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Identifying Zn-bound histidine residues in metalloproteins using hydrogen deuterium exchange mass spectrometry

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Abstract

In this work we have developed a method that uses hydrogen deuterium exchange (HDX) of C2-hydrogens of histidines coupled with mass spectrometry (MS) to identify Zn-bound histidines in metalloproteins. This method relies on differences in HDX reaction rates of Zn-bound and Zn-free His residues. Using several model peptides and proteins, we find that all Zn-bound His residues have substantially lower HDX reaction rates in the presence of the metal. The vast majority of non-Zn-binding His residues undergo no significant changes in HDX reaction rates when their reactivity is compared in the presence and absence of Zn. Using this new approach, we then determined the Zn binding site of β -2-microglobulin, a protein associated with metal-induced amyloidosis. Together these results suggest that HDX-MS of His C2-hydrogens is a promising new method for identifying Zn-bound histidines in metalloproteins.

Introduction

About one-third of all proteins require metal ions for their function. As the second most abundant transition element in biological systems, Zn is essential in diverse cellular processes, such as transcription, translation, and metabolism by either participating directly in chemical catalysis or being important for maintaining protein structure and stability.¹ Zinc binding sites in proteins are usually made up of the nitrogen of His residues and/or the sulfur of Cys residues. Less frequently the oxygens of Asp or Glu residues also contribute to the binding site.² Identification of the structure of Zn binding sites in proteins is typically essential for understanding the function of Zn metalloproteins.

There are several ways to determine the amino acid residues bound to Zn in proteins, including X-ray crystallography and nuclear magnetic resonance (NMR). Other techniques, such as extended X-ray absorbance spectroscopy (XAS), can also provide insight into the types of atoms bound to Zn and their distances. These techniques, however, have limitations.³ For example, X-ray crystallography requires large amounts of a pure sample, and the protein must be able to form a crystal. NMR has limited utility for high molecular weight or heterogeneous proteins. XAS provides very detailed information about metal-ligand distances and even geometries, but it does not identify the specific amino acid residues bound to Zn.⁴

Mass spectrometry (MS) is emerging as an alternative approach to characterize metal-protein binding at a variety of levels, including metal ion binding stoichiometry, metal oxidation states and metal ligands.⁵⁻²⁰ For example, straightforward m/z measurements can determine the number of metals bound to a given protein as has been done to measure the stoichiometry of Ca(II) binding to parvalbumin, calmodulin, and calbindin.⁵⁻⁸ Similarly, MS

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has been used to determine binding stoichiometries of transition metals, such as Fe(III),⁹ Zn(II)¹⁰⁻¹² and Cu(II),¹³ to protein and peptides. MS has also demonstrated the ability to determine the oxidation states of transition metals in proteins containing iron-sulfur clusters,¹⁴ iron-bound hemes,^{15,16} and non-heme iron-containing proteins.¹⁷ In these cases, the oxidation state of the metal was determined by accurate mass measurements. Selective chemical modification combined with MS has been applied to identify cysteine ligands in Zn binding proteins. Forest and co-workers used iodoacetamide, which is a sulfhydryl-specific alkylating reagent, to react with the Fur protein in the presence and absence of Zn. MS was then used to determine which of the Cys residues were protected from alkylation upon Zn binding, thereby identifying the Zn binding residues.¹⁸ Russell and co-workers extended this approach by developing a ratiometric pulsed alkylation method to probe the residue-specific reaction kinetics of Cys residues within each of the six zinc-finger domains of the Metal Response Element (MRE)-binding transcription factor-1 using another sulfhydryl-specific alkylating reagent, *n*-ethylmaleimide.¹⁹ The same group also further applied this method to study protein folding intermediates by monitoring the reactivity of Cys residues under different solution conditions.²⁰

While alkylation along with MS is a straightforward and powerful approach for identifying Zn-binding Cys residues in proteins, Cys represents only about 60% of the residues typically bound to Zn in proteins.² Of the remaining 40%, about 35% of the protein bound ligands are provided by His residues.² So, we set out to develop an approach for identifying His binding residues in Zn metalloproteins. To do this, we have explored hydrogen deuterium exchange (HDX) of the C2-hydrogens of His residues along with MS detection. Unlike more labile hydrogens on nitrogen, oxygen, or sulfur atoms, the imidazole C2-hydrogen can be exchanged with deuterium with a half-life around two days.²¹ This reaction is a pseudo-first-order reaction in which the rate limiting step is abstraction of the C2-hydrogen from a cationic imidazolium to form a ylide or a carbene intermediate (Scheme 1).²¹ The reaction rate is affected by several factors, including the pK_a of the His residue, solution pH, solvent accessibility, and temperature.^{21,22} Exchange at His residues has been used with MS to measure the pK_a of His residues in proteins,²³ to study protein-protein interactions,²⁴ and to examine protein folding and stability in complex mixtures.²⁵ It has also been reported that metal binding can decrease the HDX reaction rate of the C2-hydrogen of imidazole, its derivatives, and even other heterocycles in metal dependent manner.^{26,27} Given this previous observation, we envisioned that Zn-dependent decreases in the exchange of C2-hydrogens on His residues might be a means of identifying Zn-bound His residues in proteins using MS. Here, we explore this possibility by measuring the HDX reactions of His C2-hydrogens for proteins with and without Zn bound. Via studies of several proteins, we find that Zn does decrease exchange at the C2 positions on His residues, and MS can be readily used to monitor this reaction in a way that allows Zn-bound His residues to be identified in Zn-binding proteins.

