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Correlation between Protein Binding Strength on Immobilized Metal Affinity Chromatography and the Histidine-Related Protein Surface Structure

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Immobilized metal affinity chromatography (IMAC) was investigated for its ability to characterize the histidine-related surface structure of a protein, that is, a histidine residue's surface accessibility and its potential involvement in intramolecular interactions. T4 lysozyme was chosen as the model protein. Seven amino acid sites were selected on the basis of their relative surface accessibility, and they were substituted with histidine via site-directed protein mutagenesis to generate seven T4 lysozyme variants, each containing only one histidine residue on its surface, with various surface accessibility. IMAC was then used to experimentally quantify the interaction of each lysozyme variant with immobilized copper ions. A direct correlation was shown between the protein binding affinity and the surface accessibility of the histidine residue. Of all the lysozyme variants, K83H and K147H showed unusually low binding strength, as compared with variants having a histidine residue with a similar surface accessibility. However, with the aid of molecular modeling, their relatively low binding affinities were predicted to be the result of the involvement of the histidine residue in intramolecular interactions. In contrast to previously reported results, our results showed that lysozyme still binds to the IMAC column, even if its histidine residue is involved in intramolecular bonding, such as a hydrogen bond, albeit at reduced strength, as compared with the variant containing a histidine residue with a similar surface accessibility.

With advances in recombinant DNA technology, more and more therapeutic proteins are produced in heterologous expression hosts.[1] Although the growth of the biopharmaceutical industry has been very impressive (the market is estimated to be in tens of billions of dollars),[2] the increasing challenge has been to develop new and reliable techniques to characterize these recombinant therapeutic proteins.[3,4] Characterization of these recombinant proteins is necessary because biopharmaceutical production is a complex process.[5,6] For example, depending on the type of the expression host chosen, a recombinant protein may experience variation in expression, folding, posttranslational modification, and glycosylation.[7−9]

Recently, efforts have been focused on developing and extending affinity-based chromatographic techniques to characterize proteins that differ in their structural properties.[7] For example, Qiu and Regnier were able to determine, quantitatively, the extent of glycosylation in glycoproteins using a lectin-based affinity chromatographic technique.[10] Furthermore, Jiang et al. employed immobilized metal affinity chromatography (IMAC) and immobilized metal-ion affinity capillary electrophoresis (IMACE) techniques to reveal subtle changes in the surface accessibility of the histidine residues in α-chymotrypsin due to glycosylation.[11]

IMAC was developed by Porath and co-workers primarily as a protein purification technique about 3 decades ago.[12] The basic principle involved in IMAC is that certain amino acids (e.g. histidine, cysteine) present on the surface of a protein can be electron donors to form stable complexes with certain transition metal ions (e.g., Ni^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}).[13,14] Among the possible electron donors, histidine is the primary amino acid that interacts with metal ions at neutral pH.[15] The topography of histidine residues on the surface of a protein bears important structural information that can elucidate the functional properties of a protein (e.g., active site histidines in a serine protease family[16,17] and metal

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binding histidines in metalloproteins[18]). In addition, each protein has a unique distribution of surface histidine residues,[19] which can be exploited as a molecular fingerprint to identify each protein and possibly to differentiate a protein from its isoforms.

