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Detection of a Local Interaction of Hen Lysozyme under Highly Denaturing Conditions Using Chemically ^{13}C-Enriched Methionine Resonance

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Using hen lysozyme in which the ε-carbons of two methionine residues are enriched with ^{13}C nuclei, we found that there is a subtle difference in the chemical shift of the ε-carbon resonances between Met 12 and Met 105 in thermally denatured lysozyme without any reduction of disulfide bonds at pD 3.8, and also in reduced *S*-alkylated lysozyme at pD 3.8 and 35°C. The difference in the chemical shift was abolished on digestion with TPCK-trypsin and the chemical shifts of both resonances converged to that of Met 12, whose chemical shift is identical to that in the randomly coiled state. Therefore, it is suggested that the chemical shift in the ε-carbon resonance of Met 105 is different from that in the randomly coiled state due to an interaction involving Met 105. In order to locate the interaction involving Met 105, fragmentation of the reduced *S*-alkylated lysozyme into the peptides was carried out by means of chemical cleavage or specific endoprotease digestion. As a result, the local interaction of Met 105 or the residues around Met 105 with eleven residues at the C-terminus of lysozyme is suggested to occur.

Key words: ^{13}C-NMR, isotope-labeling, interaction under highly denaturing conditions, lysozyme.

Through recent structural analyses, information not only on a folded protein structure but also on a protein structure under highly denaturing conditions has been accumulating. Under highly denaturing conditions such as a high temperature, a high concentration of a denaturant and an extreme pH, a non-random coil structure has been found to be present in several proteins (*1-4*). NMR is one of the suitable techniques for such investigations (*5*). However, even if a protein has a structure under highly denaturing conditions, the chemical shift dispersion is so small that assignment of the resonances is difficult. In these circumstances, stable isotope labeling is a convenient approach for extracting information on a protein structure under highly denaturing condition from the crowded spectrum (*5*).

Lysozyme is one of the popular proteins whose structure, function, and folding have been vigorously investigated (*6-8*). Dobson's group has demonstrated, by means of NMR spectroscopy with the photo-CIDNP method, that a structure in lysozyme is present under various denaturing conditions (*9*). The results strongly suggest that aromatic residues in lysozyme form structures under various denaturing conditions. Moreover, they recently suggested that synthetic peptide 84-129, the C-terminus of lysozyme, formed a secondary structure, as judged on CD measurement (*10*). They have not yet, however, determined where the structure is present.

Lysozyme has two methionine residues. Met 12, which is located in the first α-helix, and Met 105, which is located in the hydrophobic box (*7*). The hydrophobic box is formed by Tyr 20, Tyr 23, Trp 28, Met 105, Trp 108, and Trp 111. Therefore, by means of enrichment of $^{13}CH_3$ in methionine, we may be able to reveal the structure around Met 105, if there is any structure around this region in lysozyme under highly denaturing condition as mentioned above. In this study, we demonstrated using lysozyme in which the ε-carbons of two methionine residues were chemically enriched with ^{13}C nuclei (*11*) that there is a local hydrophobic interaction of Met 105 or the residues around Met 105 with the eleven residues at the C-terminal region of lysozyme under highly denaturing conditions.

MATERIALS AND METHODS

Materials—Five-times recrystallized hen egg-white lysozyme was donated by QP. TAP-Br was kindly donated by Prof. H. Yamada of Okayama University. TPCK-trypsin was obtained from Sigma. *Staphylococcus aureus* V8

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Abbreviations: CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid; HMQC, homonuclear multiple quantum coherence; 104-nicked lysozyme, a derivative in which the peptide bond is oxidatively cleaved at Gly 104; NOESY, nuclear Overhauser enhancement and exchange spectroscopy; photo-CIDNP, photochemically induced dynamic nuclear polarization; TAP-lysozyme, reduced *S*-trimethylammoniumpropylated lysozyme; TAP-Br, trimethylammonium propyl bromide bromide salt; TPCK-trypsin, L-(tosylamino 2-phenyl)ethyl chloromethyl ketone-treated trypsin.

protease was obtained from Wako Chemicals (Osaka). Endoproteinase Asn-N was obtained from Boehringer Mannheim (USA).

