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An Improved Method for Preparing Lysozyme with Chemically ^{13}C-Enriched Methionine Residues Using 2-Aminothiophenol as a Reagent of Thiolysis

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Jones *et al.* have reported that the ε-carbons of methionine residues in myoglobin can be enriched with stable isotope (^{13}C) in two steps, *i.e.*, methylation of methionine residues with $^{13}CH_3I$ in the protein and thiolysis using dithiothreitol [Jones, W.C., Rothgeb, T.M., and Gurd, F.R.N. (1976) *J. Biol. Chem.* 251, 7452-7460]. Using their method, we failed to prepare active lysozyme in which the ε-carbons of methionine residues are enriched with ^{13}C, because many side reactions took place under the thiolysis condition (pH 10.5, 37°C). When we employed 2-aminothiophenol as a reagent for thiolysis, the reduction proceeded under a weakly acidic condition to afford fully active lysozyme, in which the ε-carbons of two methionine residues were enriched with ^{13}C, in a 30% yield. Analysis of the ^{13}C-edited NOESY spectra of ^{13}C-enriched methionine lysozyme in the absence and presence of a substrate analogue indicated the occurrence of conformational change around Met 105 in lysozyme.

Key words: chemical modification, depression of deamidation, isotope labeling, lysozyme, NMR.

NMR is a powerful tool to observe the behavior of proteins in solution, and stable isotope labeling is a convenient approach to allow straightforward assignment of signals. It is generally done by culturing *Escherichia coli* or other cells that can produce the desired protein in the presence of an amino acid labeled with the stable isotope. However, it is necessary to construct an expression system for the desired protein, and then to examine the optimum conditions for the expression of the desired protein.

Jones *et al.* (*1*) have reported a method to label ε-CH_3 of methionine residues in myoglobin with ^{13}C nuclei according to the procedure in Scheme 1. In their method, the protein was exposed to an alkaline condition for a long time in the process of thiolysis. As the deamidation of Asn or Gln residues in a protein easily occurs under the alkaline condition (*2*), it would be impossible to obtain an intact protein after such incubation. Irreversible chemical reactions such as the deamidation of Asn or Gln residues may affect the stability and the activity of the protein. Moreover, as the reduction of disulfide bond(s) occurs in the thiolysis of ^{13}C-methylated methionine in a protein with disulfide bond(s), renaturation must be carried out to obtain an active structure. However, it was reported that a decrease in the positive net charge due to deamidation of Asn or Gln residues in lysozyme, a basic protein, caused a reduction of the folding yield from its reduced form (*3*). Therefore, we must depress irreversible chemical reactions within the polypeptide chain during the thiolysis of the ^{13}C-methylated methionine residue in order to obtain intact protein in which ε-CH_3 of methionine residues are enriched with ^{13}C nuclei. As the thiol group in 2-aminothiophenol has a low pK_a, reduction of the disulfide bond of a protein using this reductant occurs under conditions from neutral pH to weakly acidic pH (*4*). Namely, when the thiolysis of the ^{13}C-methylated methionine residues is carried out using 2-aminothiophenol under a weakly acidic condition, we can depress irreversible chemical reactions. In this paper, we describe suitable conditions to obtain active lysozyme in which ε-CH_3 of methionine residues are enriched with ^{13}C nuclei, and we report a conformational change in the presence of a substrate analogue.

MATERIALS AND METHODS

Materials—Lysozyme was donated by QP company. CM-Toyopearl and BioGel P-4 were purchased from Tosoh (Tokyo) and Bio-Rad Laboratories, respectively. *Micrococcus luteus* was purchased from Sigma. Dithiothreitol and 2-aminothiophenol were purchased from Nacalai Tesque (Kyoto). Wakosil $_5C_{18}$ and Cosmosil C4 columns were obtained from Wako Pure Chemicals (Osaka) and Nacalai Tesque, respectively. $^{13}CH_3I$ was a product of ISOTEC (USA). A trimer of *N*-acetylglucosamine was purchased from Seikagaku Kogyo (Tokyo). All other chemicals were of analytical grade for biochemical use.

Reaction of Proteins with $^{13}CH_3I$—Reaction of proteins

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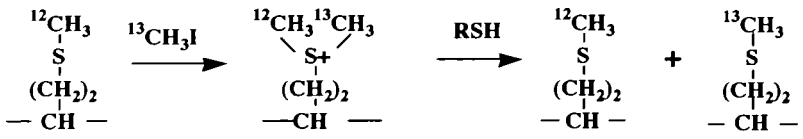
Abbreviations: NAG, *N*-acetylglucosamine; EDTA, ethylenediaminetetraacetic acid; HMQC, homonuclear multiple quantum coherence; NMR, nuclear magnetic resonance; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reversed phase-high performance liquid chromatography; TPCK, L-(tosylamino 2-phenyl)ethyl chloromethyl ketone.



