High-level expression of uniformly ^{15}N-labeled hen lysozyme in *Pichia pastoris* and identification of the site in hen lysozyme where phosphate ion binds using NMR measurements

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Abstract The non-enzymatic deamidation of Asn to Asp is known to occur in proteins and peptides and is accelerated by phosphate buffer [Tyler-Cross, R. and Schirch, V. (1991) J. Biol. Chem. 25, 22549–22556]. We attempted to identify the site in lysozyme where a phosphate ion binds by means of ^{1}H-^{15}N HSQC measurements of ^{15}N-labeled lysozyme, which was successfully obtained using *Pichia pastoris*. As a result, we found that the phosphate ion was preferentially bound to Asn-103 in hen lysozyme. The method presented here may be useful for identifying the binding site of a protein with low molecular weight substances.

Key words: Deamidation; Lysozyme; Phosphate ion; *Pichia pastoris*

1. Introduction

A protein or peptide often deteriorates due to non-enzymatic chemical reactions during storage. Among these chemical reactions, the non-enzymatic deamidation of Asn to Asp is known to occur in proteins and peptides [1–3]. Several studies have shown that the structures of the amino acids which flank Asn have a marked effect on the rate of deamidation; especially when a Gly residue follows, the reaction may be intermolecular and may proceed by way of a five-membered succinimide intermediate [4–8]. In addition, some investigations have demonstrated that the rate of deamidation is affected by both pH and ionic strength [9,10] and that certain buffers tend to increase the reaction rate. Phosphate ion especially was reported to accelerate the rate of deamidation of Asn using a series of peptides [11]. We have also demonstrated that phosphate ion accelerates the inactivation of hen lysozyme at pH 6 and 100°C [12]. This study revealed that the rate of deamidation did not significantly increase when 50 mM or 100 mM phosphate buffer was used, and this indicates that 50 mM phosphate buffer is sufficient to saturate the lysozyme site. Based on the above results, there was a possibility that phosphate ion preferentially interacted with some Asn residues in the native state. However, it is difficult to identify the lysozyme site where a phosphate ion binds in the denatured state; therefore, we attempted to do that in the native state.

In order to obtain information on the interaction between proteins and ligands, the ^{1}H-^{15}N HSQC spectrum may be suitable because of its high separation of the resonances [13,14]. To carry out the experiment, we needed an efficient expression system for recombinant proteins labeled with stable isotopes. *Escherichia coli* has been commonly employed to express recombinant proteins labeled with stable isotopes. However, the recombinant proteins produced by the *E. coli* system often possess an additional methionine residue at their N-terminals, which affect the conformation of the protein [15], and the recombinant proteins often had to be reactivated when they were expressed as inclusion bodies. The methylotropic yeast *Pichia pastoris* was reported to be an alternative host for high-level expression [16,17]. This system has the advantage that expression of the *AOX1* gene which encodes alcohol oxidase is tightly regulated and is induced by methanol to very high levels, typically over 30% of the total soluble protein [16–18]. Moreover, transformed *P. pastoris* secretes very little of its alcohol oxidase, simplifying purification of any heterologously secreted protein [19]. However, there were few reports on the utility of the expression system with *P. pastoris* for production of proteins labeled with stable isotopes. In this paper, we examined the preparation of ^{15}N-labeled hen lysozyme using the *P. pastoris* expression system and investigated the binding site of the phosphate ion with the resulting hen lysozyme by NMR measurements.

2. Materials and methods

2.1. Strains and plasmid

E. coli TG1 was used as the host strain for constructing lysozyme/pPIC9. Expression vector pPIC9 contains the alcohol oxidase (AOX) I promoter and the α-mating factor (α-MF prepro) secretion signal (Invitrogen, San Diego, CA, USA). Plasmid pHA332 was employed as the source of the lysozyme cDNA [20]. *P. pastoris* GS115 (Invitrogen) was used as the host strain for expression.

2.2. Medium

BMGY for growing *P. pastoris*, BMMY for induction of recombinant protein, and MD and MM agar plates for Mut^{s} (methanol utilization slow) screening have been described in the Invitrogen manual. FM22 (900 ml water, 42.87 g KH_2PO_4, 1 g $(NH)_2SO_4$, 1 g $CaSO_4{\cdot}2H_2O$, 14.28 g K_2SO_4, 11.7 g $MgSO_4{\cdot}7H_2O$ and 4×10^{-5}% biotin solution, at a pH 4.9 with 10 M KOH [21]) was used to prepare the ^{15}N-labeled lysozyme.