Due to the specific interactions between immobilized metal ions and the histidine residues on the surface of a protein, IMAC has been previously investigated as a tool to probe the surface topography of histidine residues,[20] including (1) their surface localization (exposed or buried), (2) their number, and (3) their density.[21] During the histidine-mediated protein-metal ion interactions in IMAC, the following factors can affect the extent of the interaction: (1) the extent of a histidine residue's surface accessibility,[17] (2) the number of surface-accessible histidine residues,[20] and (3) the microenvironment of the histidine residues, which refers to the amino acids within 15 Å of a particular histidine residue.[16] The importance of a histidine microenvironment was demonstrated by Russell and Fresht[16] who calculated the changes in the pK_a values of His-64 in subtilisin variants. Their results indicated that there was a change of 1 pK_a unit of the histidine residue when two acidic amino acids (Asp and Glu) were replaced by two basic amino acids (both Lys). Furthermore, Chicz and Regnier used a series of site-directed protein mutagenesis experiments on subtilisin (His-64) to illustrate that the histidine microenvironment had a profound effect on its retention time in an IMAC column.[8] However, our current understanding of the effects of these factors on protein binding on IMAC is still at a qualitative level. For example, Boden et al. used IMAC as a probe to characterize the subtle 3D structural differences within the serine protease family by demonstrating a qualitative correlation between the surface accessibility of the histidine residues and their corresponding elution profiles.[22] To develop IMAC as a more powerful tool in obtaining histidine-related protein structural information, it is vital to establish quantitative relationships between the measurable protein−metal binding strength and the effecting factors. Thus, the protein−metal binding strength may be used to derive histidine-related protein structural information, which is otherwise difficult to obtain.

Until now, there has been no systematic study to quantitate the relationship between the surface accessibility of histidine residues and the binding strength of a protein on IMAC. Surface accessibility or solvent accessible surface of an amino acid can be defined as the area accessible to the solvent molecules. In general, the surface accessibility values reported in the literature are the areas of amino acid residues, calculated from the X-ray crystal structure of a protein by means of computational programs. The surface accessibility of amino acids is an important concept in quantifying the protein surface structure because it elucidates the associated chemical properties of the amino acid residues (such as hydrophobic or hydrophilic).[23] However, it does not provide insights into other important aspects of the surface structure of a protein in solution (e.g., strength of a hydrogen bond). For example, His-57 in chymotrypsin was a surface-accessible residue, and therefore, it was expected to bind onto an IMAC column, but the experimental results indicated that His-57 did not bind to the IMAC column due to its involvement in intramolecular hydrogen bonds.[17] Thus, IMAC may also be used as a probe to determine the involvement of the histidine residue in intramolecular interactions.

To carry out a systematic study on the effect of the surface accessibility of a histidine residue on a protein's interaction with a metal ion, a series of T4 lysozyme variants were generated. The use of the protein variants generated from a well-studied protein would allow better interpretation of the experimental data (avoiding possible complication from protein size and other physicochemical properties) with the assistance of molecular modeling and possibly some previously reported data. In addition, it is possible to generate a series of variants to study the effect of the surface accessibility of a histidine residue over a broader range. In this article, the main objective is to use an IMAC−Cu^{2+} column to determine the binding strength of T4 lysozyme variants, each of which contains only one surface histidine residue with different surface accessibility, and to establish a correlation between the protein−metal ion binding strength and the histidine residue's accessibility. The extent of the histidine residue exposed to solvent was measured using the relative surface accessibility, which is often used to compare amino acid residues of various sizes.[24] Zonal analysis[25,26] experiments were carried out to quantify the interaction between the lysozyme variants and the immobilized copper ion of an IMAC column.

MATERIALS AND METHODS

Materials. The plasmid pHS1403[27] containing a T4 lysozyme gene was kindly donated by Dr. Brian Matthews (University of Oregon, Eugene, OR). The T4 lysozyme encoded by pHS1403 has two amino acid substitutions, C54T and C97A, yielding a cysteine-free lysozyme.[28,29] The TSK gel chelate-5PW glass column (5 cm × 5 mm, bead size 10 μm) used to perform the zonal analysis experiments was purchased from Tosoh Bioscience (Philadelphia, PA). All the other reagents were analytical grade and were purchased from either Fisher Scientific (Hampton, NH) or Sigma (St. Louis, MO).

