Adsorption of human lysozyme onto hydroxyapatite

Identification of its adsorbing site using site-directed mutagenesis

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Abstract To elucidate hydroxyapatite-protein interaction, mutant human lysozymes in which the surface charge was modified by site-directed mutagenesis were used. Five mutant human lysozymes (K1A, K13A, K33A, R10A, R14A) were expressed in yeast. The chromatographic behavior of these lysozymes was studied with a HPLC hydroxyapatite column. Elution molarities of K1A and R14A mutants were greatly lowered. While Lys-13 and Arg-10 are located around Lys-1 and Arg-14, K13A and R10A mutants bound onto hydroxyapatite stronger than K1A and R14A mutants. In combination with an X-ray crystal structure of human lysozyme, it is concluded that the adsorbing site of human lysozyme is at the back of the active site and that Arg-14, Lys-1, Arg-10 and Lys-13 play important roles in binding.

Key words: Human lysozyme; Hydroxyapatite; High performance liquid chromatography; Adsorption

1. Introduction

Hydroxyapatite has been widely used for protein and nucleic acid chromatography [1–4] since the use of hydroxyapatite columns for protein chromatography was originally introduced by Tiselius et al. in 1956 [5]. It is thought that the basic principle of hydroxyapatite chromatography is electrostatic interaction. However, the principles of ion exchange chromatography are not directly applicable to hydroxyapatite chromatography, because the elution patterns of hydroxyapatite chromatography are only loosely correlated with the isoelectric point of proteins, which is different from ion exchange chromatography. The identification of binding sites and residues of proteins is difficult although there are some theories for adsorption to and desorption from hydroxyapatite [1,2,6]. Seeing that hydroxyapatite is an important inorganic crystallite constituent in human calcified hard tissues such as bones and teeth, elucidation of the mechanism of protein-hydroxyapatite interaction has very important implications for understanding not only hydroxyapatite chromatography but also the roles of protein biomineralization in vivo. For example the incorporation of lysozyme to saliva-coated hydroxyapatite caused a reduction in the adherence of *Streptococcus mutans* [7]. Thus the investigation of lysozyme-hydroxyapatite interaction has practical significance from the viewpoint of prophylaxis.

In our previous research of hydrogen-deuterium (H-D) exchange and ^{1}H NMR [8], using hen egg white lysozyme as a model protein of protein-hydroxyapatite interaction, we concluded that the binding site of lysozyme is located at the back of the active site of the lysozyme molecule. However, the interpretation of hydrogen exchange experiments is not so simple, which results in ambiguities.

In this paper the lysozyme-hydroxyapatite interaction was studied in detail by recombinant human lysozymes. We used engineered human lysozymes in which the surface charge had been modified by site-directed mutagenesis to study directly and manifestly the effects of mutations on the adsorption.

2. Materials and methods

2.1. H-D exchange and ^{1}H NMR measurement

Human lysozyme was purified from human milk essentially as described by Jollès and Jollès [9]. Hydrogen exchange experiments were performed as described previously [8] using hydroxyapatite (Bio-Rad). For D_2O solutions, pD was the pH meter reading and was uncorrected for the isotope effect. ^{1}H NMR spectra were recorded at 400 MHz on an α400 spectrometer (JEOL). Phase-sensitive DQF-COSY spectra [10] were recorded with suppression of the water signal.

2.2. Construction of recombinant strains

Human lysozyme cDNA with signal sequence was cloned by amplification from QUICK-Clone Human Placenta cDNA (Clontech) using PCR methodology and ligated with pGEM-T vector (Promega). Mutant human lysozyme genes (K1A, K13A, K33A, R10A, R14A) were constructed by the megaprimer PCR mutagenesis method [11] or using a GeneEditor in vitro Site-Directed Mutagenesis System (Promega) with appropriate oligonucleotides. The native and mutant human lysozyme genes which were digested with restriction endonucleases and ligated with pPIC3 (Invitrogen) were confirmed by DNA sequencing and transformed into *P. pastoris* GS115 (*his4*).

