Proton Magnetic Resonance Study of Interaction between Penicillin G and Hen Egg White Lysozyme

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Interaction between penicillin G and hen egg white lysozyme has been studied using NMR and gel chromatography. It has been demonstrated that the stoichiometry of the interaction is one to one with an association constant of approximately 1.9×10^2 M^{-1} (1M=1mol dm^{-3}). It has also been shown that the phenacyl side chain of penicillin G is interacting with the tryptophan-123 residue of the lysozyme molecule.

Study of interaction of penicillin with proteins is important in order to elucidate the behavior of penicillin in biological systems such as its transportation, antibacterial activity and allergy. Proteins such as serum albumin are known to function as a transport carrier of drugs in the blood circulation.[1] The antibacterial activity of penicillin is closely related to the fact that penicillin becomes an inhibitor of enzymes which synthesize the cell wall of bacteria.[2] As for the allergy, penicillin conjugates with proteins as a hapten, acquires immunogenicity, and at the antibody-antigen reaction penicillin interacts with its antibody protein selectively with high sensitivity, causing anaphylactic shock.[3] In each case, penicillin-protein interaction plays a decisive role.

Penicillin-serum albumin interaction has been examined using NMR.[4] However, very little is known about the binding site of albumin. It was also reported that in the complex between penicillin and carboxypeptidase, the C2-H proton of one of histidine residues of the protein shows a downfield shift.[5]

In the present paper, interaction between penicillin and lysozyme, one of the best characterized proteins, will be discussed on the basis of NMR measurements. Several studies have already been published on the penicillin-lysozyme interaction by means of X-ray diffraction,[6] inhibition of lysozyme activity,[7] equilibrium dialysis,[8] radioisotope labeling[9], and pulse radiolysis.[10] It has been reported that penicillin V binds to lysozyme in the ratio of one to one in the crystalline state,[6] and that hydrolytic action of lysozyme is inhibited 50% by the presence of 24 mM penicillin G.[7] It has been reported that an irreversible binding of penicillin G with lysine-116 of lysozyme occurs when a mixture of penicillin G and lysozyme is incubated for 48 h at 37 °C.[9] It has also been reported that no complex formation between penicillin G and lysozyme can be detected by means of equilibrium dialysis, whereas naphthylazopenicillin strongly interacts with lysozyme.[8]

In the present work, stoichiometry and the binding constant of the interaction between penicillin G and lysozyme are determined, and the binding sites on both sides will be discussed on the basis of NMR and gel chromatography results.

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Experimental

Penicillin G potassium salt (Sigma, Lot No. 24C-2130) and hen egg white lysozyme (Seikagaku Kogyo, Lot No. E5210) were used without further purification. All other reagents were of analytical grade.

For NMR measurements of the NH proton of tryptophan residues, lysozyme were dissolved in H_2O (5 mM in 0.1 M NaCl, pH 5.5), to which penicillin G was added. For other NMR measurements, penicillin G and lysozyme were dissolved in 0.3 M deuterated acetate buffer in D_2O (pH 5.5). NMR measurements at 100 MHz were performed using a JEOL PS-100 spectrometer operating in the correlation[11] and in the CW mode, and 360 MHz spectra were recorded on a Bruker HXS-360 in the correlation and in the FT mode. Chemical shift values are from either external DSS or internal HDO in D_2O.[12]

Gel chromatography was performed at 25 °C on a column (1×25 cm) of Sephadex G-25 by following the procedure described by Hummel and Dreyer.[13,14] Before use, the column was equilibrated with 0.1 M acetate buffer solution (pH 5.5) containing penicillin G (0.8—8.3 mM). About 50 mg of lysozyme was dissolved in 0.5 ml of the same eluting solution, and elution was performed at flow rate of 0.25 ml/min. The effluent was collected in 2.0-ml fractions and diluted to fit the concentration of penicillin G for UV measurements. The concentration of penicillin G was determined by the color development at 325 nm.[15]

Results and Discussion

Stoichiometry and Binding Constant of the Interaction between Penicillin G and Lysozyme. Figure 1 shows a typical elution profile at 325 nm. The amount of penicillin G bound to lysozyme is estimated from the area of trough displayed in the elution profile. The extent of binding of penicillin G to lysozyme was also measured at varying concentrations of penicillin G and the result is shown as a Scatchard plot in Fig. 2. This result clearly indicates that the stoichiometry of interaction between penicillin and lysozyme is one to one. The binding constant obtained from the plot is approximately 1.9×10^2 M^{-1}.