In Silico Modeling Methods. SYBYL 6.7 (Tripos Inc., St. Louis, MO) was used to calculate the surface accessibility of all amino acid residues of the T4 lysozyme and to generate the in silico lysozyme variants (point mutants), wherein the original amino acid was replaced by a histidine residue. The PDB code for T4 lysozyme used for all calculations was 1L63. The surface accessible values of all amino acids in T4 lysozyme were calculated using a probe with a radius of 1.93 Å to mimic the metal ion accessibility in the IMAC column, as previously reported by Mrabet et al.[30] Before calculating the surface accessibility of the

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histidine residue, all the variant structures were energy-minimized with the SYBYL energy minimization program. UCSF Chimera was used to visualize and generate figures.[31] The theoretical pK_a value for the histidine residue in all the lysozyme variants was calculated using a program H++ (with both options, the Generalized Born and the Poisson−Boltzmann methods).[32]

Experimental methods: Generation of T4 Lysozyme Variants. T4 lysozyme gene in the plasmid pHS1403 was used as a template to generate all the variants. Single mutagenic[33] and overlap extension (consisting of two rounds of PCR)[33−35] PCR techniques were used to generate the PCR product containing the point mutation. The mutated gene was ligated into the vector pHS1403, replacing the original T4 lysozyme gene, and then introduced by transformation into *Escherichia coli* strain RR1.[28] In each case, the CAC histidine codon and the entire gene were confirmed by DNA sequencing.

The production and purification of the lysozyme variants was performed using a modification of a published method.[36] Overexpression of T4 lysozyme variants was induced by isopropyl β-D-thiogalactopyranoside (IPTG). *E. coli* cells containing the mutated gene were grown, induced with IPTG, centrifuged to harvest the cells, and stored at −80 °C.[36] Frozen cells were resuspended in 20 mL of 20 mM sodium phosphate buffer at pH 7.2 (buffer A). After adding a few crystals of DNase I, the cells were lysed by sonication (3 × 30 s with intermittent cooling on ice). After removal of cell debris by centrifugation at 4 °C, (13000*g* for 30 min), the supernatant fraction was dialyzed against buffer A. The dialyzed protein solution was then applied to an XK 16/20 Carboxyl Methyl Sepharose Fast Flow ion exchange column (Amersham Bioscience, Piscataway, NJ) equilibrated with buffer A. The variants were eluted by a salt gradient (they eluted at ∼0.2 M NaCl in buffer A containing 0.01% sodium azide). The eluted proteins were then dialyzed against buffer A for 45 min to lower the salt concentration below 0.2 M NaCl, and the above chromatographic step was repeated with a shallower gradient elution. Before conducting the IMAC experiments, the proteins were concentrated by ultrafiltration using a YM3 membrane (Amicon 8200, Millipore Corp., Bedford, MA), followed by a buffer-exchange step with 20 mM sodium phosphate and 1 M NaCl at pH 7.0 (buffer B) in an Econo-pac desalting column (Bio-Rad laboratories, Hercules, CA). The final protein purity of all variants was more than 95%, determined by SDS−PAGE (data not shown). The lysozyme concentration (mg/mL) was determined by measuring the absorbance at 280 nm using an extinction coefficient of 1.28.[28] The protein sequence (including the histidine residue) of all the variants except K43H was verified by electrospray ionization mass spectrometry (Table 1).

Lysozyme Activity Assay. The enzyme activity of T4 lysozyme was determined by measuring the rate of clearance of a *Micrococcus lysodeiktikus* cell suspension using a modification of a published method.[37] The *M. lysodeiktikus* cell concentration was 0.5 mg dry weight/mL, obtained by suspending the cells in 20 mM sodium phosphate and 50 mM NaCl (pH 6.0). After adding the lysozyme sample (1 μg), the rate of clearance of the *M. lysodeiktikus* cells was measured using a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) as the rate of change in the absorbance at 540 nm. One unit of T4 lysozyme activity is defined as the amount of lysozyme required to catalyze a decrease in absorbance of 0.001 in 1 min.

Table 1. Peptide Sequences of the Six Lysozyme Variants Obtained from Electrospray Ionization Mass Spectrometry[a]

variant	peptide position	peptide sequence
K19H	17−35	IY**H**DTEGYYTIGIGHLLTK
R80H	77−83	GIL**H**NAK
K83H	81−95	NA**H**LKPVYDSLDAVR
K124H	120−135	MLQQ**H**RWDEAAVNLAK
K135H	126−137	WDEAAVNLA**H**SR
K147H	146−154	A**H**RVITTFR

[a] The peptide sequences were identified by SEQUEST. The mutated histidine residues are shown in bold letters.