Experimental

Materials

Human angiotensin I (Agt I, DRVYIHPFHL), Cu/Zn SOD from bovine erythrocytes (pdb entry 1CBJ), carbonic anhydrase I from human erythrocytes (HCA I, pdb entry 2CAB), deuterium oxide (D₂O, 99.9%), dithiothreitol (DTT), glacial acetic acid, 3-morpholinopropanesulfonic acid (MOPS), trichloroacetic acid, zinc sulfate, and endoproteinase Glu-C were purchased from Sigma-Aldrich (St. Louis, MO). were purchased from Sigma-Aldrich (St. Louis, MO). Human β -2-microglobulin (β 2m, pdb entry 1JNJ) was purchased from Lee Biosolutions (St. Louis, MO), who purifies the protein from human urine. Ammonium acetate and disodium ethylenediamine-tetraacetic (EDTA) were purchased from Thermo Fisher Scientific (Waltham, MA). Immobilized trypsin and

chymotrypsin (digestion buffer triethylamine included) were purchased from Princeton Separations (Adelphia, NJ). Amicon molecular weight cutoff (MWCO) filters were purchased from Millipore (Burlington, MA). Water was prepared with a Millipore Simplicity 185 water purification system.

Preparation of apo-proteins

500 μ M holo (i.e. metal-bound) HCA I was incubated for 20 h with 10 mM EDTA in 25 mM MOPS at pH 7.4 to remove the native Zn. The protein was then buffer exchanged against 25 mM MOPS at pH 7.4 to remove the EDTA. Less than 5% metal remained after EDTA treatment, as determined by inductively coupled plasma mass spectrometry (ICP-MS). Apo (i.e. metal-free) Cu/Zn SOD was prepared in a similar manner; however, 50 mM ammonium acetate at pH 3.9 was used as the buffer, and the protein was reconstituted in 25 mM MOPS at pH 7.4. Less than 5% metal remained after EDTA treatment, as determined by ICP-MS.

Hydrogen Deuterium Exchange (HDX) of the C2-Hydrogens of Histidine

25 μ M of the protein of interest in either the apo or holo form was exchanged in D₂O (95%) at pH 7.4 and 37 °C in a 25 mM MOPS buffer. After a given reaction time, an aliquot of the sample was diluted 20-fold into H₂O, desalted, re-concentrated, digested by immobilized trypsin and chymotrypsin, and then analyzed by LC-MS. The total time in H₂O before analysis was kept at 4.5 h to ensure thorough back-exchange to hydrogen for the fast exchanging amide groups on the backbone and side chains of the protein. This step was important to ensure that deuteriums remained only at the C2 position of the His residues. In the case of the peptide Agt I, 100 μ M peptide with and without 100 μ M ZnSO₄, was exchanged in D₂O (95%) at pH 7.4 and 37 °C in a 25 mM MOPS buffer. For the protein β 2m, 100 μ M protein with and without 100 μ M ZnSO₄, was exchanged in D₂O (95%) at pH 7.4 and 37 °C in a 25 mM MOPS buffer that also contained 150 mM potassium acetate.

Proteolytic digestions

Immobilized trypsin and/or chymotrypsin or Glu-C were used to proteolytically digest proteins after the HDX reactions. A 75 μ L solution of the protein was first incubated with 15 μ L of acetonitrile at 45 °C for 45 min, and then 7.5 mM DTT was added and allowed to react with the protein at 37 °C for another 30 min. The immobilized enzymes were next added to yield a final enzyme:substrate ratio of 1:10. The protein samples were digested in a shaking water bath (VWR, Radnor, PA) at 37 °C for 2 h. The reaction was quenched by separating the digestion products from the immobilized enzymes and adding 2 μ L of glacial acetic acid. The total back exchange time was controlled to be 4.5 h. For the holo form of Cu/Zn SOD, trichloroacetic acid precipitation was applied before digestion, as a previously described, to facilitate digestion.²⁸

Liquid Chromatography and Mass Spectrometry

A Bruker AmaZon (Billerica, MA) quadrupole ion trap mass spectrometer coupled with an HP1100 series high-performance liquid chromatography system (Agilent, Santa Clara, CA) was used for all MS analyses. Typically, the electrospray needle voltage was kept at 4-4.5 kV, and the capillary temperature was set to 200 °C. Tandem mass spectra were recorded using an isolation width of 2.0-4.0 Da and excitation voltages between 0.5-0.8 V. Peptide sequences were determined from tandem MS data via *de novo* sequencing.