Preparation of ^{13}C-Enriched Methionine Lysozyme—Lysozyme in which the ε-carbons of two methionine residues are enriched with ^{13}C nuclei was obtained by the method previously reported (*11*).

Preparation of TAP-Lysozyme and Its Digestion with Various Proteases—Preparation of TAP-lysozyme was performed according to the method in our previously reported (*12*). The conditions for each protease digestion are described below. For TPCK-trypsin digestion, TAP-lysozyme (10 mg) was suspended in 600 μl of 0.05 M phosphate buffer (pH 7.0) and then digested with TPCK-trypsin (2% lysozyme weight) at 35°C for 24 h. For V8 protease digestion, TAP-lysozyme (10 mg) was suspended in 600 μl of 0.05 M phosphate buffer (pH 7.0) containing 2 M urea and then digested with V8 protease (1% lysozyme weight) at 30°C for 24 h. For Endoproteinase Asp-N digestion, TAP-lysozyme (10 mg) was suspended in 600 μl of 0.05 M phosphate buffer (pH 8.0) and then digested with Endoproteinase Asp-N (5% lysozyme weight) at 37°C for 14 h. The completion of protease digestion was confirmed by analyzing an aliquot of the digest by RP-HPLC. Each digest was purified by RP-HPLC in order to remove salts or urea according to the method in our previously reported (*13*), and then lyophilized.

Preparation of 104-Nicked Lysozyme—The 104-nicked lysozyme, a derivative in which the peptide bond is oxidatively cleaved at Gly 104, was prepared according to the literature (*14*). The 104-nicked lysozyme was enriched with ^{13}C, reduced and *S*-alkylated using TAP-Br. The resultant peptides were purified by RP-HPLC in order to remove salts and urea according to the method in our previously reported (*13*), and then lyophilized.

NMR Measurements—NMR spectra were recorded with a Varian Unity 600 plus spectrometer or a Bruker AMX 400 spectrometer. The probe temperature was calibrated with an accuracy of $\pm$0.2°C using ethylene glycol. The carbon and proton chemical shifts were internally referenced as to 1,4-dioxane (67.8 ppm for ^{13}C and 3.74 ppm for ^{1}H). NMR measurements of fragmented peptides were carried out without their separation. Reduced *S*-alkylated lysozyme and each lyophilized peptide was dissolved in 500 μl of D_2O. The pD of the solution was adjusted to 3.8 by adding 1 N DCl and/or NaOD. The pD value was taken as the pH meter reading without adjustment for the isotope effect (*15*). The sample concentration in ^{13}C-NMR of the fragmented peptide was 1 mM and that in the other measurements was 2 mM. ^{1}H-^{13}C HMQC NOESY (*16*) at pH 3.8 was carried out using the standard procedure. In the ^{1}H-^{13}C HMQC NOESY experiments, 16 transients were typically recorded for each of the 256 increments. Digital resolutions of 2.4 Hz per point in the ^{1}H dimension and 11.7 Hz per point in the ^{13}C dimension were used. The mixing time for ^{1}H-^{13}C HMQC NOESY experiments was 150 ms.