2.3. Expression and purification of lysozymes

All transformed strains were grown in a 2% MeOH BMMY medium in flasks which were then shaken at 30°C for 4 days. The culture supernatant which was clarified by centrifugation was diluted with water to a conductivity of 5 mS/cm and then adjusted with NaOH to pH 6.0. The lysozymes were retrieved by batch mode with cation exchange resin, CM-Toyopearl (Tosoh) equilibrated in 50 mM potassium phosphate buffer pH 6.0. The fractions eluted with 1 M NaCl were diluted and applied to a HiTrap SP (Pharmacia) column (5 ml) which had been equilibrated in 50 mM potassium phosphate buffer pH 6.0. The column was eluted with a gradient of 0–1 M NaCl. The fractions which had lytic activities were pooled and desalted. SDS-PAGE was performed to check the purity.

2.4. Hydroxyapatite chromatography

A 7.5×100 mm HPLC hydroxyapatite column (SH0710F, Pentax) with 950-PU system (Jasco) was used to measure the elution times of the lysozymes at room temperature. The lysozymes dissolved in 1 mM

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sodium phosphate buffer pH 6.8 were loaded on the column and equilibrated with the same buffer. The column was washed and eluted with 1–200 mM sodium phosphate buffer pH 6.8 at a rate of 1 ml/min.

3. Results and discussion

Hydroxyapatite-protein interactions have been extensively studied in order to elucidate the mechanism of hydroxyapatite chromatography. On the basis of behavior of adsorption-elution in hydroxyapatite chromatography and the adsorption isotherms of proteins, a basic protein has been proposed to adsorb onto hydroxyapatite through basic functional groups [6,12]. However, identification of the adsorption sites of protein is difficult. We previously reported the H-D exchange experiment to elucidate the adsorption sites of hen egg white lysozyme [8]. The principle of the H-D exchange experiment is that the exchange rates of amide protons of protein in D_2O buffer are dependent on their environment. For protein bound onto hydroxyapatite, most amide protons exchange to deuterons at the same rate as the free form but some amide protons positioned at adsorption sites are buried within the interface and thus protected from H-D exchange. The remaining hydrogen on individual amide sites can be measured by ^2D NMR analysis. The degree of protection, protection factor, is calculated by dividing the rate constant of the free form (k_{free}) by that of the bound form (k_{bound}). Actual interpretation of the results of a hydrogen exchange experiment is not so simple although the principle is very simple. For instance, in our experiment of hen egg white lysozyme, we concluded that Ala-9, Ala-11, and Lys-13 were candidates for a binding site. However, Leu-83, not in the side of the proposed binding site, showed a significant protection factor. This result suggested that not only residues in the binding site but residues at a great distance from the site are protected due to the remote slowing effect [13]. In addition, it is practically difficult to detect the effect of protection on some amide protons which have very small or large exchange rates. We tried to apply the same method used for hen egg white lysozyme to human lysozyme using its complete proton resonance assignments [14]. The COSY spectra of free and bound human lysozyme incubated for 24 h in D_2O (Fig. 1) show that Leu-15 and Arg-10 were protected. These two residues are positioned around Lys-13, the residue of the proposed binding site in hen egg white lysozyme. However, some residues which are not supposed to be in the binding site are weakly protected from solvent. Thus interpretation of hydrogen exchange experiments is not so simple.

We tried to construct five lysozyme variants with change in positive charge by means of site-directed mutagenesis to identify the binding site of human lysozyme onto hydroxyapatite and to investigate the mechanism of interaction between hydroxyapatite and protein. Because the three-dimensional structure of human lysozyme is very close to that of hen egg white lysozyme and Leu-15 and Arg-10 of human lysozyme were strongly protected from H-D exchange (Fig. 1), it was estimated that Lys-1, Arg-10, Lys-13, and Arg-14 were potential adsorption sites (Fig. 2). These residues were substituted by Ala to delete the positive charges. For comparison, Lys-33, which is away from the estimated binding site, was also substituted by Ala.

All lysozymes had nearly the same lytic activity. From the result of SDS-PAGE, all lysozymes were found to be sufficiently pure for these experiments. The chromatographic behaviors of native and mutant lysozymes on cation exchange column chromatography varied in accordance with its deduced charges. Only native lysozyme was eluted at higher molarities. On the other hand, the salt concentrations required to elute all mutant lysozymes were the same (Table 1) and the molarities were somewhat lower than that of native lysozyme. These results indicate that the elution molarities from ion exchange column correlated with the isoelectric points of proteins in a manner different from hydroxyapatite chromatography.