Binding Site in Penicillin G. Fischer and Jardetzky used the broadening of the phenyl and methylene peaks of penicillin G in the presence of albumin and concluded that the phenyl part of the penicillin molecule is involved in the interaction between penicillin G and albumin.[4] The phenyl and methylene peaks, which they treated as single peaks, are actually of AB and A_2B_2C spin systems, and are no longer singlet at 360 MHz. They

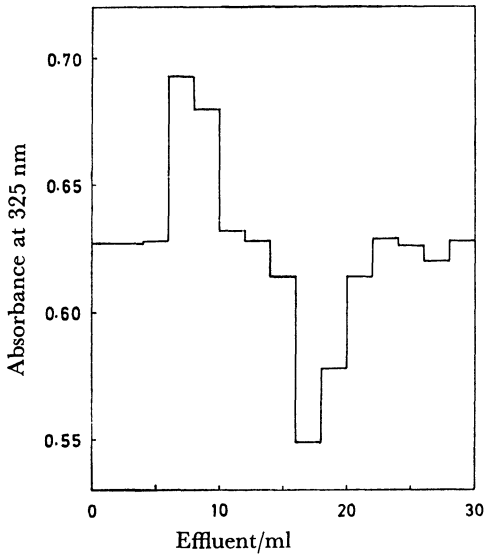

Fig. 1. Elution profile of the 325 nm absorbance accompanying the passage of lysozyme (50 mg) through a column of Sephadex G-25 which was equilibrated with 1.73 mM penicillin G at 25 °C. The collected effluent was diluted by 12.5-fold before the color development.

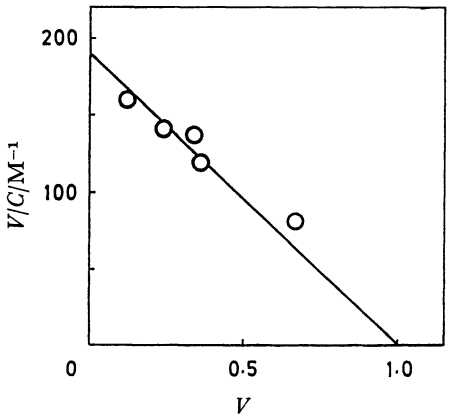

Fig. 2. Scatchard plot for the binding of penicillin G and lysozyme. *C*: The concentration of penicillin G in the eluting buffer, *V*: moles of penicillin G bound per mole of lysozyme.

have also shown that the chemical shift values of these peaks are dependent on the concentration of penicillin G. Thakkar and Wilham concluded that this concentration dependence of chemical shift values was caused by the self association of penicillin G.[16]

In the present experiment, the concentration of penicillin G is fixed at 32 mM, whereas that of lysozyme is varied from 0 to 10 mM. The change in spectrum of penicillin G under these conditions are shown in Fig. 3. With an increase in concentration of lysozyme from 0 to 10 mM, the phenyl peak shows an upfield shift of 0.098 ppm, the methylene peak an upfield shift of 0.079 ppm, and the β-CH_3 and 6-H peaks small upfield shifts of 0.035 ppm and 0.022 ppm, respectively. On the other hand, very little shift is observed in the α-CH_3, 3-H and 5-H protons. The result that 3-H is not affected by the presence of lysozyme suggests that no electrostatic

