DETERMINATION OF THE BINDING CONSTANT OF IMIDAZOLE AND HISTIDINE WITH IMMOBILIZED CU (II) BY DIFFERENTIAL UV SPECTROSCOPY*

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The binding constants of immobilized Cu (II) on PEG-IDA with imidazole and histidine at various solution pH values, salt conentrations and temperatures were determined by differential UV spectrophotometer. The bimodel binding behavior of basic solute was observed by the study of the salt effect. However, the formation of the coordinated compound is the dominated binding mechanism at the pH value higher than pKa of the deprotonation of imidazole nitrogen. There was no obvious effect of temperature on binding constant because of the complexity of binding mechanism. The binding behavior of several dipeptides and tripeptides with histidine at C- or N-terminal was also investigated and the results were explained by the "metal ion transfer" (MIT) hypothesis. Furthermore, the binding constants of synthetic heptapeptides with two histidine residues separated by different number of glycine residues were investigated to demonstrate the effect of histidine residues distance on the binding affinity. This study provides basic information of binding behavior of protein to immobilized metal ion.

Introduction

Immobilized Metal Ion Affinity (IMA) for protein separation was introduced by Porath *et al.* in 1975[16] and since then the applications have been extended to various systems such as chromatography and liquid-liquid extraction. The IMA functions by binding the accessible electron-donating pendent groups on protein surface such as histidine, cysteine, and tryptophan[16, 20, 25] to a metal ion which is held by a chelating group covalently attached on a matrix. The IMA method possesses the analogous biospecific interaction between enzyme and coenzyme, so the interaction or association between protein and immobilized metal would be dependent on the structure of individual protein. The differential binding behavior between proteins and immobilized metal as affinity ligand can be applied to proteins separation and purification. There are numerous applications of IMA method to protein separation[1, 5, 13, 14, 26, 27]. Although the interaction mechanism between protein and immobilized metal ion is still unresolved, the factors influencing the interaction include the number of electron-donating groups on protein surface, pH value of interaction environment, salt type and concentration, temperature, type of metal ion, ligand density, and type of chelating agent.

The major functional groups on protein contributing to the interaction with immobilized metal ion consist of the nitrogen atom of imidazole group of histidine residue and the sulfur atom of sulfhydryl group of the free cysteine residue. While the maintaining free cysteine residue in natural protein is rare[10], the "exposed" histidine residue is the dominant binding site in protein with immobilized metal ion. The number and the spatial position of histidine residue of a protein represent, to a certain degree, the binding ability between protein and ligand. A relationship between the increment of partition coefficient and the number of exposed histidine residues on protein had been developed by Arnold *et al.*[19, 28] in aqueous two-phase system. The retention time in proportion to the number of exposed histidine residue on protein surface in IMA chromatography had been reviewed by Sulkowske[20, 21]. The improvement of purification factor also can be reached by increasing the histidine residue number by genetic recombination technology, such as chelated peptide IMA[17, 24]. Histidine and imidazole have been applied as elution agents for IMA displacement chromatography and their concentration in the elution buffer increases according to the number of exposed histidine residue on protein surface. The effect of the number of exposed histidine residue on protein surface when interacting with IMA is clearly indicated. However, the factors about the effect of the protein conformation, the accessibility of the histidine residues and the spatial effect of histidine residus are still mysterious. The binding properties, such as the stability constant and the binding energy, of histidine-X_3-histidine (X represents different types of amino acid residues) in an α-helix of an engineered cytochrome c were determined[22]. The effects of the secondary structure rigidity to the accessibility of the histidine residues and the influence of the neighboring histidine residues were also reported[18]. Those two studies performed

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in aqueous two-phase system were all toward improving the separation factor by protein engineerin.

The interaction of amino and carboxyl groups of amino acid with immobilized metal ion is more by virtue of the electrostatic force than by the coordination force. That the nearby amino acids with aromatic side chain, such as Phe, Trp or Tyr, could enhance the Cu (II)-histidyl interactions was also demonstrated[2, 3]. Moreover, the pKa value of the imidazole group of histidine residue, i.e., the ability of donating electron to Cu (II), could also been affected by the nearby charged side chain of amino acid residues[2].