Scheme 1. **Methylation and demethylation of methione residue.**

with $^{13}CH_3I$ was carried out according to the literature (*5*). Namely, each protein (20 mg) was dissolved in 0.1 N KNO_3 (pH 3.3) containing 6 M guanidine HCl. ^{13}C-labeled methyl iodide (0.1 M) was added to the solution. The mixture was vigorously stirred at room temperature for 10 h in the dark, then dialyzed against 10% acetic acid and lyophilized.

Thiolysis of the ^{13}C-Methylated Methionine Residues in Proteins—Thiolysis of the ^{13}C-methylated methionine residues in proteins using dithiothreitol was carried out according to the literature (*1*). Thiolysis using 2-aminothiophenol was carried out as described below. Lysozyme in which methionine residues had been methylated with $^{13}CH_3I$ (10 mg) was dissolved in 1 ml of 0.1 M MOPSO buffer (pH 6) containing 8 M guanidine HCl, 1 mM EDTA, and 20% ethanol. 2-Aminothiophenol (2 ml) was added to the solution, and the reaction mixture was degassed using a vacuum pump. The vessel was then sealed. To remove 2-aminothiophenol, the reaction mixture was applied to a column of BioGel P-4 (1.5×20 cm) equilibrated with 20% aqueous ethanol containing 6 M guanidine HCl. Each protein fraction was dialyzed against 10% acetic acid at 4°C and lyophilized.

Renaturation of Reduced Protein—Renaturation was carried out according to the method of Maeda *et al.* (*6*). Namely, a protein (10 mg) was dissolved in 1 ml of 8 M urea solution (0.584 M Tris-HCl buffer at pH 8.6 containing 8.125 M urea and 5.37 mM EDTA). The solution was degassed, 5 μl of 2-mercaptoethanol was added, and the solution was incubated at 40°C for 1 h under a nitrogen atmosphere. Then 16.2 mg of oxidized glutathione dissolved in 200 μl of 8 M urea was added and the whole was incubated at 40°C for 20 min. Renaturation of reduced lysozyme which had formed a mixed disulfide with oxidized glutathione was carried out at pH 8 by slowly decreasing the urea concentration from 8 to 0 M.

Purification of the Renaturated Proteins—The purification of the renatured lysozyme was carried out according to the literature (*7*). Briefly, the pH of the renatured solution was lowered to 5.5, then 10 mM *N*-ethylmaleimide was added and allowed to react for 30 min at room temperature. To stop the reaction, the pH of the solution was lowered to 3 by adding acetic acid. After dialysis of the solution against distilled water, the dialysate was subjected to HPLC on a column of Cosmosil C4 equilibrated with 1% acetonitrile containing 1% acetic acid. The column was eluted with 50% acetonitrile containing 1% acetic acid at a flow rate of 1 ml/min. The elution of proteins was detected by measuring the absorbance at 280 nm, and the protein fraction was lyophilized. For further purification, the protein fraction was subjected to HPLC on a column of CM Toyopearl 650 S (8× 500 mm) at pH 7. The column was eluted with a gradient formed from 100 ml of 0.05 M phosphate buffer at pH 7 and 100 ml of the same buffer containing 0.5 M NaCl at a flow rate of 1.5 ml/min. The elution was monitored by measuring the absorbance at 280 nm. Each eluted protein fraction was dialyzed against water and lyophilized.

Enzymatic Activity—The enzymatic activity of lysozyme against *M. luteus* was measured turbidimetrically at 450 nm at pH 7. To a 3 ml suspension of *M. luteus* in 0.05 M phosphate buffer at pH 7 was added 100 μl of lysozyme solution, and the decrease in the turbidity was monitored with a Hitachi 150-20 spectrophotometer equipped with a thermostatically controlled cell holder.

Analytical Methods—Acid urea PAGE was carried out according to the method of Hollecker and Creighton (*8*). Analyses of the tryptic peptides of reduced *S*-carboxymethylated proteins on RP-HPLC were carried out according to a previous paper (*9*).