RESULTS AND DISCUSSION

^{13}C-NMR Spectra of Lysozyme with ^{13}C Enriched Methionines under Thermally Denaturing Conditions—Hen egg-white lysozyme has two methionines (7). One is Met 12, which is in an α-helix, and the other is Met 105, which is in the center of the "hydrophobic box." Isotope labeling of the methyl carbons of these methionine residues in lysozyme with ^{13}C nuclei was carried out by the method in the previously reported (*11*) (See "MATERIALS AND METHODS"). Using the resonances derived from ^{13}C-enriched methionine as probes, we examined the process of thermal unfolding of lysozyme. ^{13}C-NMR spectra for thermal unfolding of lysozyme with ^{13}C-enriched methionines are shown in Fig. 1. The resonance derived from the denatured state appeared as the temperature was increased. The thermal unfolding started at 66°C (Fig. 1D) and ceased at 83°C (Fig. 1H). The midpoint of the unfolding, which is due to the change in the chemical shifts of the ^{13}C resonances of the two enriched methionines, was 73$\pm$1 °C. This is consistent with the previous result of ^{1}H-NMR measurement (*17*). Under the conditions at the midpoint for the unfolding, slow exchange between the folded state and the thermally denatured state in lysozyme was observed in the ^{13}C-NMR spectrum. Based on this observation, we assigned these two resonances to the ε-CH_3 carbons of the methionines residues in lysozyme under the thermally denaturing conditions. In the ^{1}H-^{13}C HMQC-NOESY spectrum (*16, 18*) at 73°C, we could detect the magnetization transfer NOEs of slow exchange between the folded and thermally denatured states (Ex1-Ex4 in Fig. 2). We have already determined that the chemical shift of ε-CH_3 in Met 12 was 16.90 ppm and that in Met 105 was 14.06 ppm at 35°C and pD 3.8 (*11*). From these slow exchange NOEs, the chemical shift of the ε-CH_3 carbon of Met 12 under the thermally denaturing condition was

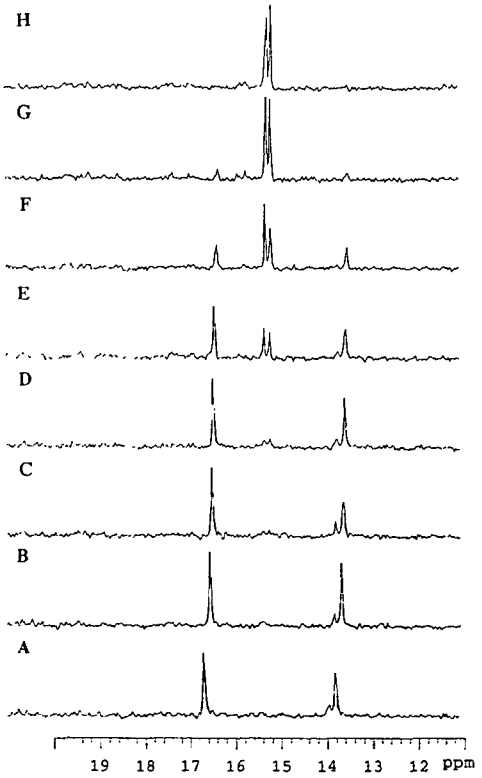

Fig. 1. Low field of the 100 MHz ^{13}C-NMR spectra of ^{13}C enriched methionine lysozyme at pD 3.8 and (A) 45.0°C, (B) 54.6°C, (C) 60.3°C, (D) 66.0°C, (E) 71.0°C, (F) 74.2°C, (G) 78.4°C, or (H) 83°C.

assigned as 15.69 ppm and that of Met 105 as 15.57 ppm at pD 3.8 and 73°C.

Interestingly, the two resonances were observed even after complete thermal unfolding (Fig. 1H). Since the errors in the chemical shifts are within 0.02 ppm under the measurement conditions, the difference in the chemical shift of about 0.1 ppm is significant. Similar separate resonances due to ε-CH_3 protons in Met in native lysozyme have been observed on ^{1}H-NMR measurement (*19*). In that report, the authors stated that separate resonances were due to an intermolecular interaction since the separate resonances become one at low pH, *i.e.* acidic denaturing conditions, and at a low concentration (0.1 mM at pH 1.8 and 53°C). On ^{13}C-NMR measurement of ^{13}C-enriched methionine lysozyme under the same dilute conditions (0.1 mM at pH 1.8 and 53°C), the two resonances remained to be observed. Therefore, the separate resonances observed on the present NMR measurement were not caused by an intermolecular interaction.