2.3. Construction of expression vector

A DNA fragment encoding lysozyme was amplified by PCR using pHA332 as a template [20], with the following oligonucleotide primers: 5′-CCCGCCTCGAGAAAAGAAAAGTCTTTGGACGATGTGAG-3′ (*Xho*I) and 5′-GTCTCCGACGGCCGACACTCTTAAGGGGCG-3′ (*Eco*RI).

The PCR reaction was carried out in 100 μl volumes using one unit of Taq polymerase with 25 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min. The PCR product was digested with *Xho*I and *Eco*RI, and inserted between the *Xho*I and *Eco*RI sites of the *P. pastoris* expression vector pPCI9. The resulting plasmid was named lysozyme/pPIC9.

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2.4. Transformation of P. pastoris

P. pastoris GS115 (His$^-$) was transformed with linear-fragmented lysozyme/pPIC9 by digestion with *Bgl*II or *Sal*I. The lithium chloride method [22] was used for transformation, and the cells were plated on MD agar plates for the selection of His$^+$ transformants. To screen for methanol utilization, the His$^+$ transformants were plated on MM agar plates, and a Mut$^+$ (methanol utilization plus) control was incubated for comparison. After 24–48 h, the Mut$^+$ colonies were visibly larger than the Mut^s transformants.

2.5. Screening for lysozyme expression

P. pastoris transformants were cultured in 5 ml of BMGY for 2 days at 30°C. Cells collected from 2 ml BMGY culture were resuspended in 2 ml of BMMY. The secretion of lysozyme into the culture medium was monitored by the lytic activity according to the literature [23].

2.6. Expression of ^{15}N-labeled lysozyme by P. pastoris [lysozyme/pPIC9]

To produce ^{15}N-labeled lysozyme, *P. pastoris* transformant cells were grown in 1 l of FM22-glycerol medium (100 ml 10×glycerol, 1 ml PTM1, 6 ml 250×biotin, and 6 ml 10 M KOH were added to 900 ml FM22) [21]. The defined minimal medium FM22, which contains [^{15}N]ammonium sulfate (99% ^{15}N; Shokotsusho Co., Ltd., Tokyo, Japan) as the sole nitrogen source, was developed. After incubation for 2 days at 30°C, the cells were collected by centrifugation and resuspended in the same medium, except that 0.5% methanol was added instead of glycerol, and were cultivated for 120 h with additional supplies of methanol every 24 h.

2.7. Purification of lysozyme

The culture supernatant was diluted 10-fold with water, and the secreted lysozyme was isolated by cation exchange chromatography on a column (4.0×15 cm) of CM-Toyopearl 650M, which was eluted with a gradient of 500 ml of 0.05 M phosphate buffer and 500 ml of the same buffer containing 0.5 M NaCl at pH 7 and 4°C. The eluted lysozyme was dialyzed against distilled water and then lyophilized. Finally, we obtained about 20 mg/l of uniformly ^{15}N-labeled lysozyme.

2.8. NMR measurements

The NMR samples were prepared to contain 1.5 mM protein in 90% H_2O/10% D_2O (v/v), and the pH was adjusted to 5.0. NMR experiments were performed at 35°C on a Varian Inova 600-MHz spectrometer equipped with a triple-resonance, pulse-field gradient probe with an actively shielded *z* gradient and a gradient amplifier unit. ^{1}H-^{15}N HSQC spectra were recorded with the combined use of water flip-back [24] and WATER-GATE [25] solvent suppression. The spectrum widths of the F_1 and F_2 dimensions were 2000 Hz and 8003.2 Hz, respectively. Each collected data set contained 256 (t_1)×2048 (t_2) complex data points.

3. Results and discussion

3.1. Expression of ^{15}N-labeled lysozyme

For the *P. pastoris* recombinant expression system, it is important to select the highest level clone from as many transformants as possible because multicopy integration naturally occurs within a transformed cell population. As a result of screening, a Mut^s strain of GS115 [lysozyme/pPIC9] was selected. The secretion yield of ^{15}N-labeled lysozyme during the fermentation, judged from the lytic activity, is shown in Fig. 1 (top). Finally, the secretion level reached 20 mg/l after a period of 5 days. After centrifugation of the cells, the supernatant was subjected to cation exchange chromatography and eluted with a salt gradient (Fig. 1, bottom). The fractions in the main peak were collected, dialyzed against water and lyophilized. The purified protein showed a single band on analysis by SDS-PAGE (data not shown). On the other hand, based on its ^{1}H-NMR spectrum, the hen lysozyme obtained here was found to be 99% enriched with ^{15}N (data not shown). In order to assign the chemical shift of the ^{15}N resonance of the main