Size Exclusion Chromatography (SEC)

Incubated solutions of β 2m were separated using a TSK-gel SuperSW2000 column (Tosoh bioscience, Prussia, PA) installed on an HP1100 series high-performance liquid

chromatography system (Agilent, Santa Clara, CA). Before analyses of samples, the SEC column was first equilibrated with a 150 mM ammonium acetate mobile phase (pH 6.8) at a 0.35 mL/min flow rate for 1 h. 20 μ L of the incubated sample or calibration standard was injected for analysis, and the variable-wavelength detector was set at 214 nm. A solution containing 5 μ M bovine serum albumin (MW= 66,000), 5 μ M ovalbumin (MW= 45,000), 5 μ M carbonic anhydrase (MW=29,040) and 5 μ M β 2m (MW= 11,731) was used for molecular weight calibration.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

After metal removal, an aliquot of the apo-protein solution was mixed with 0.5 mL of *aqua regia*, and then diluted to 10 mL with de-ionized water. Please note that *aqua regia* is highly corrosive and must be used with extreme caution! Starting with stock solutions, a series of zinc standard solutions were prepared at concentrations of 20, 10, 5, 2, 1, 0.5, and 0.2 parts-per-billion (ppb) under the same acidic conditions. The zinc standard solutions and apo-protein sample solution were measured on a PerkinElmer NexION 300X ICP mass spectrometer (Waltham, MA). All solutions were measured under the kinetic energy discrimination (KED) mode. Operating conditions were as follows: nebulizer flow rate: 1.00 L/min; RF power: 1600 W; plasma Ar flow rate: 18 L/min; dwell time: 50 ms; KED cell gas: 4.6 mL/min. The zinc concentration of apo-protein sample was calculated against the calibration curve generated from the standards.

Deuterium incorporation and HDX reaction rate constant calculations

The number of deuterium atoms added to each His residue was determined by calculating the weighted average mass of the proteolytic peptide containing the specific His residue of interest as compared to that of the unmodified peptide; isotope peaks with relative ion abundances of less than 5% were omitted from the calculation. For peptides containing more than one His residue, collision induced dissociation (CID) or electron transfer dissociation (ETD) was used to sequence the peptides and determine the number of deuteriums added to each individual His residue. The pseudo-first-order rate constants (k_ϕ) of the HDX reactions were determined by monitoring the deuterium uptake over time. The weighted average masses at time = 0 and time = t, and the number of incorporated deuteriums at time = t are presented as m_0 , m_t , and D, respectively. The mass of the peptide at time = t (i.e. m_t) can be expressed by equation 1, where $x\% = 1-D$.

$$m_t = D + 100\%m_0 = x\% m_0 + (100 - x)\% \times (m_0 + 1) \quad \text{Equation 1}$$

Because the HDX reaction follows pseudo-first-order kinetics (Equation 2), at time = t the remaining reactant [A] is x% and [A]₀ is 100%, so that k_ϕ can be determined by equation 3.

$$\ln [A] = -k_\phi t + \ln [A]_0 \quad \text{Equation 2}$$

$$k_\phi = -\ln(1 - D) / t \quad \text{Equation 3}$$

Results and discussion

Angiotensin I

As a test to see how sensitive the HDX rate of the C2 position of histidine is to Zn binding, we examined the peptide Agt I. This peptide binds transition metals at His6 and His9 as has been determined by previous NMR and MS studies.²⁹⁻³¹ Agt I was exchanged in buffered

D₂O with and without Zn for various time points, and then an aliquot of the sample was diluted 20-fold into H₂O for 4.5 h before separation by HPLC and analysis by MS and MS/MS. Figure 1a shows a typical spectrum that results after a 3-day exchange in the presence (black trace) and absence (red trace) of Zn. For comparison the isotopic distribution of the peptide that has not undergone any exchange is also shown (blue trace in Figure 1a). The weighted average number of incorporated deuteriums is found to be 0.64 ± 0.03 and 1.11 ± 0.01 in the presence and absence of Zn, respectively, which suggests that Zn binding decreases the HDX rate. Note that the extent of deuterium incorporation is low because the exchange rate at the C2 position of a fully exposed histidine is known to have a half-life around 2 d.²¹ Moreover, the 4.5 h “back-exchange” reaction in H₂O and subsequent LC separation cause all other deuteriums to be replaced by hydrogens. If the extent of deuterium incorporation is monitored over time (Figure 1b), we obtain HDX reaction rate constants of $0.101 \pm 0.005 \text{ d}^{-1}$ and $0.24 \pm 0.02 \text{ d}^{-1}$ in the presence and absence of Zn, respectively, using Equation 3. This 60% drop in rates suggests that Zn can have a notable effect on the exchange rates of His residues that are bound to it.