NMR Measurements—^{13}C-NMR spectra of lysozyme (2 mM) at pD 3.8 and 35°C in the absence or presence of a substrate analogue were recorded at 150 MHz with a Varian 600 Unity plus. Dioxane was employed as the internal standard (67.8 ppm). The pD values were the pH meter readings without adjustment for isotope effect (*10*). ^1H-^{13}C HSQC experiments were carried out using the standard procedure (*11*). ^{13}C-edited NOESY at pH 3.8 and 35°C was carried out according to the literature (*12*) under conditions where the mixing time was 150 ms. In these experiments, 16 transients were typically recorded for each of 256 increments. A digital resolution of 2.4 Hz per point in the ^1H dimension and 11.7 Hz per point in the ^{13}C dimension was used.

RESULTS AND DISCUSSION

Methylation of Methionine Residues in Lysozyme—Hen egg-white lysozyme has two methionine residues (*13*). One is methionine 12, which is in an α-helix, and the other is methionine 105 which is in the center of the "hydrophobic box" (Fig. 1). Therefore, the modification of the methionine residues in lysozyme with $^{13}CH_3I$ was carried out in the presence of 6 M guanidine HCl ("MATERIALS AND METHODS"). Methylated methionine could be detected between ammonia and histidine with a Hitachi L-8500 amino acid analyzer under routine conditions. After acid hydrolysis (110°C, 20 h) of an aliquot of the reaction mixture at an appropriate time, the number of methylated methionine residues was determined by using an amino acid analyzer. Twenty-two percent of the methylated methionine was hydrolyzed to methionine residues during acid hydrolysis. Therefore, the number of methylated methionine residues was obtained by correcting for hydrolysis (Fig. 2A). The methylation was found to be completed in 10 h. As the amino acid composition of modified lysozyme except for cystine or tryptophan, which could not be determined by means of acid hydrolysis, was identical to that of the native lysozyme, no side reaction had occurred during the reaction. After 10 h, when intact methionine had almost disappeared, the reaction mixture was dialyzed against 10% acetic acid and lyophilized. The ^{13}C-methylated lysozyme was reduced, *S*-carboxymethylated, digested with TPCK-trypsin, and subjected to peptide mapping by RP-HPLC.

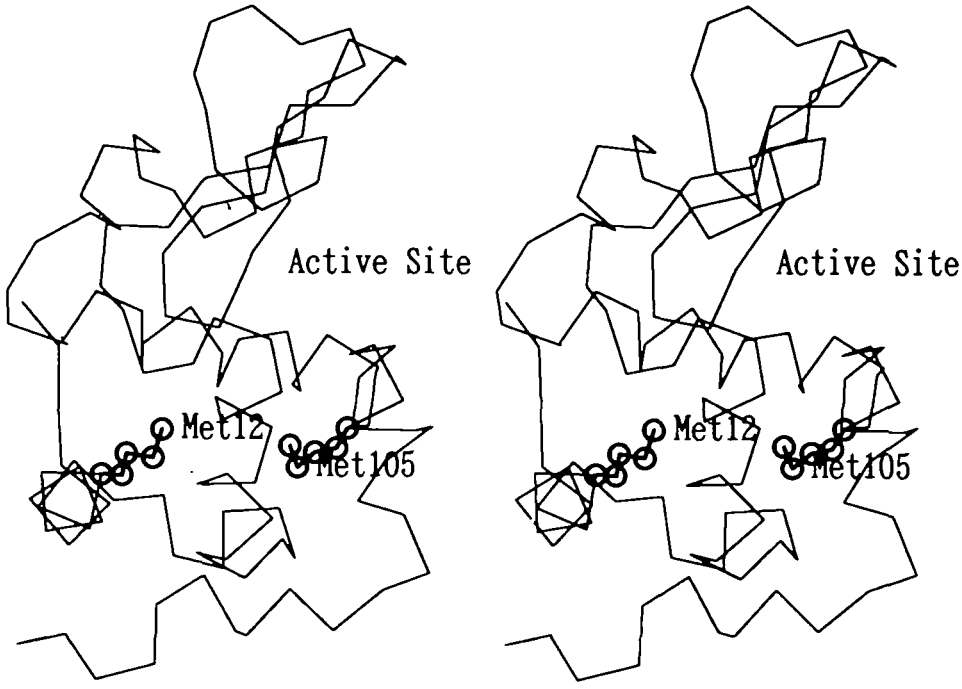


Fig. 1. **Stereoview of the crystal structure of hen lysozyme [set 16 RS, Diamonds, (*14*): Coordinates taken from Protein Data Bank (2LZT)].** The bold lines indicate methionine residues.