^{13}C-NMR Spectra of Reduced S-Alkylated Lysozyme with ^{13}C Enriched Methionines—In order to examine the effect of the disulfide bonds in lysozyme on the separate resonances, we prepared TAP-lysozyme by reduction and alkylation with TAP-Br, which is soluble even in the absence of a denaturant (*20*). It was elucidated from the ^{13}C-NMR spectrum (Fig. 3A) that the cleavage of the disulfide bonds did not affect the chemical shifts of the methionine resonances in an aqueous solution. However, when the ^{13}C-enriched methionine TAP-lysozyme was digested with TPCK-trypsin, the spectrum showed a single resonance (Fig. 3B). The chemical shift was identical to the downfield one (15.5 ppm) in Fig. 3A. Moreover, it was suggested that the chemical shift resulting from the ε-CH_3 of Met 12 is that of a methionine residue in the randomly coiled state of the polypeptide chain, because the chemical shifts of the ε-CH_3 carbons of the four methionine residues in TAP-Ribonuclease A are also 15.5 ppm under the same measurement conditions (pD 3.8 and 35°C) (data not shown). From the above results, it was concluded that the resonance at 15.4 ppm in Fig. 3A resulted from Met 105 and that the tryptic digestion of TAP-lysozyme with ^{13}C-enriched methionines abolished the difference in the environments of the two methionine residues in TAP-lysozyme.

The amino acid sequence of the tryptic peptide including Met 105 is as follows.

98 99 100 101 102 103 104 105 106 107 108 109 110
Ile-Val-Ser-Asp-Gly-Asn-Gly-**Met**-Asn-Ala-Trp-Val-Ala-
111 112
Trp-Arg

As Met 105 is not located at the N-terminus or C-terminus of tryptic peptide 98-112, the perturbation of the resonance of Met 105 (15.4 ppm) was not caused by vicinal residues. Namely, it may be due to the interaction between the Met 105 residue or some residues affecting the chemical shift of the Met 105 resonance and residues other than peptide 98-112 under the highly denatured conditions. Thus, we tried to identify the region by preparing the various peptide fragments below.

Identification of the Region Involved in the Interaction with Met 105 or the Residues around Met 105 in Lysozyme under Highly Denaturing Conditions—In hen lysozyme, Met 105, Trp 108, and Trp 111 form a hydrophobic core with Tyr 20, Tyr 23, and Trp 28 (*7*). Therefore, to examine

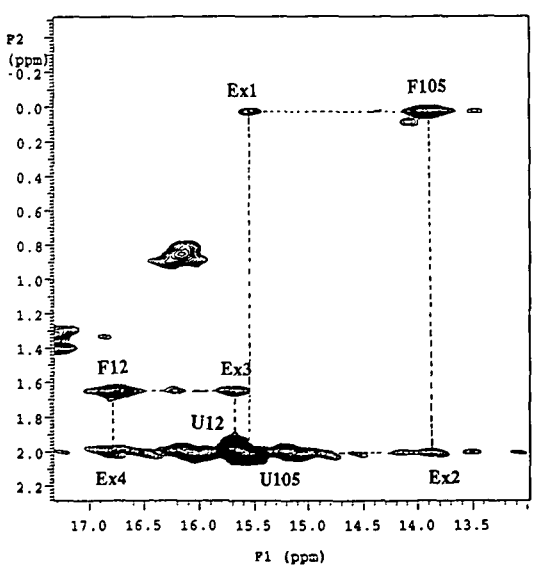

Fig. 2. **^{1}H-^{13}C HMQC-NOESY spectrum of ^{13}C-enriched methionine lysozyme at 75°C and pH 3.8 with a 150 ms mixing time.** F12 (Met 12) and F105 (Met 105) are the peaks for folded ^{13}C-enriched methionine lysozyme, U12 (Met 12) and U105 (Met 105) are those for ^{13}C-enriched methionine lysozyme under the thermal denaturing conditions, and Ex1-Ex4 are the NOEs for slow exchange between the folded and denatured states.