To confirm that deuteriums are present only at His residues, deuterated Agt I was subjected to MS/MS using CID. CID also allows determination of the deuterium incorporation into the individual His residues. It should be noted that CID does not cause deuterium scrambling because the deuterium is incorporated at a carbon. Figure 1c shows expanded regions around two of the product ions from the $(M+3H)^{3+}$ ion of Agt I and the deuterium levels in several of the product ions of interest. The overall pattern of deuterium incorporation with and without Zn is consistent with only His6 and His9 having any measurable deuterium uptake, as only product ions containing these residues (i.e. b₆, b₈, b₉, y₂, y₄, and y₅) show altered isotope patterns. Moreover, less deuterium is incorporated into these product ions after a given reaction time when Zn is present (red vs. black bars in Figure 1c). In contrast, product ions that do not contain these two His residues have no statistically significant levels of deuterium incorporation. The b₅ and y₁ product ions of Agt I are shown as examples, but all other product ions that are without His6 and His9 also show no deuterium incorporation. On the whole, our data for the peptide Agt I clearly demonstrate that HDX reaction rates of histidine C2-hydrogens can be considerably retarded by Zn binding, and this decrease in reactivity can be readily detected by MS.

Cu/Zn SOD

Bovine Cu/Zn SOD was studied next as an example of a large protein containing both Zn-free and Zn-bound His residues. Cu/Zn SOD is a native homodimer with two identical subunits that each has eight His residues.³² HDX was conducted on the apo (i.e. metal free) and holo (i.e. metal loaded) forms of the protein for varying time points. Previous studies have suggested that Zn has an important role in the process of folding of the protein, but once folded, the protein remains folded and dimeric upon removal of Cu and Zn.^{33,34}

Site specific incorporation of deuteriums at each His residue was measured after exchange and enzymatic digestion via LC/MS analysis of all the proteolytic fragments containing the His residues. Figure 2 shows example mass spectra from two peptides – one that contains a His residue (His19) that does not bind Zn (Figure 2a) and another that contains a His residue (His78) that does bind Zn (Figure 2b). The peptide, GDGPVQGTIHFEAK, which has a His residue that does not bind Zn, has 0.276 ± 0.008 and 0.27 ± 0.02 deuteriums incorporated during 3-day HDX reactions of the apo and holo forms of the protein, respectively (Figure 2a). By measuring HDX at multiple time points, we find that this peptide exchanges with essentially the same rate constants of 0.130 ± 0.002 and $0.127 \pm 0.009 \text{ d}^{-1}$ in apo- and holo-protein, respectively. In contrast, the peptide HVGDLGNVTADK shows a considerable difference in deuterium uptake between the apo and holo forms of the protein. There are

0.23 ± 0.01 deuteriums added to the peptide after a 3-day reaction in the absence of the metal, but no statistically significant level of deuterium is added when Zn is present. The exchange rate constant of the Zn-free form is $0.09 \pm 0.01 \text{ d}^{-1}$, but the exchange rate constant of the Zn-bound form is not measurable because the extent of deuterium incorporation is too low. These data are consistent with the idea that Zn binding slows HDX at His residues.

The same trend in HDX rates is seen for the other metal-bound and metal-free His residues in Cu/Zn SOD, and these results are summarized in Table 1. His69 and His78, which bind Zn, as well as His61, which bridges Cu and Zn, undergo significant decreases in HDX rates in the holo form of the protein. Interestingly, Cu binding also dramatically decreases the HDX rate of the three His residues (His44, His46, and His118) bound to this metal (Figure S1). Unfortunately, His41, which does not bind Cu or Zn and is located about 13 Å from the Cu binding site and 17 Å from Zn does not have measurable HDX in the presence and absence of Zn after a 3-day reaction.

HCA I

As a further test of whether HDX rates reliably decrease upon Zn binding, we also investigated the Zn metalloprotein human carbonic anhydrase I (HCA I). In HCA I zinc is coordinated by three His residues (His94, His96, and His119) and a bound water/hydroxyl group, and the protein contains an additional eight His residues that do not bind Zn.³⁵ Thus, this protein provides a total of 11 His residues whose HDX rates can be measured.

Apo and holo forms of HCA I were incubated in D₂O for various time points. Deuterium incorporation was monitored for each His residue by LC-MS after proteolytic digestion. From these experiments we find that the 11 His residues fall into three categories with regard to their HDX reaction rates in the apo and holo forms of the protein: (1) those that undergo no significant change in their reaction rates; (2) those that undergo slower exchange in the presence of Zn; and (3) those that exchange too slowly to confidently determine a rate. As an example of the first category, His243 has almost identical HDX reactivity with rate constants of $0.13 \pm 0.01 \text{ d}^{-1}$ and $0.128 \pm 0.007 \text{ d}^{-1}$ in the presence and absence of Zn (Figure 3(a)). His40, His67, His103, and His200 behave in a similar manner, having very similar HDX reaction rates in apo and holo forms of the protein (Table 1). The reactivity of these non-binding His residues is consistent with the results obtained for Agt I and Cu/Zn SOD; these non-binding His residues do not change in HDX reactivity when Zn is present.