Zonal Analysis. The binding strength (association constant) of the protein toward the immobilized metal ion in the IMAC column was determined using an isocratic elution method known as zonal analysis, where the inhibitor concentration in the protein sample is equal to that in the mobile phase (elution buffer).[25,26,38] All experiments were conducted at room temperature on the TSK gel column. The TSK gel column was mounted onto an HPLC system equipped with a quaternary pump, HP 1050 (Agilent Technologies, Palo Alto, CA). All solutions used were filtered and degassed using a sonicator prior to the HPLC experiments. The flow rate of the mobile phase was 0.5 mL/min.

Experimental Run: Regeneration of the Column. The TSK gel column, with iminodiacetic acid as the chelating agent, was regenerated by washing with 3 column volumes (CV) of 50 mM EDTA solution containing buffer B to elute all the metal ions, followed by washing with (3 CV) distilled water. The column was reloaded with copper ions using (4 CV) 50 mM $CuSO_4$. The column was then washed with (5 CV) 100 mM sodium acetate and 1 M NaCl (pH 4.0) to remove excess copper ions. The column was washed with distilled water (2 CV), followed by equilibration with (8 CV) buffer B containing I mM imidazole. Then a protein sample of 50 μL of lysozyme variant (1 mg/mL) in buffer B containing I mM imidazole, was injected onto the TSK column by an auto sampler on the HP 1050 system.

The column was regenerated before every experimental run. A set of four experimental runs, each of which was run with a particular imidazole concentration, [I] (I = 3, 6, 9, 12 mM), was carried out for each variant to determine the respective elution volume (V). The protein sample also contained the same concentration of imidazole (as a competitor with protein for binding in IMAC) as that in the elution buffer. This set of experiments was repeated two more times (triplicates). The elution volume in the

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Table 2. Relative Surface Accessibility[a]

variant	rSA (%) (original amino acid)	rSA (%) (histidine residue)
K19H	62	50
K43H	26	15
R80H	62	74
K83H	60	62
K124H	48	47
K135H	69	63
K147H	49	47

[a] The percentage of surface accessibility of the residue (side chain) in the folded protein divided by the SA of the residue (side chain) in the unfolded state of lysozyme variants calculated by using a probe with a radius of 1.93 Å.

absence of interaction (V_0) was obtained by performing an isocratic elution (20 mM sodium phosphate, 1 M NaCl, 0.5 M imidazole) of T4 lysozyme (50 μL) in the absence of the copper ion. The presence of NaCl and imidazole will help in preventing any nonspecific interactions that might occur between the column matrix and the lysozyme.

Determination of the Copper Concentration. The concentration of the immobilized Cu^{2+} on the TSK gel column was determined by spectral analysis using inductively coupled plasma (ICP) spectrometry (Soil Testing Laboratory, Virginia Tech, Blacksburg, VA). The total copper ion concentration was determined on the fresh column and also after a considerable number (~55) of experimental runs. To avoid any loss of immobilized Cu^{2+}, the column was recharged before every experimental run.

Quantitative Data Analysis. The protein binding strength was determined using the following eq 1,[25]

$$\frac{1}{V - V_0} = \frac{1}{K_{aP}(V_0 - V_m)B_t} + \frac{K_{aI}[I]}{K_{aP}(V_0 - V_m)B_t} \qquad (1)$$

where V = elution volume to elute half of the protein (peak maximum), V_0 = elution volume in the absence of interaction (0.78 mL), V_m = void volume in the column determined by blue dextran (0.44 mL), [I] = concentration of the inhibitor (imidazole) in the mobile phase, K_{aI} = the association constant of the imidazole-copper complex, K_{aP} = association constant for the interaction between the protein and the copper ion in IMAC (protein binding strength), and B_t = total immobilized copper ions (23.2 μmol/mL). The elution volume of the protein from the IMAC column varies with the inhibitor concentration [I]. Therefore, a plot of $1/(V - V_0)$ vs [I] would be linear. K_{aI} is equal to the ratio of the slope to the intercept, and $1/K_{aP}$ can be obtained from the intercept and the independently obtainable parameters V_0, V_m, and B_t.