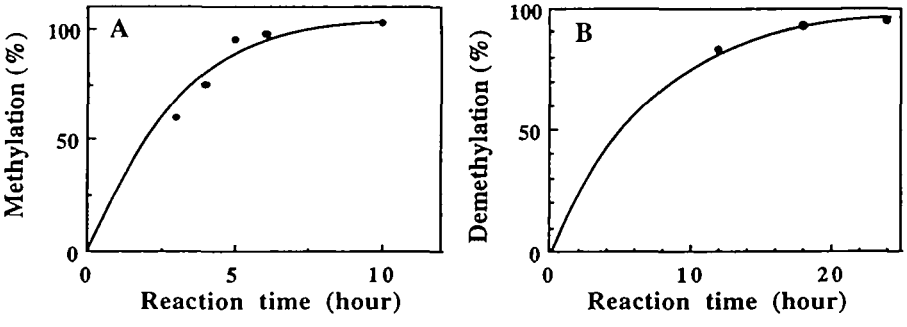


Fig. 2. **(A) Time course of the methylation of methionine in 0.1 M KNO, (pH 3.3) containing 6 M guanidine HCl and 0.1 M $^{13}CH_3I$ at room temperature. (B) Time course of thiolysis of methylated lysozyme with 2-aminothiophenol (6.24 M) in 0.1 M MOPSO buffer (pH 6) containing 1 mM EDTA, 8 M guanidine HCl, and 20% ethanol at 50°C.**

The elution pattern of the peptides thus obtained is shown in Fig. 3B. For comparison, the pattern from the intact lysozyme is shown in Fig. 3A. Methionine residues were suggested to be included in peptides a and b from the peptide analyses derived from the intact lysozyme. In ^{13}C-methylated lysozyme, these peptides disappeared and the new peptides c and d appeared. The new peptides eluted earlier than the original peptides. This indicated that the peptides had a positive charge due to the ^{13}C-methylation of a methionine residue. Similar behavior on reversed phase HPLC was observed in a peptide whose methionine residue has a positive charge due to modification by CH_3I (*15*). Acid urea PAGE, separates proteins depending on the difference in net charge (*8*). In a comparison of the mobility of the reduced *S*-carboxymethylated sample, which had reacted with $^{13}CH_3I$ for 10 h, with that of the reduced *S*-carboxymethylated unmodified lysozyme (Fig. 4, lane 2), the sample modified with $^{13}CH_3I$ had a greater positive net charge than the intact lysozyme (Fig. 4, lane 4) because the mobility of the modified lysozyme was larger than that of the intact lysozyme. From the above results, we concluded that both methionine residues were ^{13}C-methylated in the lysozyme obtained here. The yield of the modified lysozyme, in which two methionines are modified with ^{13}C at ε-CH_3 was 95%.

Demethylation of Methylated Methionine Residues in Lysozyme—Jones *et al.* (*1*) demethylated the methylated methionine residues in myoglobin using dithiothreitol. We tried to demethylate the methylated methionine residues in lysozyme according to their method (pH 10.5 in the presence of 0.5 M dithiothreitol at 37°C for 18 h). Since the amino acid composition of the demethylated lysozyme after thiolysis using dithiothreitol was identical to that of the native lysozyme, no detectable side reaction occurred during the reaction (though cystine or tryptophan could not be determined by means of acid hydrolysis). However, when we compared lane 1 and lane 2 in acid urea PAGE of the reduced *S*-carboxymethylated form, the lysozyme after thiolysis showed a broader band than the intact lysozyme with similar mobility to that of the reduced *S*-carboxymethylated intact lysozyme. The result indicated that many chemical reactions, such as destruction of the disulfide bond as suggested by Ahern and Klibalov (*16*), also occurred in addition to the deamidation of Asn or Gln residue under the alkaline condition. As a result, we could not obtain any active lysozyme when the renaturation from