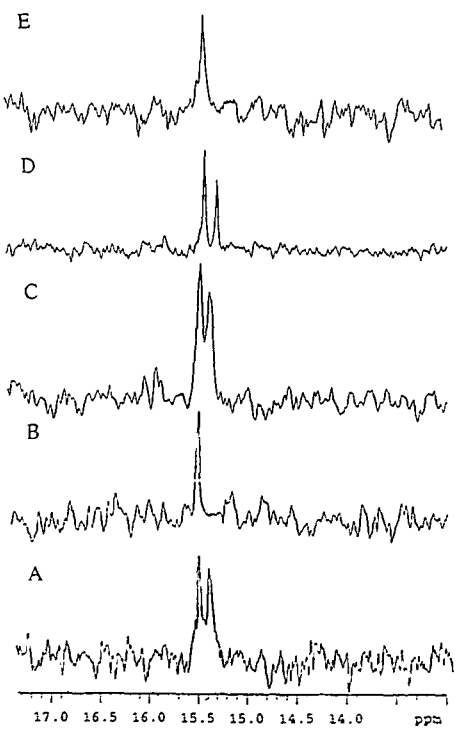

Fig. 3. **Low field of the 150 MHz ^{13}C-NMR spectra at pD 3.8 and 35°C of (A) ^{13}C-enriched methionine TAP-lysozyme in D_2O, (B) its TPCK-trypsin digest in D_2O, (C) its V8 protease digest in D_2O, (D) 104 nicked TAP-lysozyme with ^{13}C-enriched methionines in D_2O, and (E) its Endoproteinase Asp-N digest in D_2O.** The conditions for each measurement are given under "MATERIALS AND METHODS."

the involvement of Tyr 20, Tyr 23, and Trp 28 in the interaction in TAP-lysozyme, V8 protease digestion of TAP-lysozyme with ^{13}C-enriched methionines was carried out, which cleaved the peptide bonds at the C-terminus of Glu 7 and Glu 35 in TAP-lysozyme (Fig. 4). As the difference in the chemical shifts between the two resonances abolished in the peptide mixture of tryptic digests (Fig. 3B), the coexistence of a peptide without a favorable interaction was found not to affect the difference in the chemical shifts between the two resonances. Moreover, the difference in the chemical shifts between the two resonances was very small (about 0.1 ppm). Therefore, we carried out the subsequent NMR measurements of fragmented peptides without their separation, because we could reveal the difference in the chemical shifts between them. The ^{13}C-NMR spectrum of the mixture of the digested peptides is shown in Fig. 3C. Since separate resonances were still observed in the spectrum of the mixture of digests, the involvement of Tyr 20, Tyr 23, and Trp 28 in the interaction under highly denaturing conditions was eliminated. The efficiency of the digestion was evaluated by RP-HPLC (data not shown). Therefore, residues 36-129 retained the interaction of lysozyme under highly denaturing conditions.

Next, 104-nicked lysozyme, which has a nick at residue 104 (*13*), was prepared to eliminate N-terminal peptide 1-103 (Fig. 4). After ^{13}C enrichment of the methionines in the 104-nicked lysozyme according to the method in the previously reported (*11*), it was reduced and *S*-alkylated with TAP-Br. The spectra of the mixture of peptides 1-103 and 105-129 remained separate (Fig. 3D). Therefore, the interaction of lysozyme under highly denaturing conditions was confirmed to occur even in residues 105-129. On the other hand, digestion of the ^{13}C-enriched methionine TAP-lysozyme with Endoproteinase Asp-N was carried out. This treatment gave peptide 101-118, which includes Met 105 (Fig. 4). The chemical shift of the ε-CH_3 resonance from Met 105 in the ^{13}C-NMR spectrum of the mixture of the peptides derived on Endoproteinase Asp-N digestion converged to that of Met 12 (Fig. 3E). Since the ^{13}C-NMR measurements of fragmented peptides described above were carried out with similar concentrations, we considered that the difference in the chemical shifts between the two resonances resulted from the cleaved mainchain. Therefore, residues 119-129 were concluded to be involved in the interaction with Met 105 or the residues around Met 105 in TAP-lysozyme in an aqueous solution.