In contrast, His64 and His94 undergo slower exchange in the holo protein as compared to the apo protein, and thus these sites represent a second category of His residues. In particular, the reaction rate constant of His94 drops by about one order of magnitude in the presence of Zn ($< 0.01 \text{ d}^{-1}$ vs. $0.072 \pm 0.002 \text{ d}^{-1}$). This significant drop is consistent with the fact that this residue binds to Zn. His64 also undergoes slower exchange in the presence of Zn, but the drop is only about 50%. Interestingly, this residue does not bind Zn; however, it undergoes a conformational change that causes it to become less solvent exposed upon Zn binding even though it is about 9 Å from the Zn binding site.³⁶⁻³⁸ This drop in solvent accessibility and likely change in $\text{pK}^{\text{a}23,24}$ might explain why His64 undergoes slower exchange in the presence of Zn.

The third category of His residues, which includes His96, His107, His119, and His122, exchanged too slowly to confidently determine the exchange rate. Because both increased temperature and pH accelerates the exchange rate of His side chains, we investigated increasing the reaction temperature to 45 °C and pH to 8.5 as a means of obtaining exchange rates for these residues. At these conditions, HCA I is known to maintain its structure in both the apo and holo forms.³⁹ Under these new reaction conditions, we find that the exchange rates for His96 and His119 are now measurable. Furthermore, we find that both of

these residues now fall into category 2 in that they undergo much slower exchange in the presence of Zn (Table 1). Importantly, both of these residues bind Zn, so there slower exchange in the presence of this metal is consistent with the other Zn-binding His residues described earlier in this work. Unfortunately, we still did not see sufficient deuterium incorporation in His107 and His122, probably due to the very low solvent accessible surface areas of these residues (0 Å² for both His107 and His122). All the other His residues that had measurable rates at the lower temperature and pH retain the same general HDX behavior as before (i.e. they fall into the same category) even when elevated temperature and pH conditions are used (see Table S1).

β2m

Based on the promising data with a peptide and two well-characterized Zn-binding proteins, we examined whether HDX of His residues could be used to determine the Zn binding site of the protein β2m, whose Zn binding site is unknown. The idea is to identify which His residue(s) have slower HDX exchange in the presence of Zn. β2m was chosen because it forms amyloid fibrils in the joints and connective tissues of patients undergoing dialysis treatment as a result of kidney disease. Deposition of β2m amyloids leads to a condition referred to as dialysis-related amyloidosis (DRA).^{40,41} Several different conditions can be used to induce β2m amyloid formation *in vitro*, including incubation with stoichiometric amounts of Cu(II).⁴¹⁻⁴³ Interestingly, while the protein readily forms amyloids in the presence of Cu(II), binding by Zn does not cause amyloid formation, although it does cause β2m to oligomerize and form amorphous aggregates.^{42,44} While the Cu(II)-β2m binding site is known,^{45,46} the Zn site is not. Determining where Zn binds β2m should help us understand the different effect that Zn has on β2m oligomerization and aggregation.

β2m was exchanged in D₂O with and without Zn, and deuterium incorporation into its four His residues was determined by LC/MS. Because β2m forms dimers in the presence of Zn, even after 1 day under the conditions used (see Figure S5), we could not reliably measure the effect of Zn binding on His HDX at longer exchange times. Table 2 shows deuterium incorporation at the four His residues after 1-, 2- and 4-day exchange times. From the data it appears that His51 is the only His residue that has a reliably lower extent of exchange in the presence of Zn. Using a t-test, the extent of exchange of His51 at 1, 2, and 4 days is statistically different with and without Zn at a 99% confidence interval. His13 and His84 are not statistically different at any of the time points. His31 is only statistically different with and without Zn at day 4, but this is at a 90% confidence interval. Because some dimer begins to form after 1 day (Figure S5), the exchange data at longer time points is complicated by increasing levels of oligomers. The higher levels of oligomers result in decreases in solvent accessibility at certain sites on the protein, and this might explain the decreased reactivity of His31 at the 4-day time point. It should be noted here that there is only a modest change in the exchange rate of His51 as compared to the data for Cu/Zn SOD and HCA I. This modest change is most likely caused by the incomplete loading of the protein by Zn under the conditions used. Based on the concentrations at which Zn causes β2m oligomerization, we estimate a K_d value of 30 μM. If this value is correct, then under the conditions used to acquire the data in Table 2 only about 50 % of the protein is loaded by Zn. The HDX measurements in the presence of Zn are then influenced by a significant percentage of Zn-free protein. The change in the extent of exchange will then not be as great as if the protein were fully loaded with Zn, as is the case in the Cu/Zn SOD and HCA I experiments.

Taken as a whole, we conclude that Zn binds to His51 and not at any other His residue. Because metals like Zn typically bind proteins through multiple amino acids, additional data would be necessary to fully elucidate the Zn binding site. Even so, the data obtained here are

somewhat enlightening with regard to the different effect of Zn and Cu(II) on β 2m aggregation. Cu(II) binds β 2m via His31, the N-terminus, the amide between Ile1-Gln2, and Asp59,^{42,45,46} while Zn binds at a different site (i.e. His51). The full ramifications of these different binding modes clearly require further study.