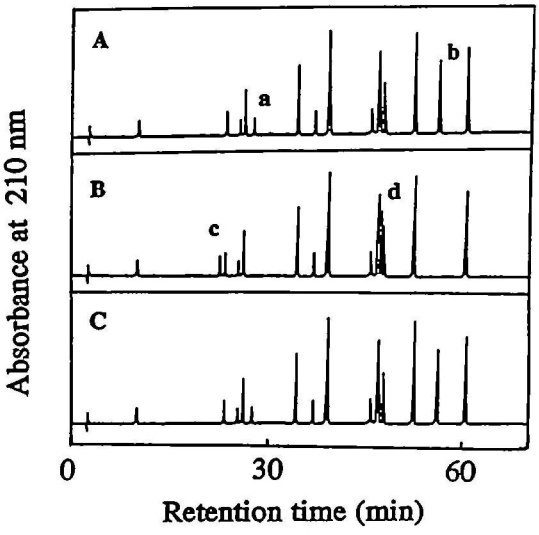


Fig. 3. **Reversed phase HPLC of tryptic peptide from reduced and *S*-carboxymethylated (A) intact lysozyme, (B) lysozyme with ^{13}C-methylated methionine residues, (C) lysozyme after the thiolysis of the protein with ^{13}C-methylated methionine residues using 2-aminothiophenol.** The Wakosil $_5C_{18}$ column (4.6×250 mm) was eluted with a gradient of 40 ml of 1% acetonitrile and 40 ml of 40% acetonitrile both containing 0.1% HCl at a flow rate of 0.6 ml/min.

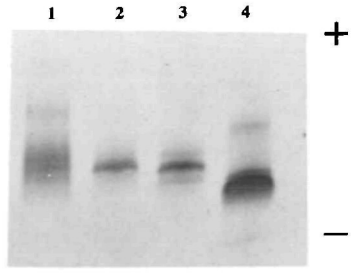


Fig. 4. **Acid urea PAGE of reduced and *S*-carboxymethylated lysozymes.** Lane 1, from lysozyme after thiolysis using dithiothreitol under an alkaline condition; lane 2, from intact lysozyme; lane 3, from lysozyme after the thiolysis using 2-aminothiophenol under a weakly acidic condition; and lane 4, from lysozyme with ^{13}C-methylated methionine residues.

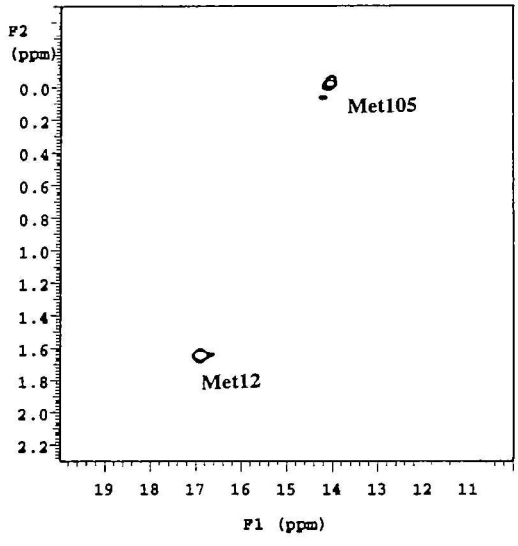


Fig. 5. **^{1}H-^{13}C HMQC spectrum of ^{13}C-enriched methionine lysozyme at pD 3.8 and 35°C.** The ^{1}H-^{13}C crosspeaks are labeled with the residue number.

its reduced form was carried out at a concentration of 0.1 mg/ml according to the method of Maeda *et al.* (*6*), whereas the folding yield of the intact lysozyme from its reduced form was 80% under the same conditions.

2-Aminothiophenol has reducing power under weakly acidic conditions (*4*). The lysozyme with ^{13}C-methylated methionine residues was dissolved in 0.1 M MOPSO buffer at pH 6 containing 6 M guanidine HCl and 1 mM EDTA and incubated at 50°C in the presence of 6.24 M 2-aminothiophenol. The reaction mixture was applied to a column of BioGel P-4 (1×40 cm) equilibrated with 20% aqueous ethanol containing 6 M guanidine HCl in order to remove 2-aminothiophenol, then amino acid analysis of the eluted lysozyme was carried out. The number of methionines increased with the reaction time and reached a plateau within 24 h (Fig. 2B). Therefore, the thiolysis of methylated methionine in lysozyme was found to be completed in 24 h. Although we tried to carry out the thiolysis at lower temperature and lower pH, the thiolysis of methylated methionines in lysozyme was incomplete within 24 h (data not shown). Since the amino acid composition of the lysozyme after thiolysis using 2-aminothiophenol was identical to that of the native lysozyme, no side reaction occurred during the thiolysis using 2-aminothiophenol. The protein fraction was dialyzed against 10% acetic acid and lyophilized. Acid urea PAGE of the reduced *S*-carboxymethylated protein after the thiolysis of the lysozyme with ^{13}C-methylated methionine residues using 2-aminothiophenol is shown in lane 3 in Fig. 4. The mobility of the major band was the same as that of the intact lysozyme, but some minor bands were observed. These results indicated that the irreversible chemical reactions seen in the case of 2-aminothiophenol were greatly depressed as compared with the case of dithiothreitol. A reduced *S*-carboxymethylated sample after thiolysis of lysozyme with ^{13}C-methylated methionine residues using 2-aminothiophenol was digested with TPCK-trypsin, and subjected to peptide mapping by RP-HPLC. The elution pattern of the peptides thus obtained is shown in Fig. 3C. The pattern was almost identical to that of the intact lysozyme. Moreover, we obtained active lysozyme when the renaturation from the reduced form at a concentration of 0.1 mg/ml was carried out according to the method of Maeda *et al.* (*6*). After RP-HPLC and ion-exchange chromatography of the renatured solution, the total yield of active lysozyme in which ε-CH_3 of methionine residues is enriched with ^{13}C nuclei was 30%.