Using a HPLC hydroxyapatite column, the strengths of hydroxyapatite-lysozyme interactions were measured (Table 1). All mutant lysozymes were eluted at lower molarities than native lysozyme. These changes suggested that interaction between lysozymes and hydroxyapatite results from the positive charges of lysozymes. Especially, the elution molarities of K1A and R14A mutants were greatly lowered. In addition, those of R10A and K13A mutants were moderately lowered. These results show that native lysozyme binds onto the hydroxyapatite surface around these residues. In our H-D exchange experiment of hen egg white lysozyme [8], some residues showed a protection factor considerably larger than 2. If a protein has multiple binding sites, its protection factor is smaller than 2 because protons protected while bound at one site will be exchanged when it is bound at another site. Our results for cytochrome *c* reveal that its protection factors of binding sites are less than around 2 because of multiple site binding although it binds to hydroxyapatite more strongly than lysozyme (Kamakura et al., unpublished). These results indicate that hen egg white lysozyme binds onto hydroxyapatite with only one side of the protein surface. Assuming that the binding mechanisms of human and hen egg white lysozymes are the same, it is concluded that Lys-1, Arg-14, Arg-10 and Lys-13 form the binding site in human lysozyme and that this site is the only site for binding. The effectiveness of deletion of positive charges which exist in the same face on bind-

Table 1
Elution of native and mutant lysozymes

Lysozyme	Ion exchange		Hydroxyapatite	
	Molarity (mM)	Difference[a] (mM)	Molarity (mM)	Difference[a] (mM)
Native	390	–	101	–
K1A	350	40	81	20
R10A	340	50	87	14
K13A	345	45	86	15
R14A	350	40	76	24
K33A	350	40	97	4

[a]Difference is calculated by subtracting the elution molarity of native lysozyme from that of each mutant lysozyme.

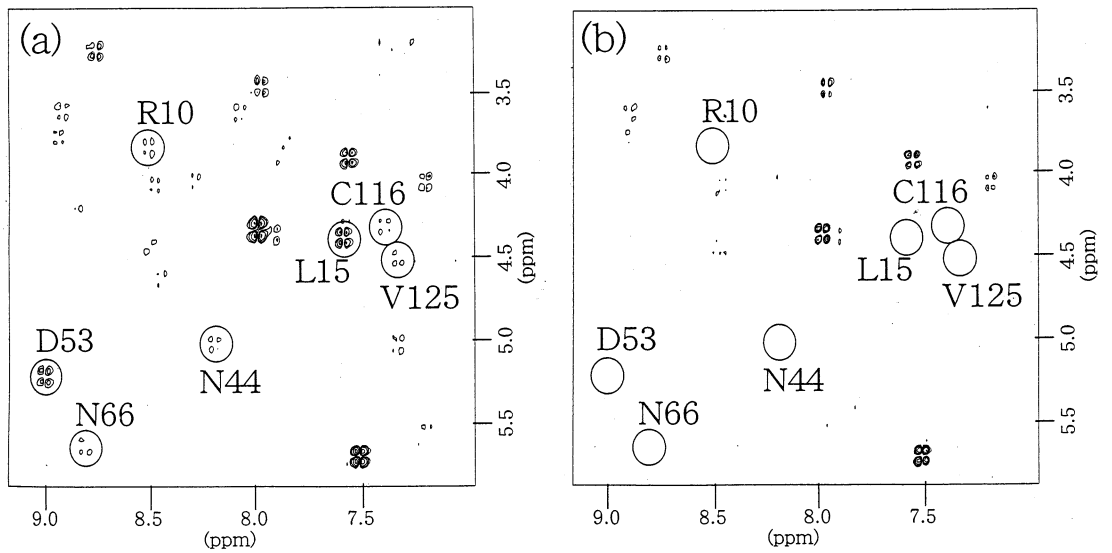

Fig. 1. Part of the ^{1}H NMR COSY spectra of native human lysozyme recorded at pD 3.8 at room temperature after partial H-D exchange for 24 h (a) in the presence and (b) in the absence of hydroxyapatite. The cross peaks of L15 and R10, which are supposed to be binding sites, were clearly protected. However, some peaks of residues at a great distance from the binding site were also protected.