Conclusions

Using a model peptide and two well-characterized Zn-binding proteins, we have demonstrated that Zn binding substantially slows the HDX rates of hydrogens at the C2 position of His residues, and this effect is readily detectable by MS. For non-binding His residues, HDX does not change unless the residue undergoes a substantial change in its solvent accessibility. His residues with very low solvent accessibilities fail to undergo any deuterium exchange, but in some cases HDX of these residues can be measured by moderately increasing the temperature and pH. Overall, our results suggest that monitoring HDX of the C2 position on His residues might be a method for identifying Zn binding His residues in proteins, especially those that are difficult to study by more traditional means. Although, it should be noted that completely buried His residues (e.g., His107 and His122 in HCA I) may provide ambiguous results. As an initial test of this method's ability to elucidate Zn binding in a protein's whose binding site has not been previously characterized, we provide evidence that His51 binds Zn in β 2m. While this approach can only provide information about the Zn binding status of His residues, it should be noted that His residues represent about 35% of the Zn binding sites in proteins.⁴⁷ The remaining 60% typically involve mostly Cys residues, and previous work has illustrated how covalent labeling together with MS can be used to determine Zn-bound Cys residues.¹⁷⁻¹⁹ We envision that the two methods, His-specific HDX and Cys-specific covalent labeling, could be combined in the same experiment to more fully characterize Zn-protein binding sites by MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. McCall KA, Huang C, Fierke CA. J. Nutr. 2000; 130:1437S–1446S. [PubMed: 10801957]
2. Zheng H, Chruszcz M, Lasota P, Lebioda L, Minor W. J. Inorg. Biochem. 2008; 102:1765–1776. [PubMed: 18614239]
3. Patel K, Kumar A, Durani S. Biochim. Biophys. Acta. 2007; 1774:1248–1253.
4. Debeer S. X-ray absorption spectroscopy. Methods Mol. Biol. 2011; 766:165–176. [PubMed: 21833867]
5. Hu P, Buckel SD, Whitton MM, Loo JA. Eur. Mass Spectrom. 1995; 2:69–76.
6. Hu P, Loo JA. J. Mass Spectrom. 1995; 30:1076–1082.
7. Lafitte D, Capony JP, Grassy G, Haiech J, Calas B. Biochemistry. 1995; 34:13825–13832. [PubMed: 7577976]
8. Veenstra TD, Johnson KL, Tomlinson AJ, Naylor S, Kumar R. Biochemistry. 1997; 36:3535–3542. [PubMed: 9132004]
9. Petillot Y, Forest E, Mathieu I, Meyer J, Moulis JM. Biochem. J. 1993; 296:657–661. [PubMed: 8280064]
10. Yu X, Wojciechowski M, Fenselau C. Anal. Chem. 1993; 65:1355–1359. [PubMed: 8517548]
11. Surovoy A, Waidelich D, Jung G. FEBS Lett. 1992; 311:259–262. [PubMed: 1397325]