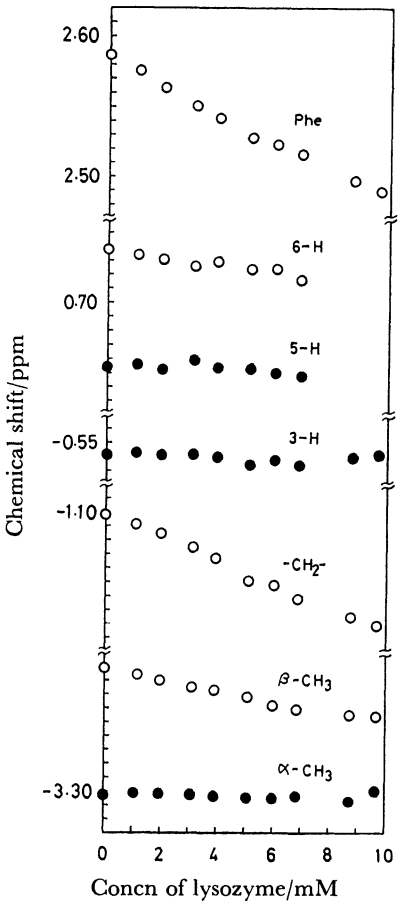

Fig. 3. A change in chemical shift values of penicillin G at varying concentrations of lysozyme in 0.3 M deuterated acetate buffer (pH 5.5). 28 °C. The concentration of penicillin G: 32 mM. Chemical shift values are in ppm from internal HDO whose chemical shifts are independent of the concentration of lysozyme within experimental error.

interaction involving the carboxyl group at C-3 of penicillin G occurs between penicillin G and lysozyme.

Among the groups of protons affected by the addition of lysozyme, the shifts observed for the phenyl and methylene peaks in the side chain are probably caused by the interaction with lysozyme; small shift in the β-CH_3 protons appears to be due to a change in intramolecular interaction of penicillin G molecule caused by interaction with lysozyme. This is supported by the experiments of the temperature dependence of the chemical shifts of penicillin G. As shown in Fig. 4, the β-CH_3 signal shows large temperature dependent shift. In contrast to this, 6-aminopenicillanic acid (6-APA), which has no side chain, does not show any such temperature dependent shift. Therefore, it may be concluded that the temperature dependent shift of β-CH_3 of penicillin G is primarily caused by the phenacyl side chain. Furthermore, the temperature dependent shift is much larger in the β-CH_3 group as compared to the α-CH_3 group. This result suggests that in an aqueous solution the penicillin G molecule tends to be less extended in shape, with the phenacyl side chain approaching from the side of the β-CH_3 group to the heterocyclic ring.

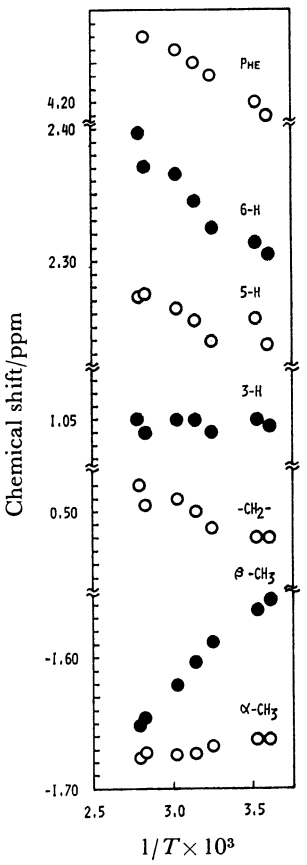

Fig. 4. Temperature dependence of chemical shift values of penicillin G in 0.3 M deuterated phosphate buffer (pH 7.0). Chemical shift values are in ppm from internal tetramethylammonium.

We assume that the shift caused by the addition of lysozyme is due to the fast exchange between the free and bound penicillin molecules. Then, the binding constant (K) and the chemical shift difference between the two states can be estimated by a least-squares method;[17] K was estimated to be in the range 10—250 M^{-1}, and the chemical shift differences for the phenyl and methylene groups in the ranges 0.4—1.3 ppm and 0.3—1.1 ppm, respectively.

In the presence of lysozyme, broadening of the methylene peak of penicillin G is observed at 100 MHz as Fischer and Jardetzky have already reported.[4] This peak is observed as an AB quartet at 360 MHz. Therefore, the broadening observed at 100 MHz is not quite helpful in obtaining information about the interaction of penicillin G with lysozyme.

Binding Site in Lysozyme. The result that the aromatic side chain of penicillin G molecule is the binding site suggests that aromatic amino acid residues of lysozyme may be involved in this interaction. Even in a relatively small protein such as lysozyme (M.W. 14300), proton signals observed separately are limited at the present resolving power of an NMR spectrometer.[18] Among those, C2-H proton of histidine-15 and NH protons of the indole ring of tryptophan residues can be used as probes for the investigation of the interaction site of lysozyme. The C2-H proton of His-15 does not show any change in chemical shift in the presence and absence of penicillin G, indicating that this histidine residue is not responsible for the interaction of penicillin G with lysozyme.