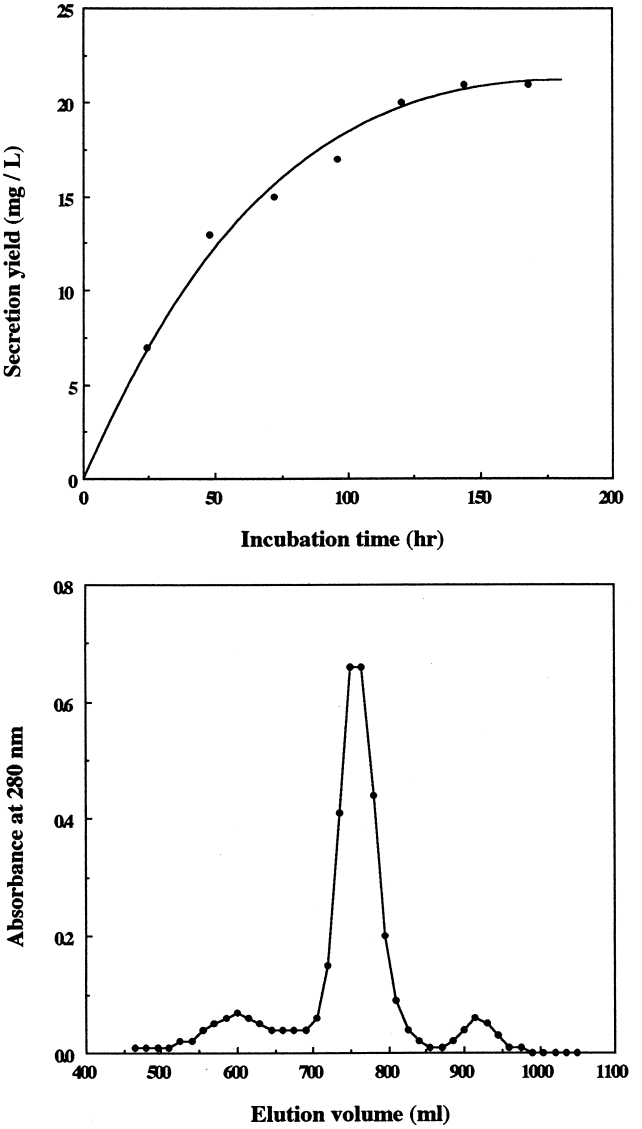

Fig. 1. Top: The amount of secreted lysozyme in the culture medium of *P. pastoris* transformant cell followed by lytic activity. Bottom: Purification of lysozyme by cation exchange chromatography at pH 5.0. The culture supernatant was applied to a column (4.0×15 cm) of CM-Toyopearl equilibrated with 0.05 M AcONa at pH 5.0. The protein was eluted with a NaCl gradient from 0 to 0.5 M in 0.05 M AcONa at pH5.0.

chain by reference to the literature [26], we measured the ^{1}H-^{15}N HSQC spectra under the same conditions. The present ^{1}H-^{15}N HSQC spectrum was completely consistent with the previous one [26]. In a recent report, we showed that the residue attached to the N-terminus of hen lysozyme caused the chemical shift perturbation [15]. Therefore, the consistency of these results revealed that the α-MF prepro-peptide was removed from the N-terminus of the secreted lysozyme.

This system is comparable with higher eukaryotic expression systems in protein processing, protein folding, and post-translational modification, while it is as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. This system also has the merits of lower cost, greater convenience and efficiency compared to other eukaryotic expression systems such as baculovirus or mammalian tissue culture. Furthermore, we obtained a stable isotope-labeled lysozyme at 20 mg/l which is sufficient