Tomizawa *et al.* (*17*) have demonstrated that inactivation of lysozyme occurred owing to a decrease in the folding yield because of the formation of the succinimide intermediate at the Asp-Gly sequence during incubation under the weakly acidic condition. However, the formation of succinimide could not always be detected by peptide mapping with RP-HPLC under the routine conditions. Therefore, the reason why the folding yield was lower than expected from the population of the major band in acid urea PAGE is presumably that such a side reaction occurred in the major fraction. Nevertheless, we were able to obtain

enough active lysozyme to measure its ^{13}C-NMR spectra.

Conformation of the Renatured Lysozyme That Contains ^{13}C-Enriched Methionine Residues—Purification of the renatured lysozyme was carried out using ion-exchange chromatography and RP-HPLC ("MATERIALS AND METHODS"). The retention time of the renatured lysozyme on ion-exchange chromatography and its activity were identical to those of intact lysozyme. Furthermore, in order to examine the tertiary structure, the ^1H-^1H phase-sensitive DQF-COSY spectrum of the renatured lysozyme was measured (data not shown). When the spectrum of the renatured lysozyme was compared with that of intact lysozyme, there was no difference in the fingerprint region.

Signal Assignment of ^{13}C-Enriched Methionine Lysozyme—The ^1H-^{13}C HMQC spectrum of the ^{13}C-enriched methionine lysozyme at pD 3.8 and 35°C is shown in Fig. 5. There were two major crosspeaks. As the ε-CH_3 proton resonances of both methionine residues have already been assigned (*18*), the resonances at 16.09 ppm and at 14.06 ppm were predicted to be due to the ε-carbons of methionine 12 and methionine 105, respectively, based on the ^1H-^{13}C HMQC spectrum. There was a minor cross peak around the major cross peak of Met 105 (Fig. 5). The minor one may have originated from Met 105 because the signal volume from Met 12 was equal to that of the major one of Met 105 plus the minor one. After measurement for 24 h at pD 3.8 and 35°C, the sample was applied to a CM Toyopearl 650S ion-exchange HPLC column (5×400 mm) at pH 7. The column was eluted with a gradient formed with 100 ml of 0.05 M phosphate buffer and 100 ml of 0.05 M phosphate buffer containing 0.5 M NaCl at a flow rate of 1.5 ml/min. The minor peak that eluted later than the major peak of native lysozyme on the ion-exchange HPLC was identified as 101-succinimide lysozyme where the aspartylglycyl sequence at Asp 101–Gly 102 formed a cyclic imide. The isomerization from the native lysozyme to the 101-succin-

