Note

State of Imidazole Side Chain of Hen Lysozyme Modified with Histamine and Japanese Quail Lysozyme. A Study by Immobilized Metal Ion Affinity Chromatography

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Hen lysozyme modified with histamine (HML) and Japanese quail lysozyme (JQL) were treated with immobilized metal ion affinity chromatography to analyze the states of their imidazole groups. When Ni(II) was used as the metal ion immobilized, JQL was strongly retained in a Ni(II)-chelating Sepharose column, while hen lysozyme and HML were hardly retained in the same column. All of these lysozymes have a histidine imidazole group at the 15th position, while JQL has an additional histidine imidazole group at the 103rd position and HML has an additional imidazole group covalently attached to Asp101. Thus, I concluded that the imidazole group at the 103rd position of JQL is exposed to the solvent and recognized by the metal ion, but that the imidazole group attached to Asp101 in HML is localized to a hydrophobic region and not recognized by the metal ion.

Key words: immobilized metal ion affinity chromatography; hen egg white lysozyme; Japanese quail lysozyme; histamine; imidazole side chain

Several avian c-type lysozymes have amino acid sequences highly similar to each other and share similar tertiary structures. Each of these lysozymes has a variable number of histidine residues and all histidine residues are localized on the surface of the lysozyme molecule. Hen egg white lysozyme (HEWL) has only one histidine residue at the 15th position. The lysozyme from Japanese quail egg white (JQL) has a histidine residue at the 103rd position, in addition to His15 (Araki et al., PIR database, accession No. IU00237). Hist103 of JQL is located in the upper part of the substrate binding cleft (subsite B or C), and the state of its imidazole group is of interest from the viewpoint of the substrate binding mechanism. An analogous situation is found in hen lysozyme modified with histamine (HML) prepared in our laboratory. Histamine was covalently attached to the carboxyl side chain of Asp101 of hen lysozyme by water-soluble carbodiimide, and the state of the imidazole group was investigated by kinetic analysis and NMR spectroscopy. Nuclear Overhauser effects (NOE) were observed on the proton signals of the imidazole group attached to Asp101 when saturating the signals of Trp63 and Ile98, which are in the hydrophilic box most proximal to the saccharide binding cleft. This suggests that the imidazole group attached to Asp101 is not fully exposed to the solvent, but localized to the hydrophobic region. However, further evidence on the state of the imidazole group is needed because the state of the additional imidazole side chain in the saccharide binding cleft may significantly affect the enzymatic activity. On the other hand, the surface topography of histidine residues in the lysozymes was investigated by immobilized metal ion affinity chromatography (IMAC), and the varied affinity of the lysozymes for IMAC has been rationalized in terms of the presence, multiplicity, and microenvironments of histidine residues. This method seems to be most appropriate for investigating the state of additional imidazole group of JQL or HML. In this study, the states of the imidazole group of these lysozymes were analyzed by IMAC, and compared to each other.

The selective modification of Asp101 with histamine was done by the method of Yamada et al. The reaction mixture containing 1.4 mM hen egg white lysozyme (Seikagaku Kogyo), 10 mM 1-ethyl-(3,3-dimethyl-aminopropyl)carbodiimide (Wako Pure Chemicals), and 40 mM histamine was incubated for 3 h at pH 5.0 and room temperature. The modified lysozyme was purified by ion-exchange chromatography on Bio-Rex 70 (1×120 cm). A linear gradient system of 0.1–0.4 mM sodium phosphate, pH 7.0, was used for elution of the modified lysozyme. The modification was confirmed by HPLC peptide mapping and 1H-NMR spectroscopy. The JQL preparation was kindly donated by Dr. Torikata, Kyushu Tokai University, Japan. Chelating Sepharose 6B was purchased from Pharmacia. Other reagents were of analytical grade commercially available.

At first, the lysozymes were examined with a Cu(II)-chelating Sepharose column. Chelating Sepharose 6B was washed with distilled water, and equilibrated with 0.1 M sodium acetate buffer, pH 4.0, containing 1 M NaCl. The gel was poured into a chromatographic column (1.0×8 cm) and charged with 50 ml of CuCl2 solution dissolved in the same buffer (5 mg/ml). Unbound

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Abbreviations: IMAC, immobilized metal ion affinity chromatography; IDA, iminodiacetate; IDA-Me(II), chelate of transition metal ion (Me++) with IDA; HEWL, hen egg white lysozyme; JQL, lysozyme from Japanese quail egg white; HML, hen lysozyme of which Asp101 is modified with histamine; NOE, nuclear Overhauser effects
metal ion was washed out of the column with 25 ml of the metal-free buffer, and then the column was equilibrated with 20 mM sodium phosphate buffer, pH 7.0, containing 1 M NaCl. Each of the lysozyme preparations (HEWL, JQL, and HML) was dissolved in the phosphate buffer, and put on the IDA-Cu(II) column. Elution of the proteins was monitored by ultraviolet absorption at 280 nm. All of these lysozymes were strongly retained in the column, and not eluted under these conditions. Thus, an imidazole gradient elution (from 2 mM to 40 mM) was done to elute the protein. Although the proteins were eluted at 10–20 mM of imidazole, no clear difference in the elution position was found between these proteins. The gradient elution seems to be inappropriate for discrimination of a fine difference in microenvironment of imidazole side chains.