The amino acid sequence of residues 98-129 is as follows.

98 99 100 101 102 103 104 105 106 107 108 109 110
Ile-Val-Ser-Asp-Gly-Asn-Gly-Met-Asn-Ala-Trp-Val-Ala-
111 112 113 114 115 116 117 118 119 120 121 122
Trp-Arg-Asn-Arg-Cys-Lys-Gly-Thr-Asp-Val-Gln-Ala-
123 124125 126 127 128 129
Trp-Ile-Arg-Gly-Cys-Arg-Leu

Residues 119-129 include some hydrophobic residues (Val 120, Trp 123, Ile 124, and Leu 129). As judged from the X-ray structure of lysozyme, the 3_{10}-helix is present between residues 119 and 122 (*6, 7*). Moreover, Dobson's group has reported that non-native helical structure was observed in the region of 117-124 in the presence of trifluoroethanol (*21*). Therefore, the eleven residues at the C-terminal of lysozyme may have a potential for a helical structure. On the other hand, the hydrophobic residues (Trp 108, Val 109, and Trp 111) lie around Met 105. Thus, under highly denaturing condition, in lysozyme the long range local hydrophobic interaction of Met 105 or the residues around Met 105 with residues 119-129 may occur. As a result of the formation of the clustered structure on the local interaction, the resonance of $^{13}CH_3$ from Met 105 shifted under the highly denaturing conditions. Since Wüthrich (*5*) suggested that the equilibrium among the denatured structures is rapid, the chemical shift in the resonance of $^{13}CH_3$ from Met 105 of lysozyme under the conditions employed here may reflect the average of many chemical shifts derived from many structures, which are evoked by local hydrophobic interactions, including the randomly coiled structure.

^{13}C-NMR Spectrum of the Reduced S-Alkylated Lysozyme with ^{13}C Enriched Methionines in the Presence of Trifluoroethanol—In order to confirm the interaction, we measured the ^{13}C-NMR spectrum of the reduced *S*-alkyl-

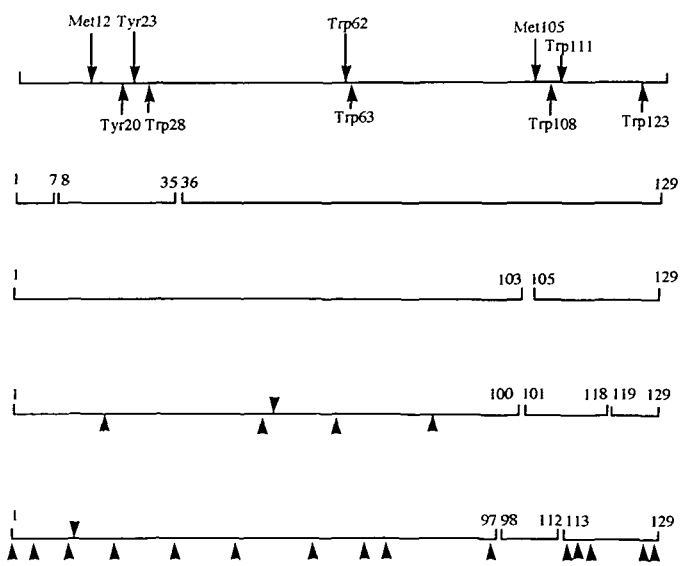

Fig. 4. **The fragmentation of TAP-lysozyme using specific proteases or a selective chemical modification.** Since there are many cleavage sites for trypsin and Endoproteinase Asp-N, these cleavage sites are indicated by arrowheads except for in the region in question, in which they are shown as the cleaved bonds. The positions of methionine and tryptophan residues are also indicated.