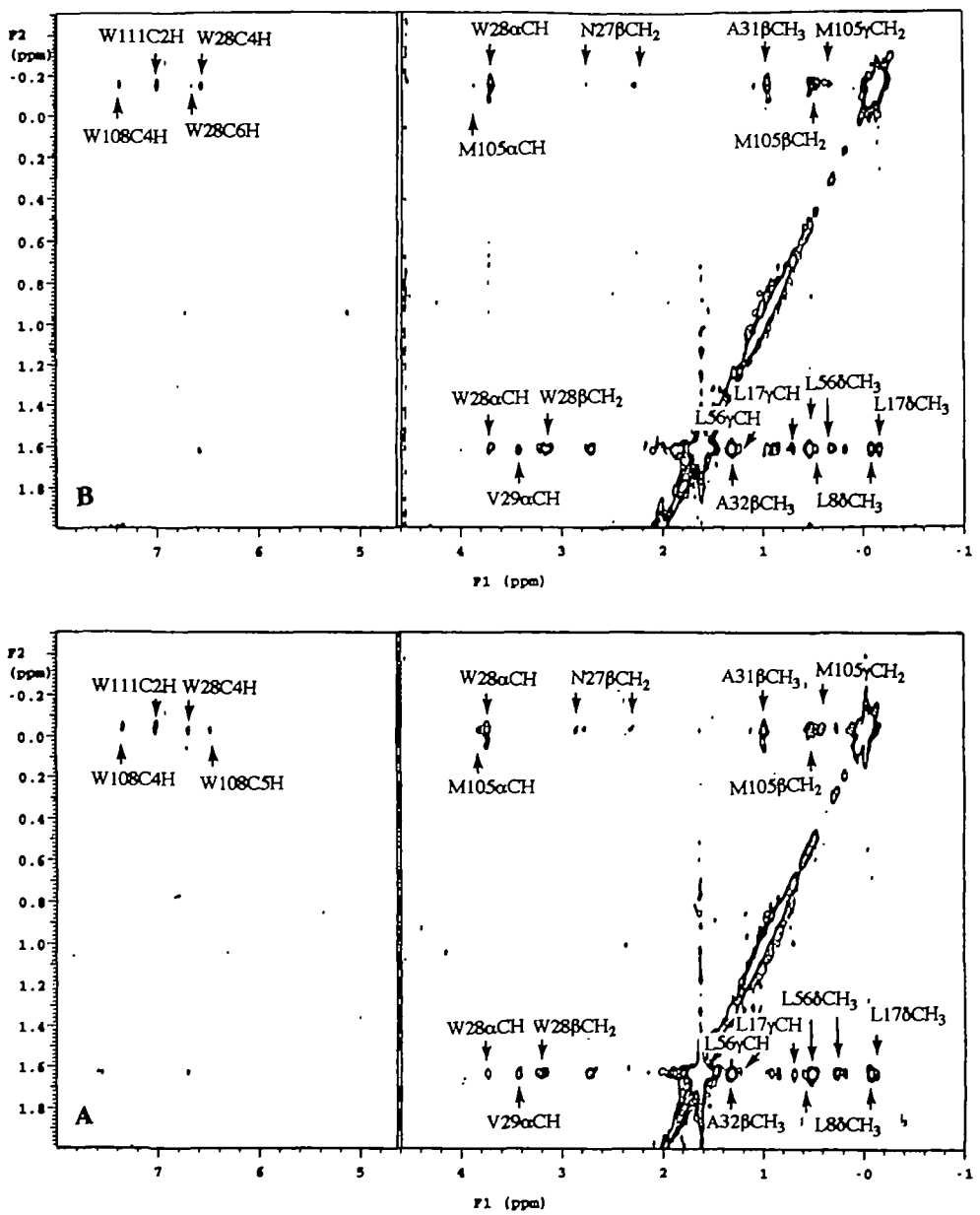


Fig. 6. **^{13}C-edited NOESY spectra of ^{13}C-enriched methionine lysozyme in the absence (A) and presence (B) of 1.61 mM (NAG)$_3$ at pH 3.8 and 35°C with a 150 ms mixing time.** Protons at which NOE is observed with ε-proton of Met 12 and Met 105 were labeled on the chart.

imide lysozyme was suggested to occur non-enzymatically under acidic conditions at 40°C (*17*). In our previous report, the ^1H chemical shift of ε-CH_3 of Met 105 in lysozyme was shown to be altered by 0.04 ppm by isomerization from the α-aspartylglycyl sequence at Asp 101-Gly 102 to the β-one (*19*). Therefore, the local conformational change around Asp 101 may affect the chemical shift at Met 105. From these results, we consider that the minor signal in the ^{13}C-NMR spectrum of ^{13}C-enriched methionine lysozyme may result from the formation of the 101-succinimide lysozyme during the signal accumulation.