12. Zirah S, Rebuffat S, Kozin SA, Debey P, Fournier F, Lesage D, Tabet JC. *Int. J. Mass Spectrom.* 2003; 228:999–1016.
13. Loo JA, Hu P, Smith RD. *J. Am. Soc. Mass Spectrom.* 1994; 5:959–965. [PubMed: 24226385]
14. Johnson KA, Verhagen MF, Brereton PS, Adams MW, Amster IJ. *Anal. Chem.* 2000; 72:1410–1418. [PubMed: 10763234]
15. Li YT, Hsieh YL, Henion JD, Ganem B. *J. Am. Soc. Mass. Spectrom.* 1993; 4:631. [PubMed: 24227666]
16. He F, Hendrickson CL, Marshall AG. *J. Am. Soc. Mass Spectrom.* 2000; 11:120–126. [PubMed: 10689664]
17. Lei QP, Cui X, Kurtz DM, Amster IJ, Chernushevich IV, Standing KG. *Anal. Chem.* 1998; 70:1838–1846. [PubMed: 9599583]
18. Gonzalez de Perodo A, Saint-Pierre C, Adrait A, Jacquamet L, Latour LM, Michaud-Soret I, Forest E. *Biochemistry.* 1999; 38:8582–8589. [PubMed: 10387106]
19. Apuy JL, Chen XH, Russell DH, Baldwin TO, Giedroc DP. *Biochemistry.* 2001; 40:15164–15175. [PubMed: 11735399]
20. Apuy JL, Park ZY, Swartz PD, Dangott LJ, Russell DH, Baldwin TO. *Biochemistry.* 2001; 40:15153–15163. [PubMed: 11735398]
21. Vanghan JD, Mughrabi Z, Wu EC. *J. Org. Chem.* 1970; 35:1141–1145.
22. Mullangi V, Zhou X, Ball DW, Anderson DJ, Miyagi M. *Biochemistry.* 2012; 51:7202–7208. [PubMed: 22901083]
23. Miyagi M, Nakazawa T. *Anal. Chem.* 2008; 80:6481–6487. [PubMed: 18665614]
24. Miyagi I M, Wan Q, Ahmad MF, Gokulrangan G, Tomechko I SE, Bennett B, Dealwis C. *PLoS ONE.* 2011; 6:e17055. [PubMed: 21359214]
25. Tran DT, Banerjee S, Alayash AI, Crumbliss AL, Fitzgerald MC. *Anal. Chem.* 2012; 84:1653–1660. [PubMed: 22185579]
26. Buisson DH, Jones JR, Taylor SE. *J.C.S. Chem. Comm.* 1975; 20:856–856.
27. Buncel E, Clement O, Onyido I. *Acc. Chem. Res.* 2000; 33:672–678. [PubMed: 11041831]
28. Ambler RP, Brown LH. *Biochem. J.* 1967; 104:784–825. [PubMed: 4860639]
29. Lim J, Vachet RW. *Anal. Chem.* 2003; 75:1164–1172. [PubMed: 12641237]
30. Bridgewater JD, Lim J, Vachet RW. *Anal. Chem.* 2008; 78:2432–2438. [PubMed: 16579630]
31. Arnold AP, Stanley DM, Collins JG. *FEBS Lett.* 1991; 289:96–98. [PubMed: 1654278]
32. Hough MA, Hasnain SS. *J. Mol. Biol.* 1999; 287:579–592. [PubMed: 10092461]
33. Potter SZ, Zhu HN, Shaw BF, Rodriguez JA, Doucette PA, Sohn SH, Durazo A, Faull KF, Gralla EB, Nersissian AM, Valentine JS. *J. Am. Chem. Soc.* 2007; 129:4575–4583. [PubMed: 17381088]
34. Banci L, Bertini I, Cramaro F, Conte RD, Viezzoli MS. *Biochemistry.* 2003; 42:9543–9553. [PubMed: 12911296]
35. Vedani A, Hubta DW, Jacober SP. *J. Am. Chem. Soc.* 1989; 111:4075–4081.
36. Fisher Z, Prada H, Tu CK, Duda D, Yoshioka C, An HQ, Govindasamy L, Silverman DN, McKenna R. *Biochemistry.* 2005; 44:1097–1105. [PubMed: 15667203]
37. Shimahara H, Yoshida T, Shibata Y.; Shimizu M, Kyogoku Y, Sakiyama F, Nakazawa T, Tate S, Ohki S, Kato T, Moriyama H, Kishida K, Tano Y, Ohkubo T, Kobayashi Y. *J. Biol. Chem.* 2007; 282:9646–9656. [PubMed: 17202139]
38. Avvaru BS, Busby SA, Chalmers. M. J.; Griffin PR, Venkatakrishnan B, Agbandje-McKenna M, Silverman DN. *Biochemistry.* 2009; 48:7365–7372. [PubMed: 19583303]
39. Chegwiddden, WR.; Carter, ND.; Edwards, YH. *The Carbonic Anhydrases: New Horizons.* Springer: 2000. p. 241–262.
40. Wearsch PA, Cresswell P. *Curr. Opin. Cell. Biol.* 2008; 20:624–631. [PubMed: 18926908]
41. Mena C, Eser E, Sprague SM. *Kidney Int.* 2008; 73:1275–1281. [PubMed: 18368032]
42. Eakin CM, Attenello FJ, Morgan CJ, Miranker AD. *Biochemistry.* 2004; 43:7808–7815. [PubMed: 15196023]
43. Antwi K, Mahar M, Srikanth R, Olbris MR, Tyson JF, Vachet RW. *Protein Sci.* 2008; 17:748–759. [PubMed: 18305198]

44. Calabrese MF, Miranker AD. *Prion*. 2009; 3:1–4. [PubMed: 19377278]
45. Srikanth R, Mendoza VL, Bridgewater JD, Zhang GS, Vachet RW. *Biochemistry*. 2009; 48:9871–9881. [PubMed: 19754160]
46. Eakin CM, Knight JD, Morgan CJ, Gelfand MA, Miranker AD. *Biochemistry*. 2002; 41:19646–10656.
47. Sousa SF, Lopes AB, Fernandes PA, Ramos MJ. The zinc proteome: a tale of stability and functionality. *Dalton Trans*. 2009:7946–7956. [PubMed: 19771357]

Scheme 1.
Mechanism of the Hydrogen Deuterium Exchange Reaction of the C2-Hydrogen of Histidine Residues

Figure 1.

Selective deuterium incorporation at His residues in Agt I. (a) ESI mass spectrum of the $(M+3H)^{3+}$ ion of Agt I [DRVYIHPFHL] $^{3+}$; (b) Number of deuteriums incorporated into the His residues of Agt I over the course of a 9-day exchange reaction with and without Zn; (c) Number of deuteriums incorporated into selected b- and y-ions of Agt I after CID of samples that had undergone exchange for 3 days with and without Zn.

Figure 2.