The pH value moderating the donating power of the lone-pair of imidazole group to Cu (II) directly affects on the binding ability. Adsorption of proteins to immobilized metal ion is performed at a pH in which the electron donor groups on the protein surface is partially unprotonated. The varying pH value and the pKa value of imidazole group can strongly govern the adsorption and desorption process of protein to immobilized metal ion. The temperature effects on the binding behavior of protein would be primarily on the protein structure and the dissociation ability of electron donating group. For small solute, such as amino acid, di- or tri-peptide, temperature dependence would reveal the nature of the electrostatic adsorption. But, unfortunately, no effect has been devoted to a systematic study on the temperature effect. The nonspecific adsorption of protein to immobilized metal ion can be reduced by adding salt to the buffer solution. The salt effect is complex in the protein adsorption with immobilized metal ion. Both the concentration and the type of salt affect the electrostatic attraction and/or repulsion; they also affect the hydrophobic interaction of protein with matrixes (since most of the matrix for immobilized metal ion are hydrophilic materials). in addition, they weaken the coordination bonding of water to the metal ion. Moreover, the competition between protein and salt with immobilized metal ion[12] would also make the adsorption process of protein difficult to analyze.

In order to gain an insight into the physico-chemical basis of the binding behavior between immobilized metal with protein, an understanding of the interaction of amino acid and peptides with immobilized metal is imperative. The interaction of imidazole and histidine with immobilized metal ion at various pH values, at various salt concentrations and at various temperatures were studied. In this study, the Cu (II) was chelated by polyethylene glycol (PEG) (Mr 5,000)-Iminodiacetic acid (IDA) and the binding constants were determined in PEG aquieous solution by differential UV spectoscopy.

1. Material and Method

MPEG 5000 (polyethylene glycol 5000 monomethyl ether) was purchased from Fluka (Switzerland). Histidine, imidazole, iminodiacetic acid (IDA), and all other chemicals were all analytical grade and from Merck (Germany). The equilibrium buffer solution is 20 mM phosphate ion