ated lysozyme with ^{13}C enriched methionines at 35°C and pD 3.8 in the presence of 30% trifluoroethanol. Separate resonances derived from the ε-$^{13}CH_3$ of Met 12 and Met 105 were observed. Interestingly, the difference in the chemical shifts of the resonances of ε-$^{13}CH_3$ between Met 12 and Met 105 was 53 Hz, which was larger than that in an aqueous solution (14 Hz). This indicated that the local interaction in the reduced *S*-alkylated lysozyme increased on the addition of trifluoroethanol. This may be considered to be due to the increased hydrophobic interaction of Met 105 or the residues around Met 105 with the hydrophobic surface of helix 117-124 with an increase in the population of the helical structure in peptide 117-124 on the addition of trifluoroethanol. Therefore, the increase in the difference in the chemical shifts between them in the presence of 30% trifluoroethanol supported the idea that there was a local interaction of Met 105 or the residues around Met 105 with the eleven residues at the C-terminal of lysozyme.

Meaning of the Effect of the Local Interaction under Highly Denaturing Conditions on the Protein Folding—Alpha-lactalbumin has a tertiary structure similar to that of lysozyme. Recently, Shulman *et al.* (*22*) demonstrated that a residual structure for the unfolding is located at the D-helix (residues 105-109) and the 3_{10}-helix (residues 115-119) in α-lactalbumin in the presence of 10 M urea at 50°C. They suggested that a hydrophobic region formed by both the D-helix (residues 105-109) and the 3_{10}-helix (residues 115-119) in α-lactalbumin remains collapsed under extremely denaturing conditions, and that the geometrically local hydrophobic core is collapsed at the early stage of folding, and the core provides a template for correct assembly of the global peptide (*22*). The hydrophobic region formed by the D-helix (residues 105-109) and the 3_{10}-helix (residues 115-119) in α-lactalbumin corresponds to the C-terminal region of lysozyme. Therefore, based on the analogy between α-lactalbumin and hen lysozyme, the interaction of the Met 105 residues or the residue around Met 105 with the eleven residues at the C-terminal of lysozyme may provide a template for correct assembly at the early stage of lysozyme. This idea is consistent with the finding that peptide 84-129 in lysozyme could have a residual structure but that the other peptides could not (*10*).

On the other hand, aggregation was reported to be induced by the unfavorable hydrophobic interactions in the reduced state (*23*). If there is an intrapeptide hydrophobic interaction in the reduced state of lysozyme, the aggregation may be as little as possible. Namely, the local interaction in lysozyme might be involved in the depression of the aggregation of reduced lysozyme.