Correlation NMR technique is employed to observe exchangeable NH protons in H_2O. As shown in Fig. 5, the indole NH protons of five among six tryptophan residues are resolved. Peak I which is at the lowest field shows an upfield shift in the presence of penicillin G. Figure 6 gives chemical shift values of these five NH protons at varying concentrations of penicillin G. Only peak I shows an upfield shift with an increasing concentration of penicillin G. This result indicates that the tryptophan residue giving rise to peak I is involved in the interaction with penicillin G. Glickson, Phillips, and Rupley have reported that peaks III and V show a large shift in the presence of N-acetylglucosamine (NAG), an inhibitor of lysozyme.[19] The result of the present experiment suggests that penicillin G is different from NAG in its way of interaction with lysozyme.

On the basis of experiments such as chemical modification, binding of paramagnetic ions, inhibitors, and spin labels and solvent exchange, Cassels, Dobson, Poulsen, and Williams assigned the NH proton peaks from downfield as Trp-123, Trp-111 (or Trp-28), Trp-63, Trp-62, and Trp-108. In addition to this, they found the sixth peak in the upper field and assigned it as Trp-28 (or Trp-111).[20-22] Lenkinski, Dallas, and Glickson independently reported the assignments which agree with one of the two possibilities reported by Cassels *et al.*[23] Following the above assignments, Trp-123 is

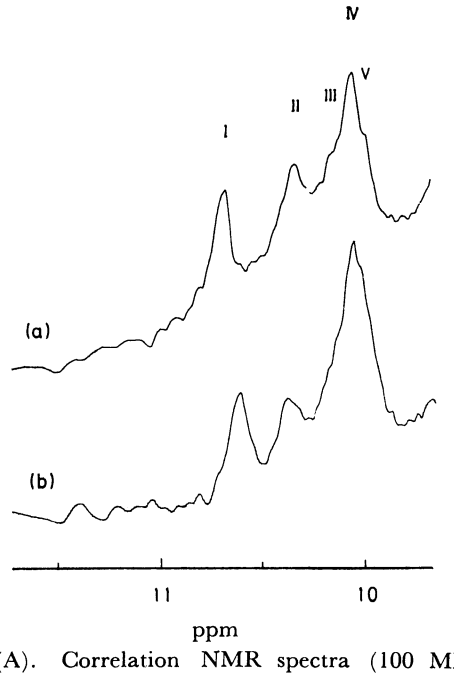

Fig. 5(A). Correlation NMR spectra (100 MHz) of indole NH protons of tryptophan residues of hen egg white lysozyme in the absence (a) and presence (b) of penicillin G. (a): 5.0 mM lysozyme in H_2O (0.1 M NaCl), pH 5.5, 48 °C. (b): 5.0 mM lysozyme in H_2O (0.1 M NaCl) containing 31 mM penicillin G. pH 5.5, 48 °C. Chemical shift values are in ppm from external DSS in H_2O. sweep rate: 417 Hz/s, sampling time: 0.512 s, repetition time: 0.8 s, sampling point: 512, number of accumulation: 2048, filter: 125 Hz.

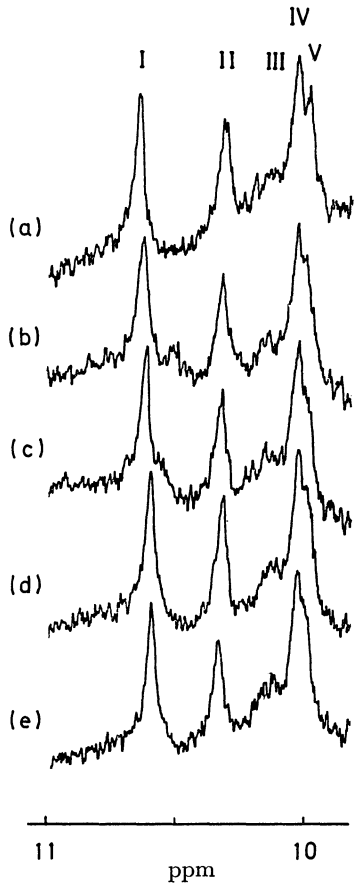

Fig. 5(B). Correlation NMR spectra (360 MHz) of indole NH protons of tryptophan residues of hen egg white lysozyme in the absence (a) and presence (b—e) of penicillin G. 5.0 mM lysozyme in H_2O (0.1 M NaCl). pH 5.5, 53 °C. The concentration of penicillin G: (b) 6 mM, (c) 12 mM, (d) 17 mM, (e) 26 mM. Chemical shift values are in ppm from external DSS in H_2O. sweep width: 1200 Hz, sampling interval: 0.2 ms, sampling point: 4096, number of accumulation: 256.