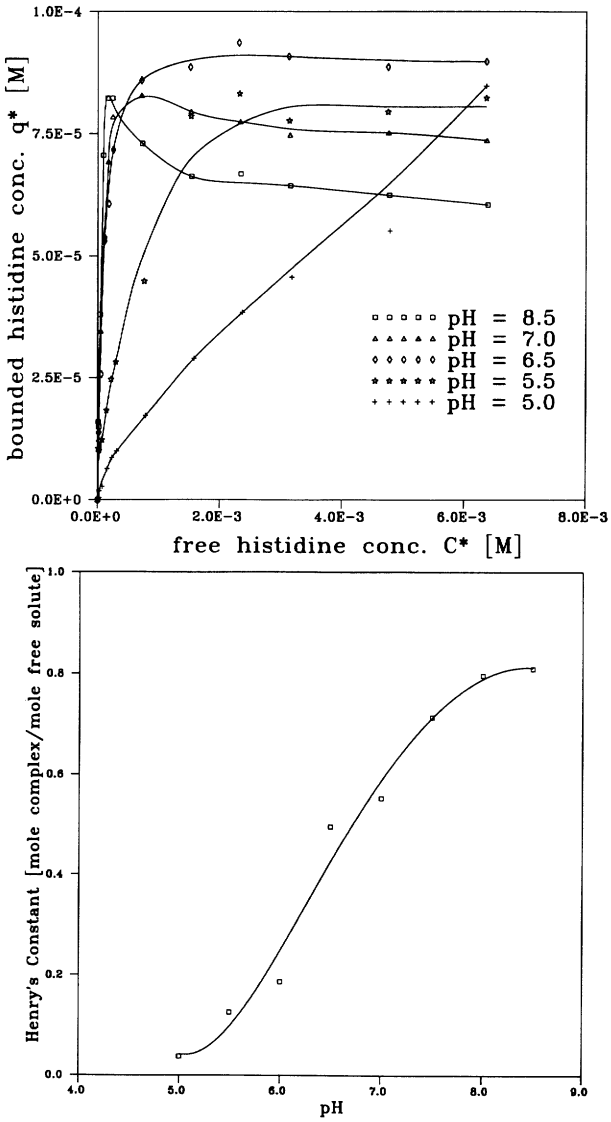

Fig. 1 The isotherm of histidine at various pH values

on various pH value and/or various NaCl concentrations. The synthesis of PEG (5000)-IDA referred to Buckmann *et al.*[4] and Van Dam *et al.*[23]. The loading Cu (II) content of the PEG-IDA determined by 0.2M EDTA solution was 8.4×10^{-5} mole Cu (II)/g PEG-IDA. The UV differential spectroscopy method[15] performed to evaluate the binding constants of immobilized Cu (II) with solutes was described as the following: (1) Various concentration of solutes at a certain pH value and salt concentration were prepared and added to the buffer solution with and without Cu (II) PEG-IDA. The volume ratio of solute solution to buffer solution was 1:1. The mixed solution was set for 24 hrs at a designed temperature in a constant temperature bath for equilibrium. (2) UV scanning was performed for the solutions and the wavelength exhibiting the highest difference of UV absorbance between the scanning spectrums was selected to calculate the binding constant. (3) The UV absorbance at the wavelengh was measured for the solutions of (i) the solutes with Cu (II) PEG-IDA, (ii) the solutes without Cu (II) PEG-IDA and (iii) Cu (II) PEG-IDA with buffer. The

difference of the absorbance of (i)-(ii+iii) represented the amount of formation of coordimated complex. Similar study for binding constant measurement of vancomycin with Methoxypolyethylene glycol (5000) was reported by Godbole *et al.*[6]. the UV spectroscopy used in this study was Spectronic 601 (Milton Roy) (U.S.A.) with water jacketed UV cell. In this study, in stead of adsorption isotherm model, Henry's constant was proposed to represent the binding ability of solutes with immobilized metal ion in various binding conditions. Since the adsorption mechanism is still unclear and the phenomena of "metal ion transfer"[3, 21] (the stripping of the immobilized metal ion) may happen at higher solutes concentration or higher pH value of adsorption buffer.

2. Results and Discussions

The pH effects

The adsorption isotherm of histidine and imidazole be UV differential spectroscopy at various pH values were determined as shown in **Figs. 1a** and **2a**. All the experiments were carried out under the conditions of 0.5M NaCl and 298K. The pKa values of the imidazole group of histidine and imidazole agent are 6.5 and 6.95, respectively, and the studied pH range covers the pKa values of solutes. The adsorption isotherm follows the Langmuir isotherm when the pH value is lower than the pKa values of both solutes. However, the amount of coordination compound drops when the concentration of histidine in solution is higher than a certain concentration and when the pH value is higher than pKa value of its imidazole group. In addition, Henry's constant of the adsorption at the "infinite" dilution of solutes increases as the pH value increases, as shown in Figs. 1b and 2b. The formation of the complex between the solutes and immobilized Cu (II) is reasonably made by the nitrogen atom of the imidazole group and the oxygen atom of two water molecules; those three atoms provide lone pair electrons to the tridentated Cu (II) by IDA. Therefore, the formation of the complex or the Henry's constant of the adsorption should be strongly dependent on the donating ability of the lone pair electrons. The higher the pH value is, the higher the dissociation degree of the proton from nitrogen atom of the imidazole group is, and consequently the higher the binding constant is. The steeper increment of the binding constant happens at the pH value near by pKa value. The change of the Henry's constant in histidine is more pronounced than in imidazole, since the adsorption forces of the histidine include not only the coordination interaction but also the electrostatic interaction between solute and the chelated ion[2, 8]. The degree of the deprotonation of the histidine's amino group might influence the adsorption ability since the possible complex formation of the amino group with immobilized ion has also been proposed[3, 8, 9]. The amount of the total complex formation decreases along with higher histidine concentration when pH value is higher than pKa value, as shown in Fig. 1a. This phenomenon could be referred to