RESULTS AND DISCUSSION

On the basis of the surface accessibility values, seven sites with varying relative surface accessibility (rSA) were chosen to generate the seven lysozyme variants (Table 2). Six of the seven native amino acids are lysines (K), and the other is arginine (R). The distribution of the seven selected sites on the T4 lysozyme surface, in relation to the amino acids that are catalytically active,[39]

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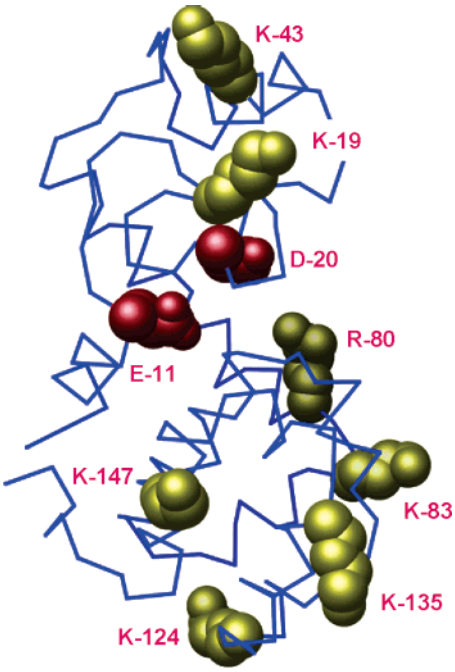

Figure 1. Sites selected for histidine replacement (amino acid substitutions) on the surface of T4 lysozyme are shown in yellow. The amino acid residues that are catalytically active in T4 lysozyme (Glu-11 and Asp-20) are shown in red. This figure was generated using UCSF Chimera.

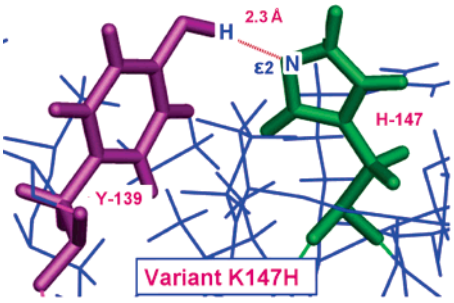

Figure 2. Hydrogen bond (distance 2.32 Å) between the imidazole Nε2 atom of His-147 (donor) and the hydroxyl hydrogen atom of Try-139 (acceptor). The figure was generated using UCSF Chimera. This is a sectional view of the molecule around His-147.

is shown in Figure 1. The rSA values of the histidine residue on each variant range from 15 to 74% (Table 2). Although the rSA of K147H was equal to that of K124H, both were chosen for this study, because molecular modeling predicted the presence of a hydrogen bond between the unprotonated imidazole nitrogen (Nε2) of His-147 and the hydroxyl hydrogen of Tyr-139 in K147H (Figure 2). Therefore, the goal for analyzing variant K147H, in addition to K124H, was to understand the behavior of the protein molecule in an IMAC column, when its donor amino acid is predictably involved in an intramolecular hydrogen bond. The enzymatic activity of all the variants is shown in Table 3. All the variants were enzymatically active, indicating that their 3D structures were largely intact. These results are consistent with the results reported by Dao-Pin et al., wherein the 3D structures of the T4-lysozyme variants containing K83H were intact.[40]

The microenvironment effects of a histidine residue can be divided into two components: (a) the involvement of the histidine

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Table 3. Enzymatic Activities of Purified T4 Lysozyme Variants[a]

variant	% activity
wild type	100
K19H	112
K43H	110
R80H	126
K83H	131
K124H	116
K135H	162
K147H	102

[a] The activities of all the lysozyme variants are compared with the wild type.

residue in intramolecular bonds (hydrogen bonds or salt bridges)[41] and (b) the effects of the electrostatic environment, which involves short- and long-range charge−charge interactions.[42] Although the in silico pK_a value of the histidine residue in all the variants was calculated (data not shown), we were not able to draw any inferences regarding microenvironment effects. Nevertheless, the present set of IMAC experiments was able to reveal the effects of the microenvironment due to the first component (the intramolecular interactions), as discussed in the following sections. However, to understand the effects due to the second component, further experimental work is required and is currently underway.