ing is different, and this result indicates that the position of each residue for specific interaction is strictly restricted. Kawasaki proposed a model of lysozyme binding to hydroxyapatite on the basis of his theory of hydroxyapatite chromatography [6]. In his model, he could not determine the adsorption site. He estimated, however, only two or three positively charged residues of lysozymes could be candidates for binding. In addition, some studies reveal that the net positive charge is not sufficient for binding on hydroxyapatite and a sufficient density of charged groups must be on the surface of the protein [15,16]. For instance, it is known that denatured forms of proteins that can bind hydroxyapatite in their native forms do not bind to hydroxyapatite. Our conclusion for binding sites is further supported by these investigations.

Gorbunoff studied the lysozymes in which all lysine residues were chemically modified by non-selective acetylation [16]. This modified lysozyme was no longer retained on a hydroxyapatite column. Because mutation of Arg-14 and Arg-10 showed that they play important roles in binding, the loss of binding ability in acetyllysozyme was not only because the specific interactions of Lys-1 and Lys-13 were blocked. These results suggest that not only the specifically interacting residues but other residues as well participate in the interaction. Positive charges which do not interact specifically with the negative charges on the surface of hydroxyapatite equilibrated with phosphate buffer can counteract negative charges of protein found around these positive charges. As a result of this counteraction, electrostatic repulsion between negative charges of hydroxyapatite and protein may be attenuated. In the case of acetyllysozyme, although it has Arg residues, the number of positively charged residues may be not enough to counteract negative charges of lysozyme.

It is interesting to note that K33A was eluted at a lower molarity than native lysozyme although this residue is not in

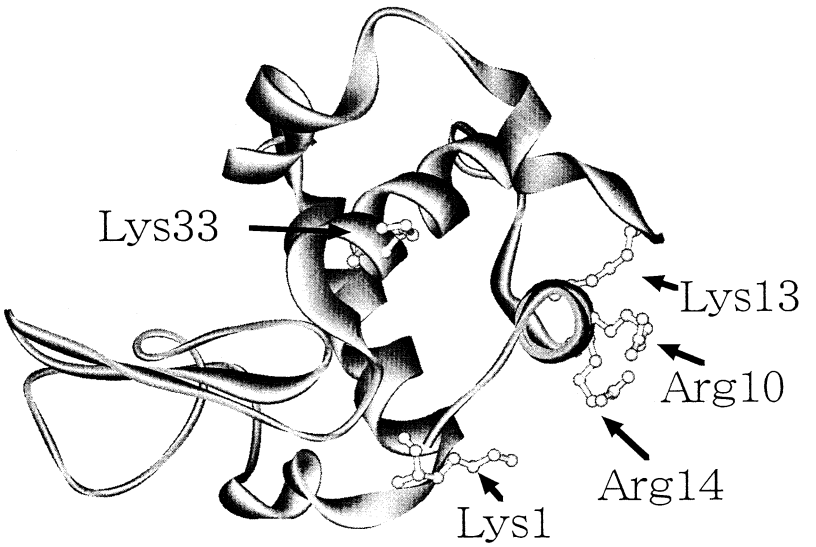

Fig. 2. Structural positions of mutations in this study. Drawing was performed using atomic coordinates obtained from Brookhaven Protein Data Bank (entry set 1LZ1, Artymiuk and Blake, 1984) and a computer program, WebLab Viewer (Molecular Simulations Inc.).

the proposed binding site. This fact can be explained on the basis that there may be non-specific interaction of protein with hydroxyapatite which occurred during elution through a column after desorbing from the primary adsorbing layer of hydroxyapatite.

Many experiments, including chemical modification, have been performed to elucidate protein-hydroxyapatite interaction. In almost all chemical modification experiments, proteins were, however, non-specifically modified. These experiments give little information about the specific binding site of protein. On the other hand, proteins specifically modified by using site-directed mutagenesis were used in our experiment. We show not only that the adsorbing site of human lysozyme is at the back of the active site but also that the residues which take part in binding are Lys-1, Lys-13, Arg-14 and Arg-10. Furthermore, our results suggest that studying mutant proteins can be used as a complementary approach with hydrogen exchange and ^{1}H NMR experiments for analysis of the mechanism of adsorption.

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