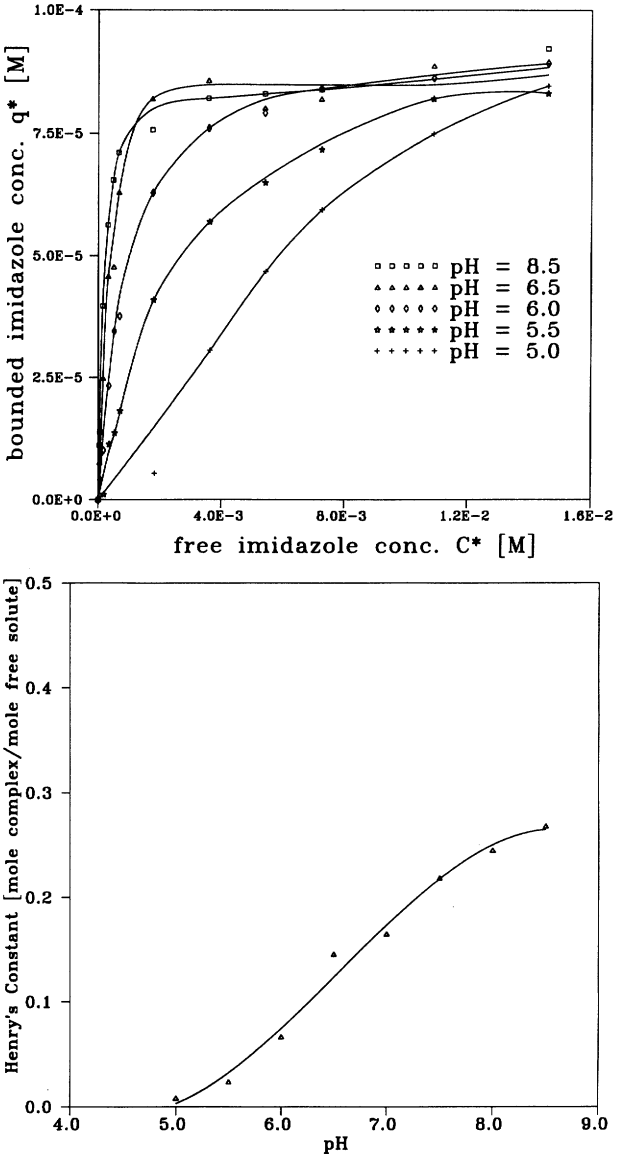

Fig. 2 The isotherm of imidazole at various pH values

the so-called "metal ion trransfer" (MIT) hypothesis proposed by Belew and Porath[3]. However, the phenomenon that the adsorbed amount changes along with differnet pH values and along with different solute concentrations has not been reported. That the formation of a coordination compound of metal ion by the α-amino group, imidazole group and one peptide bond nitrogen atom is stronger than that of by IDA would be the reason of MIT phenomenon in which the Cu (II) is stripped from Chelating Superose gel. The MIT phenomenon was also supported by the retention time of peptides in chromatography[7, 17] as well as by the partition coefficient of cytochrome *c* variant in aqueous two-phase system[22]. The partition coefficient dropped as pH value increased. In this study, histidine has no peptide bond nitrogen. Nevertheless, the highly deprotonated oxygen atom of carboxyl group at high pH value could possibly provide a free pair of electron for d-orbital of the immobilized Cu (II) and form a coordinated compound. This MIT behavior of histidine

Table 1 Henry's constant [mole of comple/mole of free solute] of histidine and imidazole at various salt concentrations

NaCl Conc. [M]	Histidine pH = 7.0	Histidine pH = 5.5	Imidazole pH = 7.0	Imidazole pH = 6.0
0	0.501	0.163	0.172	0.068
0.1	0.551	0.137	0.167	0.087
0.5	0.552	0.126	0.165	0.063
1.0	0.561	0.095	0.210	0.091

could also explain the difference between the effects of pH on the adsorption of histidine and imidazole, as shown in Figs. 1a and 2a. The imidazole has no deprotonated oxygen atom of carboxyl group and no MIT phenomenon is observed at the pH value higher than its pKa value.