Zonal Analysis Using IMAC. The total concentration of copper ions immobilized on the TSK gel was 23.2 μmol/mL, which is within the concentration range given by the manufacturer (20 μmol/mL). In addition, the total copper ion concentration after a number of experimental runs did not change significantly (~2 μmol/mL). The elution profiles of the variants, R80H and K147H, are shown in Figure 3. Clearly, these elution profiles are highly reproducible. The time required to half-elute the protein (V) for all the variants (the time at which the maximum absorbance occurs) was obtained from the elution profiles. The binding strength, K_{aP}, was calculated from the intercept of the plot of $1/(V - V_0)$ vs [I] (Figure 4). The K_{aP} indicates the strength of the binding between the immobilized copper ion and the protein (via a histidine residue). The K_{aP} value for all the variants is shown in Table 4. It is important to note that the wild-type T4 lysozyme, which contains a histidine residue (His-31) that is buried and engaged in an intramolecular salt bridge,[43] did not bind to the Cu^{2+} column. The binding strength and the rSA values are plotted in Figure 5. In general, as the surface accessibility of the (lone) histidine residue increases, the binding strength of the protein to immobilized copper ions increases; however, both K147H and K83H variants showed significantly lower binding strength as compared with the variants containing a histidine residue with a similar surface accessibility.

Variant K147H. In the case of variant K147H, the binding strength is ~50% that of K124H; however, through molecular

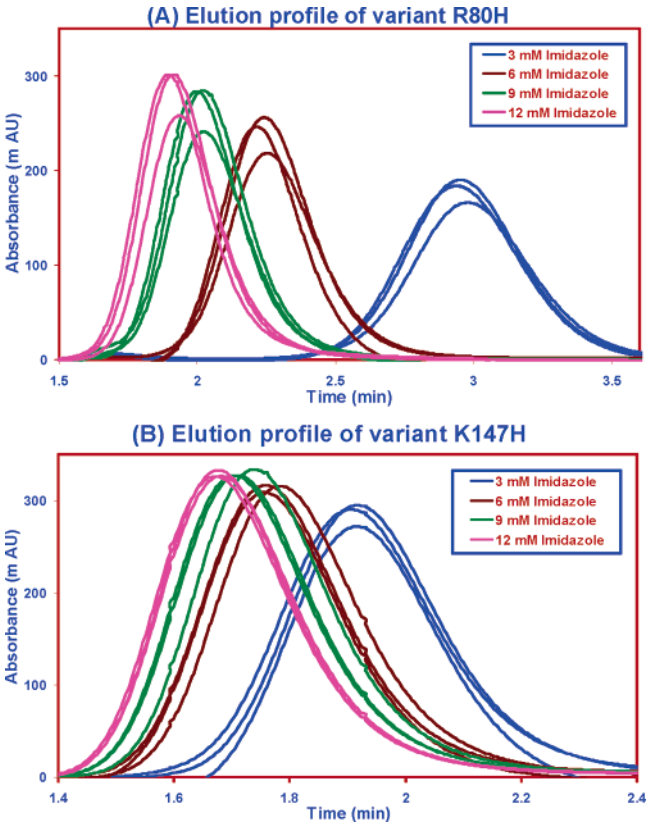

Figure 3. Elution profiles of variants (A) R80H and (B) K147H, obtained in triplicate, for the four imidazole concentrations, 3, 6, 9 and 12 mM, in the mobile phase. The IMAC column was regenerated for each chromatographic run. The differences among the peak heights are due to the unnormalized baselines, which drifted slightly from run to run.

