties from native lysozyme in saccharide binding ability, stability, and so on. Further experiments about the nature of 101-β-lysozyme are under way.

Registry No. L-Asp, 56-84-8.

References

Battersby, A. R., & Robinson, J. C. (1955) *J. Chem. Soc.*, 259–269.

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C., & Sarma, V. R. (1967) *Proc. R. Soc. London, Ser. B 167*, 378–388.

Bornstein, P. (1970) *Biochemistry 9*, 2408–2421.

Buchanan, D. L., Haley, E. E., & Markiw, R. T. (1962) *Biochemistry 1*, 612–620.
Canfield, R. E. (1963) *J. Biol. Chem. 238*, 2691–2697.
Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.
Imoto, T., Okazaki, K., Yamada, H., Fujita, K., Yamato, T., & Koga, D. (1981) *J. Biochem. (Tokyo) 90*, 991–995.
Kuramitsu, S., Ikeda, K., Hamaguchi, K., Fujio, H., Amano, T. Miwa, S., & Nishina, T. (1974) *J. Biochem. (Tokyo) 56*, 432–440.
Le Quesne, W. J., & Young, G. T. (1952) *J. Chem. Soc.*, 24–28.
Meerwein, H., Hinz, G., Hofmann, P., Kroning, E., & Pfeil, E. (1937) *J. Prakt. Chem. 147*, 257–285.
Okazaki, K., Imoto, T., Yamada, H., Kuroki, R., & Fujita, K. (1982) *J. Biol. Chem. 257*, 12559–12562.
Parsons, S. M., & Raftery, M. A. (1969) *Biochemistry 8*, 4199–4205.
Parsons, S. M., & Raftery, M. A. (1972a) *Biochemistry 11*, 1623–1629.
Parsons, S. M., & Raftery, M. A. (1972b) *Biochemistry 11*, 1630–1633.
Parsons, S. M., & Raftery, M. A. (1972c) *Biochemistry 11*, 1633–1638.
Parsons, S. M., Jao, L., Dahlquist, F. W., Borders, C. L., Jr., Groff, T., Racs, J., & Raftery, M. A. (1969) *Biochemistry 8*, 700–712.
Yamada, H., Imoto, T., Fujita, K., Okazaki, K., & Motomura, M. (1981) *Biochemistry 20*, 4836–4842.
Yamada, H., Imoto, T., & Noshita, S. (1982) *Biochemistry 21*, 2187–2192.
Yamada, H., Kuroki, R., Hirata, M., & Imoto, T. (1983) *Biochemistry 22*, 4551–4556.
Yamada, H., Ito, Y., Fukumura, T., & Imoto, T. (1985) *Anal. Biochem. 146*, 71–74.

Structural Studies on Acid Unfolding and Refolding of Recombinant Human Interferon γ

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ABSTRACT: Interferon γ is distinguished from other types of interferons in its instability upon acid treatment, as demonstrated by a loss of antiviral activity. Acid unfolding and refolding experiments were performed with recombinant DNA derived human interferon γ. When the protein was subjected to unfolding and refolding, the refolded protein showed two peaks (peaks I and II) in gel filtration, which have been shown to differ in size, structure, and antiviral activity. When the smaller, peak II, form was unfolded by dialysis against 0.01 M HCl containing 0.1 M NaCl (pH 2) and refolded by dialysis against various solvents at neutral pH, it re-formed as peak II but also generated peak I, and the ratio of the two forms was dependent on protein concentration and solvent conditions. Higher protein concentrations and higher ionic strength led to a greater ratio of peak I to peak II. Phosphate buffers caused precipitation of peak I. Since peak II is 4–8 times more active than peak I in the antiviral bioassay, generation of peak I by acid treatment of peak II should lead to a decrease in antiviral activity.

Immune interferon (IFN-γ)[1] differs from leukocyte (IFN-α) and fibroblast (IFN-β) interferons in a variety of biological and chemical properties (Wheelock, 1965; Green et al., 1969; Epstein et al., 1971; Falcoff, 1972; Stewart et al., 1980; Blalock et al., 1980; Rubin & Gupta, 1980; Yip et al., 1981a, 1982; Nakamura et al., 1984). In particular, instability of IFN-γ upon acid treatment distinguishes it from other IFNs (Wheelock, 1965; Green et al., 1969; Epstein et al., 1971; Yip et al., 1981a). For example, Yip et al. (1981b) observed that the antiviral activity of natural IFN-γ drops by approximately 10-fold upon dialysis against a pH 2 solution and then a neutral phosphate buffer. Conversely, other IFNs (IFN-α and IFN-β) maintain antiviral activity when treated in the same way. This suggests that IFN-γ is denatured in acid and cannot be refolded into the native structure. However, no structural studies of the protein have been carried out, in part due to difficulty in obtaining sufficient quantities of purified protein for structural studies.

We have constructed the gene for IFN-γ designed for expression in *Escherichia coli* (Alton et al., 1983), which lacks three N-terminal residues, Cys-Tyr-Cys, predicted from the originally published gene sequence (Gray et al., 1982; Devos et al., 1982). Recombinant IFN-γ, when expressed in *E. coli*, differs from natural IFN-γ (Rinderknect et al., 1984; Kelker et al., 1983, 1984) in that recombinant IFN-γ has an N-terminal Met residue (P. Lai, unpublished result) and glycosylation does not occur. In addition, the natural protein is known to undergo C-terminal processing (Rinderknecht et al., 1984). Therefore, natural IFN-γ usually lacks C-terminal residues whereas the amino acid sequence of recombinant IFN-γ is intact (P. Lai, unpublished result). In spite of these differences, structural studies on the recombinant protein should help elucidate the mechanism of acid instability of the natural protein.

We have carried out extensive chemical and physicochemical

[1] Abbreviations: IFN, interferon; Gdn·HCl, guanidine hydrochloride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NH_4OAc, ammonium acetate; CD, circular dichroism; UV, ultraviolet; Tris, tris(hydroxymethyl)aminomethane.

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