The salt effects

The interactions of the solutes with immobilized Cu (II) consist of formation of coordinated compound, the hydrophobic or van der Waals' force and electrostatic interaction. The hydrophilic packing material was used to promote the adsorption of peptides or proteins in IMA chromatography[25]. Peptides which were more retarded on Superose column without NaCl in the equilibration buffer exist coulombic interaction with column packing. The effect of salt concentration on the binding ability between the immobilized metal and the solutes is also considered in this study in which the water soluble polymer PEG (5000) is used as immobilizing matrix for Cu (II). The effects of NaCl concentration on the binding ability were investigated varying from 0.0 to 1.0 M at the pH value higher or lower than pKa of imidazole groups of the solutes as listed in **Table 1**. That the immobilized metal ions exhibiting the net negative charge have been proposed[3, 9] at neutral pH is used as a fundamental condition to explain the results in Table 1. The effects of salt concentration on the binding constant of histidine are suppressed when the formation of complex dominates the binding interaction at neutral pH. The hydrophobic or electrostatic contribution could be ignored. However, about 40% dropping of binding constant was observed from no salt to 1.0 M salt added at the pH value lower than pKa. The negative charge nature of the immobilized Cu (II) promotes the electrostatic attraction, since histidine exhibits positive charge at pH = 5.5. Therefore, the effects of salt concentration on binding ability of histidine with immobilized Cu (II) is more marked. Besides, the higher salt concentration would reduce the coulombic force between charged particles. In other words, the bimodel character is the adsorption of basic histidine with immobilized metal ion and can be manipulated by varying the salt concentration. On the other hand, from the listing of imidazole binding constants with salt concentration, not much changes was observed in pH = 6.0 and pH = 7.0, indicating that the formation of coordinated compound is apparently predominant the binding mechanism.

Table 2 Henry's constant [mole of comple/mole of free solute] of histidine and imidazole at various temperature

Temperature [K]	Histidine	Imidazole
278	0.761	0.251
283	0.744	0.177
288	0.742	0.150
293	0.761	0.178
298	0.707	0.159
303	0.832	0.116

The temperature effects

The adsorption heat of amino acids, peptides, and proteins with immobilized metal ion has not been reported, nor has a systematic study of the temperature effects on the binding behavior been investigated. The complexity of adsorption mechanism could be a possible reason. The experimental conditions were set at the pH = 7.0 and 7.5 for histidine and imidazole respectively and the salt concentration is 0.5 M NaCl to resolve the temperature effects on the formation of coordination compound in this study. Little, if any, change with temperature was observed for both histidine and imidazole as listed in **Table 2**. Apparently, both the solutes are able to form coordination compound with immobilized Cu (II) at the pH value. Therefore, the effect of temperature on binding ability will be diminished since the dependence of the deprotonation of imidazole nitrogen on temperature is insignificant. The temperature effect on the retention of no histidine-containing oligopeptides had also been demonstrated from 0°C to 50°C in IDA-Ni (II) chromatography[9]. The oligopeptides have the highest retardation at 15°C. Explanation had not been given since the mechanism of the binding of the oligopeptides with immobilized Ni (II) is not clear. Besides, the temperature presented different effect on lysozyme and ribonuclease a in chelating Sepharose column[11]. While, in this work, temperature has little effect on the interaction of the small solutes such as imidazole and histidine with immobilized metal ion in this study.