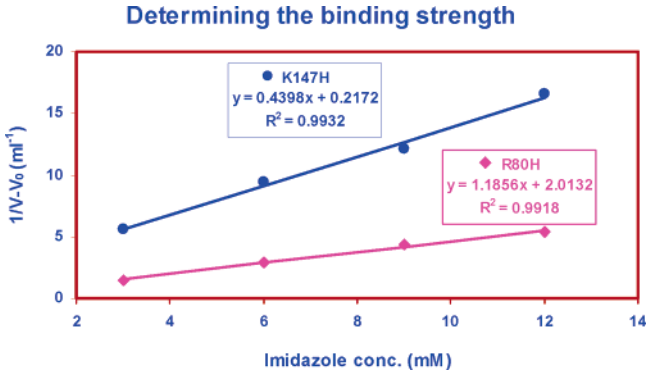

Figure 4. Determination of the $1/(V - V_0)$ intercept required to calculate the binding strength of variants R80H and K147H.

modeling, we predicted that His-147 would engage in a hydrogen bond with Tyr-139, as shown in Figure 2. Thus, the involvement of a surface histidine residue in a hydrogen bond appears to significantly reduce the interaction between the protein and the immobilized metal ions. On the other hand, the fact that K147H binds to the IMAC column is a little surprising. Berna et al. showed that in chymotrypsin, His-57 (surface accessible area ~63 Å^2) had no interaction with the immobilized copper ions due to its participation in intramolecular hydrogen bonds. In contrast, His-41 in chymotrypsin was shown to interact with copper ions, although His-41 has a smaller surface accessible area (~20 Å^2).[17] These conflicting results (between ours and that reported by

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Table 4. Protein−Metal Ion Binding Strength and the Relative Surface Accessibility Values for T4 Lysozyme Variants

variant	rSA (%) (histidine residue)	protein binding strength (K_{aP}) (10^4 M^{-1})
K19H	50	28.4
K43H	15	1.3
R80H	74	59.6
K83H	62	11.4
K124H	47	17.8
K135H	63	74.3
K147H	47	6.4

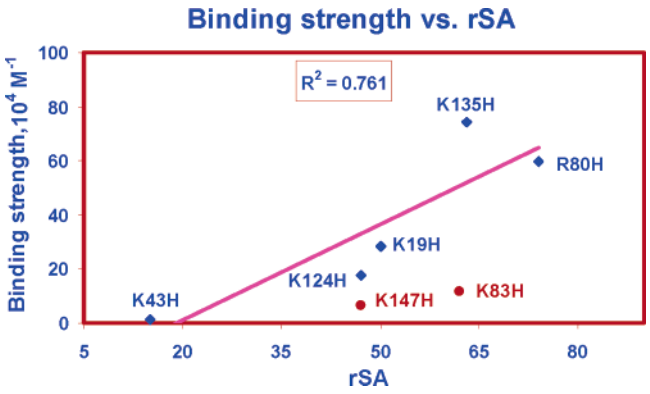

Figure 5. Correlation between the relative surface accessibility (rSA) and the binding strength of T4 lysozyme variants, excluding the two variants K147H and K83H. The histidines of these two variants are involved in intramolecular hydrogen bonds, shown as circles.

Berna et al.) can be explained by the fact that (1) the number of hydrogen bonds involved may differ, and (2) not all hydrogen bond strengths are equal.[44] The strength of a hydrogen bond depends on a number of factors, such as (1) the microenvironment of both donor and acceptor atoms;[44,45] (2) the type and the stereoelectronic effects of the donating group (e.g., syn- or anti-orientation of the carboxylate ions);[46] (3) the distance and the particular angle of the donor and the accepting atoms;[44,47] and (4) in the case of hydrogen bonds involving histidine residues, the proper orientation and the tautomeric state of the imidazole ring.[44]

Variant K83H. For variant K83H, which has a rSA almost equivalent to that of K135H, the binding strength is ~15% that of K135H. As predicted by molecular modeling, there was no evidence of His-83 being involved in a hydrogen bond with any of the neighboring amino acid residues because there was neither a donor nor an acceptor group within 3 Å, which is considered to be the distance threshold for the formation of a hydrogen bond.[48] Nevertheless, results reported by Dao-Pin et al., from the X-ray crystal structure of a T4-lysozyme variant that contained K83H, implied that the Nδ1 hydrogen of His-83 was involved in a weak