REFERENCES

1. Robertson, A.D. and Baldwin, R.L. (1991) Hydrogen exchange in thermally denatured ribonuclease A. *Biochemistry* **30**, 9907-9914
2. Neri, D., Billeter, M., Wider, G., and Wüthrich, K. (1992) NMR determination of residual structure in a urea-denatured protein, the 434-repressor. *Science* **257**, 1559-1563
3. Kuwajima, K. (1989) The molten globule state as a clue for understanding the folding and cooperativity of globular protein structure. *PROTEINS: Struct. Funct. Genet.* **6**, 87-103
4. Shortle, D. (1996) The denatured state (the other half of the folding equation) and its role in protein stability. *FASEB J.* **10**, 27-34
5. Wüthrich, K. (1994) NMR assignment as a basis for structural characterization of denatured states of globular proteins. *Curr. Opin. Struct. Biol.* **4**, 93-99
6. Blake, C.C.F., Koenig, D.F., Mair, G.A., North, A.C.T., Philips, D.C., and Sarma, V.R. (1965) Structure of hen egg-white lysozyme: a three dimensional Fourier synthesis at 2.0 Å resolution. *Nature* **206**, 757-761
7. Imoto, T., Johnson, L.N., North, A.C.T., Phillips, D.C., and Rupley, J.A. (1972) Vertebrate lysozyme in *The Enzymes* 3rd ed. (Boyer, P.D., ed.) Vol. 7. pp. 665-868, Academic Press, New York
8. Dobson, C.M., Evans, P.A., and Radford, S.E. (1994) Understanding how proteins fold: the lysozyme so far. *Trends Biochem. Sci.* **19**, 31-37
9. Broadhurst, R.W., Dobson, C.M., Hore, P.J., Radford, S.E., and Rees, M.L. (1991) A photochemically induced dynamic nuclear polarization study of denatured state of lysozyme. *Biochemistry* **30**, 405-412
10. Yang, J.J., Buck, M., Pitkeathly, M., Kotik, M., Haynie, D.T., Dobson, C.M., and Radford, S.E. (1995) Conformational properties of four peptides spanning the sequence of hen lysozyme. *J. Mol. Biol.* **252**, 483-491
11. Abe, Y., Ueda, T., and Imoto, T. (1997) An improved method for preparing lysozyme with chemically ^{13}C-enriched methionine residues using 2-aminothiophenol as a reagent of thiolysis. *J. Biochem.* **122**, 1153-1159
12. Okazaki, K., Imoto, T., and Yamada, H. (1985) A convenient protein substrate for determination of protease specificity: reduced and *S*-3-(trimethylated amino) propylated lysozyme. *Anal. Biochem.* **145**, 87-90
13. Yamada, H., Yamashita, T., Domoto, H., and Imoto, T. (1990) Reaction of hen egg-white lysozyme with tetranitromethane: A new side reaction, oxidative bond cleavage at glycine 104, and sequential nitration of three tyrosine residues. *J. Biochem.* **108**, 432-440
14. Ueda, T., Nakashima, A., Hashimoto, Y., Miki, T., Yamada, H., and Imoto, T. (1994) Formation of α-helix 88-98 is essential in the establishment of higher-order structure from reduced lysozyme. *J. Mol. Biol.* **235**, 1312-1317
15. Bundi, A. and Wüthrich, K. (1979) ^1H NMR parameters of the common amino-acid residues measured in aqueous solutions of the linear tetrapeptides H-Gly-Gly-X-Ala-OH. *Biopolymers* **18**, 285-297
16. Nicholson, L.K., Kay, L.E., Baldisseri, D.M., Arango, J., Young, P.E., Bax, A., and Torchia, D.A. (1992) Dynamics of methyl groups in proteins as studied by proton-detected ^{13}C-NMR spectroscopy. Application to the leucine residues of staphylococcal nuclease. *Biochemistry* **31**, 5253-5263
17. Dobson, C.M. and Evans, P.A. (1984) Protein folding kinetics from magnetization transfer nuclear magnetic resonance. *Biochemistry* **23**, 4267-4270
18. Feng, Y. and Roder, H. (1988) Relayed magnetization transfer by isotropic mixing in exchanging systems. *J. Magn. Reson.* **78**, 597-602
19. Evans, A.E., Topping, K.D., Woolfson, D.N., and Dobson, C.M. (1991) Hydrophobic clustering in nonnative states of a protein: interpretation of chemical shifts in NMR spectra of denatured states of lysozyme. *PROTEINS: Struct. Funct. Genet.* **9**, 248-266
20. Yamada, H., Seno, M., Kobayashi, A., Moriyama, T., Kosaka, M., Ito, Y., and Imoto, T. (1994) An *S*-alkylating reagent with positive charges as an efficient solubilizer of denatured disulfide-containing proteins. *J. Biochem.* **116**, 852-857
21. Buck, M., Schwalbe, H., and Dobson, C.M. (1996) Main-chain dynamics of a partially folded protein: ^{15}N NMR relaxation measurements of hen egg white lysozyme denatured in trifluoroethanol. *J. Mol. Biol.* **257**, 669-683
22. Shulman, B.A., Kim, P.S., Dobson, C.M., and Redfield, C. (1997) A residue-specific NMR view of the non-cooperative unfolding of a molten globule. *Nature Struct. Biol.* **4**, 630-634
23. Goldberg, M.E., Rudolph, R., and Jaenicke, R. (1991) A kinetic study of the competition between renaturation and aggregation during the refolding of denatured-reduced egg white lysozyme. *Biochemistry* **30**, 2790-2797