the one which is involved in the interaction with penicillin G. Measurements of solvent exchange rates and X-ray structure studies have shown that Trp-123 is exposed to solvent;[20,24] penicillin G is presumably interacting with the Trp-123 residue on the surface of the lysozyme molecule.

Felsenfeld and Handschumacher have suggested that penicillin G binds to all tryptophan residues.[7] On the contrary, the present experiment indicates that penicillin G-lysozyme binding is more specific. Corran and Waley have reported that penicillin G is irreversibly bound to Lys-116 of lysozyme at its ε-amino group. However, this irreversible binding was observed after incubation of both substances for 48 h at pH 7.4 and 37 °C,[9] under which condition penicillin G is known to degrade forming various products.[25] In the present experiments, NMR measurements were performed immediately after penicillin G and lysozyme were mixed, so that it is quite unlikely that the covalent bond is formed.

The binding constant of $1.9\times10^2\ M^{-1}$ obtained for penicillin G is larger than that for NAG ($K=39\ M^{-1}$)

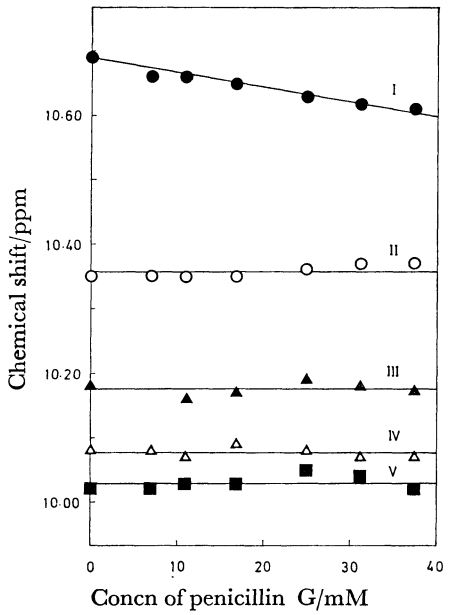

Fig. 6. A change in chemical shift values of indole NH protons of tryptophan residues of lysozyme (5.0 mM) at varying concentrations of penicillin G (pH 5.5, 48 °C).

and other saccharide monomers; however, it is smaller than those for dimers and trimers such as $(NAG)_2$ and $(NAG)_3$.[26] It is known that various pigments such as Biebrich scarlet, which have aromatic rings in the molecular structure, bind to lysozyme with high affinity.[27] Our result that the aromatic side chain of penicillin G is interacting with lysozyme with higher affinity than monosaccharides is consistent with these facts.

On the basis of an X-ray structural study of lysozyme which is diffused by penicillin V, Johnson has concluded that penicillin V binds specifically to the cleft on the surface of lysozyme. He has also shown that the penicillin binding site is apart $\approx$12 Å from the binding site of NAG and suggested that the binding is weaker than in the case of NAG.[6] Our results are not consistent with the conclusion reached by Johnson; *i.e.*, Trp-123 is not in the cleft of lysozyme molecule and the binding of penicillin G is stronger than that of NAG. There are two possibilities for this discrepancy. One is the difference in the molecular structure of penicillin G and penicillin V; in the penicillin V molecule the phenoxymethyl side chain is substituted for the benzyl side chain of penicillin G. All of our experiments were carried out in solution, whereas the X-ray structure was obtained in the crystalline state. This difference is another possibility for the above discrepancy. It is likely that in the crystalline state, where the lysozyme molecules are presumably more closely packed than in the case of solution, the penicilline molecule is hindered from approaching to Trp-123, which is in the dominant binding site of lysozyme.

In conclusion, interaction between penicillin G and lysozyme is one to one with a binding constant of approximately $1.9\times10^2\ M^{-1}$, and the phenacyl side chain of penicillin G and tryptophan-123 in lysozyme are involved in the binding.

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