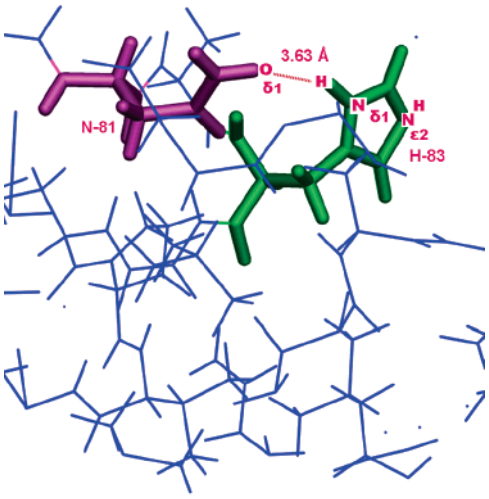

Figure 6. The presence of a weak intramolecular hydrogen bond between the Oδ1 oxygen of Asn-81 and the Nδ1 hydrogen of His-83, a distance of 3.63 Å. The figure was generated using UCSF Chimera. This is a sectional view of the molecule around His-83.

hydrogen bond (3.5 Å) with the Oδ1 oxygen of Asn-81.[40] Consequently, the 3D structure (by molecular modeling) of the variant K83H in this study was scrutinized, and the presence of a possibly weak intramolecular hydrogen bond (3.63 Å) was discovered between the Oδ1 oxygen of Asn-81 and the Nδ1 hydrogen of His-83 (Figure 6). This argument is valid because the imidazole group of the histidine residue can exist in the doubly protonated form,[49] which may be stabilized by the electronegative Oδ1 oxygen of Asn-81 (Figure 6). Thus, this results in the decrease of the binding strength of the variant K83H when compared to K135H.

Histidine-Related Protein Surface Structure Derived from Protein Binding Strength. Because the histidine residues in K83H and K147H are involved in intramolecular interactions, the correlation between the protein binding strength and the rSA is, thus, generated excluding these two variants. As shown in Figure 5, there is a direct correlation ($R^2 = 0.76$) between the rSA and the protein binding strength. Using this correlation, we can predict not only the surface accessibility of a histidine residue on a protein surface but also the presence of intramolecular hydrogen bonds involving the reporter amino acid residue, histidine, from the measured protein−metal ion binding strength. These intramolecular bonds may or may not be captured by molecular modeling and often can only be confirmed by X-ray crystallography or NMR analysis. In addition, K147H and K83H showed slightly different binding strength with immobilized copper ions. In combination with the reported result that the presence of a hydrogen bond in chymotrypsin (His-57) completely prevented it from interacting with copper ions,[17] it is possible that IMAC, at least with immobilized copper ions, may also be used as a tool to detect the strength of a hydrogen bond involving a histidine residue.

CONCLUSIONS

On the basis of the surface accessibility, seven lysozyme variants were generated, each containing one surface histidine residue. An IDA-Cu^{2+} IMAC column was used to determine the

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binding strength of these lysozyme variants. The binding strength was shown to have a direct correlation with the rSA of the histidine residues, except for variants K83H and K147H, whose histidines were shown to be involved in intramolecular hydrogen bonds. Thus, on the basis of the binding strength of a protein, IMAC can be used to determine the histidine-related protein surface structure, including the surface accessibility of histidine residues and the presence of hydrogen bonds, which generally requires an X-ray or NMR structure. It is also possible that this method could be used to evaluate the strength of a hydrogen bond. The results shown in this report strongly suggest that IMAC can be used as a quantitative tool to characterize proteins to derive their histidine-related surface structures. Finally, the experiments reported in this paper were designed to study the influence of surface accessibility on the binding strength of a protein containing only one surface histidine residue. Our future efforts would be focused on studying the effects of the microenvironment of the histidine residue on the binding strength of a protein and also consider proteins having multiple surface histidines.

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