Binding constant of amino acids, peptides and oligopeptides

The peptides selected were based on the investigation of the role of the individual group of histidine and the position of histidine in peptides in this study. The peptides are: (1) histidine with α-amino group (N-t-Boc-His) and carboxyl group blockd (histidine methyl ester), (2) dipeptides with histidine as amino or carboxyl terminal combined with different types of second amino acid residues, (3) tripeptides with histidine as the second or third residue and (4) synthetic heptapeptides with one histidine residues at amino terminal and the other histidine at different poisition with various number glycine residues between both histidines. Henry's constants of these peptides with immobilized Cu (II) are listed in **Table 3**. The experiments were carried out at 298K, pH = 7.0, in 0.5M NaCl, 20mM sodium phosphate buffer. Of particular interest is that there is no differential UV absorbance of the dipeptides with histidine at carboxyl terminal and the tripeptide with histi-

Table 3 Henry's constant [mole of comple/mole of free solute] of amino acids and peptides

Amino Acid/Peptide	Henry's Constant
Histidine	0.552
Imidazole	0.165
Histidine Methyl Ester	0.641
His-Glu	0.667
his-Pro	0.609
His-Tyr	0.836
His-Gly	1.602
His-Ala	0.708
His-Leu	0.963
His-Arg	0.817
His-Lys	0.935
His-Phe	0.648
β-Asp-His	0.497
Gly-Gly-His	0.407
Histamine	***
N-t-Boc-im-Tosyl-$_L$-Histidine	***
Ala-His	***
Gly-His	***
Tyr-His	***
Gly-His-Gly	***
His-His-Gly-Gly-Gly-Gly-Gly	***
His-Gly-His-Gly-Gly-Gly-Gly	2.581
His-Gly-Gly-His-Gly-Gly-Gly	4.045
His-Gly-Gly-Gly-His-Gly-Gly	1.887

dine at the second residue. These results correspond to the no retardation phenomenon in column experiments[3, 17]. The MIT hypothesis can aptly be adopted here to describe the phenomenon of no differential UV absorbance. As a whole, the dipeptides have a higher binding affinity than histidine except β-Asp-His, where modified N-terminal residue presents negative contribution of binding interaction. However, the dipeptide results do not follow the added formula of amino acid residues to dipeptide as proposed in the column retention time[29]. In this study, His-Gly has the highest Henry's constant among the dipeptides. This result disagrees with the notion that amino acids of aromatic side chain having the highest promotion of the binding of His-X observed in the column studies[3, 29]. The discrepancy can be attributed to the solution adsorption in this study while in the column experiments the steric hindrance may be presented by the solid matrix of the column packing material. One other important observation is the results of the heptapeptides. The binding pattern of the heptapeptides with free Cu (II) prevails over the binding of IDA with immobilized Cu (II) as described in MIT, when the histidine is the second residue. The spatial position between the two histidines profoundly affects binding ability. Arnold *et al.*[18, 22] have make a significant contribution to the study of the binding behavior of His-X_3-His (X represents different amino acids) in the α-helix region as a synthetic metal binding site by genetic engineering. However, due to the inadequacy length in the synthetic heptapeptides, any secondary structure formation in this study is unable to be shaped. So, that the binding behavior of the random structure peptides may be different from Arnold's results[18, 22].

Conclusion

This investigation demonstrates the feasibility of the determination of the binding constants between solutes and immobilized metal ion on polymer by UV differential absorbance spectroscopy. The study has confirmed the bimodel binding mechanism of the basic solutes by observing the salt concentration effects. The formation of coordinated compound is the major binding mechanism at the pH value higher than the pKa value of deprotonation of N-imidazole. When the behavior of dipeptide with histidine residue on the carboxyl terminal and that of tripeptide with histidine residue on the second residue happen, no differential UV absorbance was observed. The results were explained by the metal ion transfer hypothesis. The binding constants of the five heptapeptides with two histidine residue separated by different number of glycine residues demonstrate the effect of the distance between the two histidines on the binding affinity.

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Nomenclature

C^* = mole of solute per unit volume in solution phase [M] ≡ [mole/L solution]

q^* = mole of solute per unit volume in adsorbed phase [M] ≡ [mole/L gel]

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