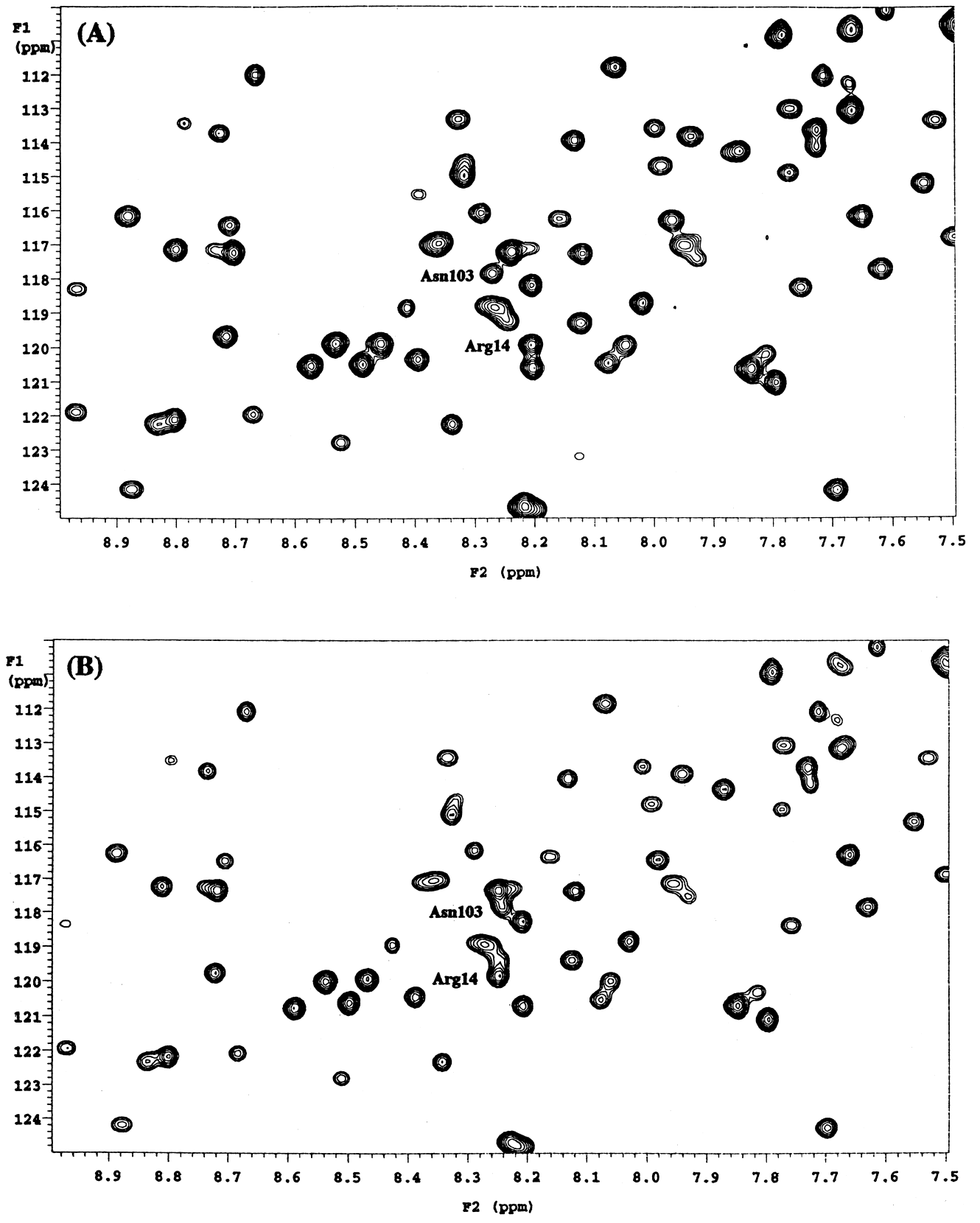

Fig. 2. Region of the 2D ^{1}H-^{15}N HSQC spectrum at a phosphate ion concentration of (A) 0 mM and (B) 19 mM. The peaks corresponding to the backbone amides Arg-14 and Asn-103 are displaced significantly.

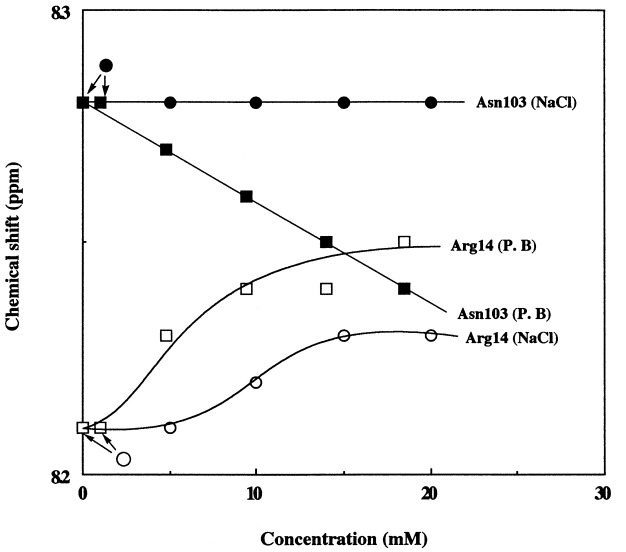

Fig. 3. The chemical shift of NH proton of Arg-14 and Asn-103 vs. the concentration of phosphate ion (P.B) and NaCl, respectively.