Then Co(II) was used instead of Cu(II). An IDA-Co(II) column was prepared as described above except that Chelating Sepharose 6B was charged with CoCl₂ instead of CuCl₂. Isocratic elution was done with the phosphate buffer to elute the proteins. The results are shown in Fig. 1. HEWL, having only a single histidine residue at the 15th position, was not retained in the IDA-Co(II) column, but JQL, having two histidines at the 15th and 103rd positions, displayed a weak but distinct retardation in this column. On the other hand, HML having two imidazole groups at the 15th and 101st positions did not have any retardation. This indicates that the additional imidazole group of HML is in a microenvironment different from that of His103 in JQL.

Further analysis was done with IDA-Ni(II) column, which was prepared by charging Chelating Sepharose 6B with NiCl₂ solution. The lysozyme preparations were put on, separately, and the results are shown in Fig. 2. In this case, a more distinct retardation of JQL relative to HEWL and HML was observed. HML was not retained in the IDA-Ni(II) column as in the case of IDA-Co(II). The retardation of JQL was not caused by the affinity for the Sepharose itself but by the affinity for IDA-Ni(II). This is because the sugar-binding ability of JQL is not higher, but rather slightly lower than that of HEWL (Torikata et al., unpublished). Thus, I concluded that the imidazole group covalently attached to Asp101 in HML is not recognized by IDA-metal ion, hence it is localized to the hydrophobic region adjacent to the substrate binding cleft. In contrast, His103 of JQL is exposed to the solvent, being recognized by the IDA-metal ion.

In the report by Sulkowski,³ the rules of engagement between imidazolyl side chain and IDA-Me(II) are established. A protein with a single histidine residue is retained by IDA-Cu(II), and a protein with two histidine residues is more strongly retained by it. As expected from the rule, all of the proteins tested in this study were retained in the IDA-Cu(II) column. But the imidazole gradient elution from this column could not give any information on the difference in microenvironment of imidazole side chains. More complicated processes might be involved in the displacement reaction of the proteins with imidazole in the gradient elution. In fact, the imidazole interacts with tryptophan residues of the lysozyme to inhibit the lytic activity.⁷ This situation might result in an unexpected behavior in IMAC with imidazole gradient elution. On the other hand, IDA-Co(II) or IDA-Ni(II) cannot retain proteins having a single histidine residue. Nevertheless, these adsorbents might be convenient for investigating fine differences in microenvironment of imidazole side chains of proteins. As shown in Figs. 1 and 2, a difference in elution position between HML and JQL is distinct even though each of these proteins has two imidazole groups, indicating that these adsorbents are convenient for this purpose. As reported by Sulkowski,³ IDA-Ni(II) can retain proteins that have two histidine residues in any locations, but IDA-Co(II) cannot always retain the proteins with two histidine residues. Proteins are retained by IDA-Co(II) only in the presence of two vicinal histidine
residues, or only when two histidine residues are located in an α-helical segment. In this case, however, HML and JQL have two imidazole side chains each, which are far separated from each other in their locations (15th and 101st or 103rd positions). Thus, in IDA-Co(II) column, the difference in elution position between HML and JQL is not as distinct as in the case of IDA-Ni(II).

The results obtained from IMAC are consistent with the results of HML reported in our previous paper. As described above, NOE connectivity of the proton signal of imidazolyl ethyl moiety in HML showed that the imidazolyl ethyl moiety is not fully exposed to the solvent but localized to a hydrophobic region. This situation of the imidazolyl ethyl moiety of HML should impair its interaction with IDA-Me(II). The $pK_a$ of the imidazolyl ethyl group of HML was reported to be 7.1. This relatively high $pK_a$ would be due to such a hydrophobic environment of the imidazolyl ethyl moiety. On the other hand, we have analyzed the $^1H$-NMR spectrum of JQL, and obtained some information on His103 of the enzyme (Torikata and Fukamizo, unpublished). When N-acetylglucosamine trisaccharide was added to JQL, the His103 C2H signal was shifted upfield, and a weak line-broadening of the signal was observed in the bound state of JQL. In the case of HML, however, the imidazolyl ethyl C2H and C4H signals completely disappeared in the trisaccharide-bound state by a strong line-broadening. This indicates that motion of the imidazolyl ethyl moiety of HML is considerably suppressed by binding of N-acetylglucosamine trisaccharide but the suppression is not so strong in the His103 imidazole of JQL. The trisaccharide binding data are consistent with the IMAC results presented in this study; that is, the movement suppression by trisaccharide binding is intensive in the imidazole moiety localized to the hydrophobic region, but weak in the imidazole exposed to the solvent.

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**References**