Binding of Substrate Analog to ^{13}C-Enriched Methionine Lysozyme—Lysozyme is one of the β-1,4-*N*-acetylmuramidases. A trimer of *N*-acetylglucosamine, $(NAG)_3$, is a substrate analogue and nonproductively binds to the upper part of the active site cleft in lysozyme (*12*). Using ^{13}C-enriched methionine lysozyme, it is possible to detect any change in the local structure around the methionine residues when the substrate analog binds to the lysozyme. From the ^{13}C-NMR spectra in the absence or presence of $(NAG)_3$ (data not shown), the chemical shifts of both ε-CH_3 resonances were found not to change. In the ^{13}C-edited NOESY spectrum in the absence of $(NAG)_3$, NOEs between the ε-proton of enriched Met 105 and protons around Met 105 (within about 5 Å) were observed. The assignments of the resonances are described in Fig. 6. All these protons were spatially located within 5 Å from the ε-proton of enriched Met 105 based on X ray crystallographic data (*14*). NOEs were also observed in the absence of $(NAG)_3$ as well in the presence of $(NAG)_3$ (Fig. 6B). In particular, when the NOEs from the two-dimensional ^{13}C-edited NOESY spectra were examined at pH 3.8 and 35°C for the signal corresponding to the ε-CH_3 proton of Met 105 in the absence or presence of $(NAG)_3$, the intensity of NOE between the ε-CH_3 proton of Met 105 and the C5-proton of Trp 28 increased (154%) and that between the ε-CH_3 proton of Met 105 and the C5-proton of Trp 108 decreased (57%), while the intensity of NOE between the ε-CH_3 proton of Met 105 and the other protons did not change (within $\pm$15%). Therefore, these results indicated that a subtle conformational change in the hydrophobic box, which includes Met 105, occurred in the presence of $(NAG)_3$. As regards Met 12, there were some NOEs between the ε-CH_3 proton of Met 12 and surrounding residues within 5 Å. However, in the presence or absence of $(NAG)_3$, there was no difference in the NMR signals of Met 12. The result may imply that Met 12 is apart from the active site cleft. From the above results, it was concluded that the enrichment of ε-CH_3 of the methionine residues with ^{13}C nuclei was useful for obtaining information about the local conformation of lysozyme molecule.

Implications of the Improved Method to Enrich Methionine Residues in Proteins with ^{13}C—We have obtained lysozyme in which ε-CH_3 of methionine residues is enriched with ^{13}C nuclei using 2-aminothiophenol as a reagent for thiolysis. It was reported that information on the relationship between protein structure and function could be obtained by labeling ε-CH_3 of methionine residues in proteins with ^{13}C (*20-22*), and such enrichment has been carried out using *E. coli* or mammalian cells. On the other hand, an analysis of the iron coordination of ferric cytochrome *c* has recently been carried out using a chemical labeling method (*23*). The chemical labeling method has the advantage that a special expression system to produce a labeled protein is not required. On the other hand, the efficiency of incorporation of a stable isotope into a protein in the case of using *E. coli* or mammalian cells is generally superior to that of the chemical labeling method (50%). However, we were easily able to obtain sufficient incorporation to allow measurement of the NMR spectra of a protein obtained by chemical labeling.

A novel feature of the present labeling method is the suppression of the irreversible chemical reactions in the course of ^{13}C-labeling in comparison with the previous method (*1*). Myoglobin (*1*), cytochrome *c* (*23*), and α-chymotrypsin (*24*) in which ε-CH_3 of methionine residues are labeled with ^{13}C have been prepared according to the method of Jones *et al.* (*1*), but they contain Asn and Gln residues, and deamidations at Asn and Gln residues in these proteins would have occurred during thiolysis at pH 10.5 and 37°C. Thus, it is important to develop an improved method to give an intact protein with ^{13}C-enriched methionine at ε-CH_3. Moreover, undesirable side reactions such as deamidation would decrease the yield of the labeled protein or might affect protein function. This may be the reason why the previous labeling method has been applied to only a few proteins. Our method makes it possible to obtain ^{13}C-methionine labeled intact proteins. This approach should also be applicable to other proteins. Indeed, we have already obtained intact ribonuclease A in which ε-CH_3 of four methionine residues is enriched (Ueda *et al.*, unpublished results).

Prospects for Chemical Labeling in the Analysis of Protein Structure—As the population of methionine residues in a protein is smaller than those of other amino acids, the assignment and analysis of the resonances from methionine residues are relatively easy. Moreover, methionine is a good reporter to analyze complex system (interaction with ligands, denatured protein, *etc.*). In addition, further methionine residues could be introduced by using gene engineering techniques at the positions of leucine or isoleucine residues, because the sizes of these residues are almost identical to that of the methionine residue, and this would allow other regions of the protein to be probed. Since the relaxation mechanism of ^{13}C nuclei in proteins is simpler than that of ^1H nuclei, extensive information on the mobility of the methionine residues in a protein, for example the mobility of the side chain in the presence of a ligand, may be obtained rather easily.

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