for heteronuclear NMR measurements. Laroche et al. reported the preparation of the ^{15}N-labeled tick anticoagulant peptide (TAP) using the *P. pastoris* expression system, which was the first report of production of an isotopically labeled protein using *P. pastoris* [27]. They used an expression vector containing a new chimeric leader sequence which consisted of both a PHO1 secretion signal and a synthetic 19 amino acid prosequence, whereas we used an αF signal for the expression vector. Furthermore, it is reported that the αF secretion signal sequence is comparatively good for secretion of recombinant protein by yeast [28,29]. In addition, although they used a high culture density method and the defined minimal medium FM22 which contained 5 g/l of [^{15}N]ammonium sulfate as the sole nitrogen source, we used a different culture method (see Section 2) and FM22 which contained only 1 g/l of an isotopic compound. Therefore, in the production of ^{15}N-labeled protein, our method may be more economic. Consequently, the present method has an advantage over the previous one and should be widely applicable to other proteins.

3.2. Phosphate ion binding to lysozyme

In order to identify the binding sites of phosphate ion in hen lysozyme, concentrated phosphate buffer was added stepwise to the uniformly ^{15}N-labeled lysozyme solution (1.5 mM) so that the total concentrations of phosphate ions were 1, 5, 9, 14, and 19 mM. Fig. 2 shows part of the ^{1}H-^{15}N spectra of hen lysozyme in the absence or presence of 19 mM phosphate ion. The chemical shifts of many resonances were almost unaltered by the addition of phosphate ion, whereas considerable chemical shift changes were observed in the amide nitrogens of Arg-14 and Asn-103. In order to examine whether phosphate ion was preferentially bound to Asn-103 and Arg-14, we carried out the same experiment using NaCl, which has been reported to have no effect on the deamidation of the Asn residue [11], as a control. Fig. 3 shows the chemical shift of Arg-14 and Asn-103 against the concentration of phosphate ion or NaCl and strongly suggested that the chemical shift of Asn-103 by adding phosphate ion depended not on ionic strength but on preferential binding of the phosphate ion to Asn-103. On the other hand, the chemical shift of Arg-14 may be due to preferential binding of the anion.

Asn-103 is located at the loop region and is one of the residues possessing high accessibility to the solvent (PDB 1HEL). However, the accessibility would not be the determinant by which phosphate ions bound preferentially, because the chemical shift of Asn-77, whose accessibility is almost the same as that of Asn-103, did not change on addition of phosphate ion. Asn-103 lies between Gly-102 and Gly-104, and this is a unique sequence in lysozyme. Therefore, phosphate ion may preferentially bind to Asn-103 for the sake of less steric hindrance due to the unique sequence (Gly-102–Asn-103–Gly-104).

On the other hand, Arg-14 is also exposed to the solvent and spatially covers a neighboring residue, His-15. The distance between the side chain guanidinium of Arg-14 and the Nε2 of His-15 was 4.36 Å from the X-ray structure (PDB 1HEL), and the pK_a of His-15 was 5.66 [30]. Therefore, these residues should form a positively charged cluster at pH 5. Thus, both the negatively charged phosphate and chloride ions may bind to Arg-14 by electrostatic interaction. Other candidates among the continuous positive amino acid residues of lysozyme are the peptides Lys-96 and Lys-97. However, because the distance between the nitrogen atoms of the side chain was 9.43 Å based on the X-ray structure (PDB 1HEL), they could not form a positively charged pocket. Furthermore, according to the crystal structure of lysozyme, the electron density map of Arg-14 was one of the most well-defined arginine residues (PDB 1HEL). This indicates that the motions are restricted. Consequently, it may be considered that both phosphate and chloride ions could bind to the residue (Arg-14) in the positively charged cluster with less mobility.

In conclusion, using a ^{15}N-labeled lysozyme, we identified the binding site of phosphate ion to hen lysozyme where the deamidation preferentially occurs. These results showed that chemical shift perturbations were a reliable and sensitive probe for ligand binding to a protein. Currently, increasing numbers of proteins and peptides are being employed as drugs. The present procedure for both highly efficient expression of a ^{15}N-labeled protein and analysis of the ligand binding to the resulting protein may be useful to identify the binding site of a protein with low molecular weight substances.

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