Selective deuterium incorporation at His residues in Cu/Zn SOD. (a) ESI mass spectra of the $(M+3H)^{3+}$ ion of the proteolytic peptide GDGPVQGTHFEAK, which contains His19 that does not bind Zn; (b) ESI mass spectra of the $(M+2H)^{2+}$ ion of the proteolytic peptide HVGDLGNVTADK, which contains His78 that does bind Zn. The resulting mass spectra from the 3-day exchange reactions of the apo form are shown in red and the holo form are shown in black. For comparison, the mass spectra from the unexchanged sample are shown in blue.

Figure 3. Selective deuterium incorporation at selected His residues in apo and holo forms of HCA I over the course of 11-day exchange. (a) His243; (b) His94; and (c) His64.

Table 1 HDX Reaction Rate Constants for His Residues in Apo and Holo Forms of the Proteins Studied in This Work

His	Metal binding	Proteolytic peptide fragment	HDX reaction rate constant / d ⁻¹	
			apo (-Zn)	holo (+Zn)
Cu/Zn SOD				
His19		KGDGPVQGT II HF	0.130 ± 0.002	0.127 ± 0.009
		GDGPVQGT II FEAK	0.125 ± 0.006	0.121 ± 0.009
His41		GD II HGFHVHQFGDNTQGCTS AGPHFNPLSKKHGGPKDEE ^a	< 0.01 ^b	< 0.01 ^b
His44	Cu	HV HQ F ^a	0.026 ± 0.006	< 0.01 ^b
His46	Cu	HV HQ F ^a	0.0870 ± 0.0005	< 0.01 ^b
His61	Cu, Zn	GDNTQGCTSAG PH FNPL	0.074 ± 0.009	< 0.01 ^b
His69	Zn	H GGPKDEER	0.14 ± 0.01	< 0.01 ^b
		H GGPK	0.16 ± 0.02	< 0.01 ^b
His78	Zn	H VGDLGNVTADK	0.09 ± 0.01	< 0.01 ^b
His118	Cu	TMVV H EKPDDLGR	0.41 ± 0.03	< 0.01 ^b
HCA I				
His40		H DTSLKPISVSYNPATAK	0.201 ± 0.001	0.19 ± 0.02
		TSET K HDTSLKPISVSY	0.17 ± 0.02	0.18 ± 0.01
		NPATAK		
His64		NPATAKEIINV G HSF	0.0249 ± 0.0001	0.0130 ± 0.0003
His67		HV NF	0.240 ± 0.009	0.214 ± 0.005
His94	Zn	G F H F	0.072 ± 0.002	< 0.01 ^b
His96	Zn	H WGSTNEHGSEHTVDGVKY ^a	0.108 ± 0.002 ^c	0.0345 ± 0.007 ^c
His103		GSTNE H GSEHTVDGVKY ^a	0.18 ± 0.06	0.20 ± 0.05
His107		GSTNE H GSEHTVDGVKY ^a	< 0.01 ^b	< 0.01 ^b
His119	Zn	HV A H W ^a	0.0248 ± 0.0007 ^c	< 0.01 ^{b,c}
His122		HV A H W ^a	< 0.01 ^b	< 0.01 ^b
His200		T HPPLYESVTW	0.30 ± 0.01	0.326 ± 0.001
		TYPGSL T HPPLY	0.29 ± 0.02	0.32 ± 0.02
His243		SLLSNVEGDNAVPMQ H NNR	0.128 ± 0.007	0.13 ± 0.01
		PTQPLK		

^aThe individual reaction rate constants for the His residues in these peptides were determined from MS/MS data (See Figures S1, S2, S3, and S4 in the Supporting Information).

^bThe HDX reaction rate constants for these His residues were too low to determine. We estimate that the lowest rate constant we can determine reliably is 0.01 d⁻¹.

^cThese rate constants were obtained from samples incubated at 45 °C and pH 8.5. See text for details.

Table 2

Number of Deuteriums Incorporated into His Residues of $\beta 2m$ After 1, 2 and 4 Days of Exchange with and without Zn Present

Reaction time	Zn	His13 ^a	His31	His51	His84
1d	□	0.171 ± 0.004	0.104 ± 0.008	0.195 ± 0.007	< 0.05 ^b
	+	0.161 ± 0.009	0.10 ± 0.01	0.14 ± 0.02	< 0.05 ^b
2d	□	0.32 ± 0.02	0.25 ± 0.01	0.33 ± 0.01	< 0.05 ^b
	+	0.31 ± 0.01	0.241 ± 0.001	0.24 ± 0.01	< 0.05 ^b
4d	□	0.568 ± 0.005	0.46 ± 0.02	0.52 ± 0.02	0.051 ± 0.001
	+	0.53 ± 0.03	0.41 ± 0.01	0.44 ± 0.03	0.054 ± 0.006

^a Number of deuteriums incorporated into His13 is the average from two different proteolytic peptides.

^b Number of deuteriums incorporated into His84 at 1 d and 2 d were too low to be confidently measured.