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Slow Histidine H/D Exchange Protocol for Thermodynamic Analysis of Protein Folding and Stability using Mass Spectrometry

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Abstract

Described here is a mass spectrometry based protocol to study the thermodynamic stability of proteins and protein-ligand complexes using the slow H/D exchange reaction of the imidazole C_2 proton in histidine side chains. The protocol, which involves evaluating the denaturant dependence of this slow H/D exchange reaction in proteins, allows the global and/or subglobal unfolding/refolding properties of proteins and protein-ligand complexes to be probed. The protocol is developed using several model protein systems including: ribonuclease (Rnase) A, myoglobin, bovine carbonic anhydrase (BCA) II, hemoglobin, and the hemoglobin-haptoglobin protein complex. The compatibility of the protocol with conventional mass spectrometry-based proteomic sample preparation and analysis methods is also evaluated in an experiment in which the protocol is applied to proteins in a yeast cell lysate and used to detect the binding of Zn to superoxide dismutase in the yeast cell lysate sample. The yeast cell sample analyses also helped define the scope of the technique, which requires the presence of globally protected histidine residues in a protein's three-dimensional structure for successful application.

INTRODUCTION

Thermodynamic measurements of protein folding and ligand binding are important for understanding biological processes from basic cellular functions to drug mode-of-action. Experimental approaches for making thermodynamic measurements on proteins and protein-ligand complexes in solution have traditionally relied on the use of calorimetry- or optical spectroscopy-based methods. More recently, a number of mass spectrometry-based methods have been developed to study the thermodynamic properties of proteins and protein-ligand complexes in solution. For example, the so-called native mass spectrometry experiment, in which a protein-ligand complex of interest is introduced into the mass spectrometer under native solution phase conditions, has been useful for evaluating the solution-phase K_d values of protein-ligand complexes.[1–4] Chemical labeling techniques, such as amide H/D exchange,[56–8] methionine oxidation,[9] and lysine amidination[10] have also been used in

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ASSOCIATED CONTENT

Supporting Information. Supplemental Text, two additional tables, and four additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

combination with mass spectrometry to characterize the thermodynamic properties of protein folding and ligand binding interactions.

Mass spectrometry-based methods for the thermodynamic analysis of protein folding and ligand binding interactions are attractive because they require small amounts of protein for analysis, are often general with respect to the protein and the ligand class, and can be used to characterize protein-ligand complexes with a wide range of binding affinities. Some mass spectrometry-based methods have also proven useful for the analysis of unpurified proteins in complex biological mixtures such as cell lysates.[5,10,11–13] The experimental capability to characterize unpurified proteins is not only experimentally convenient because time consuming purification steps can be eliminated from the sample preparation, but it is also fundamentally important because the ability of proteins to fold and interact with ligands can be impacted by the presence of other proteins.

Described here is an H/D exchange- and mass spectrometry-based protocol for making thermodynamic measurements of protein folding and ligand binding that exploits the slow H/D exchange reaction of the C_2 proton in the imidazole ring of histidine residues. The protocol is similar to that used in the SUPREX (stability of unpurified proteins from rates of H/D exchange) technique,[5] which exploits the amide H/D exchange reaction in proteins. In both SUPREX and the histidine H/D exchange protocol described here, the denaturant dependence of the H/D exchange reaction is probed using a mass spectral readout in order to evaluate the more global thermodynamic parameters associated with the more global unfolding/refolding reactions in proteins and protein-ligand complexes.

The half-life of the H/D exchange reaction of an unprotected histidine residue is on the order of ~2 days,[14] which is considerably longer (~400,000 times longer) than that of the H/D exchange reaction of an unprotected amide proton.[15] This means that much longer H/D exchange times are required in the histidine H/D exchange protocol than in SUPREX, typically days compared to hours. It also means that the extent of back-exchange during the mass spectral sample preparation and analysis is relatively small, even when conventional proteomic sample preparation and analysis methods are used. Thus, unlike SUPREX, the histidine H/D exchange protocol developed here can be interfaced with standard mass spectrometry-based proteomics platforms.

As part of this work the histidine H/D exchange protocol is developed and applied to a series of model protein systems including: ribonuclease (Rnase) A, myoglobin, bovine carbonic anhydrase (BCA) II, hemoglobin (Hb), and the hemoglobin-haptoglobin (Hb-Hp) complex. The compatibility of the protocol with conventional mass spectrometry-based proteomic sample preparation and analysis methods is evaluated in an experiment in which the protocol is applied to the proteins in a yeast cell lysate sample both in the absence and in the presence of added Zn^{2+} in order to test the ability of the protocol to detect the binding of Zn^{2+} to unpurified superoxide dismutase. The results obtained on proteins in the yeast cell lysate samples also help define the scope of the technique, which relies on the presence of at least one globally protected histidine residue in a protein's three-dimensional structure for successful analyses.

EXPERIMENTAL

Materials

The following materials were purchased from Sigma-Aldrich (St. Louis, MO): Rnase A from bovine pancreas (≥ 60 wt %), myoglobin from equine skeletal muscle (≥ 95 wt %), BCAII from bovine erythrocytes (≥ 80 wt %), trypsin from porcine pancreas (proteomic grade), deuterium oxide (D_2O; 99.9 atom % D), sodium deuteroxide (35 wt % in D_2O, 99.9

atom % D), deuterium chloride (20 wt % in D_2O, 99.9 atom % D), 2-(N-morpholino)ethanesulfonic acid (MES), Tris(2-carboxyethyl)phosphine hydrochloride (TCEP•HCl), and *S*-methyl methanethiosulfonate (MMTS). Guanidine hydrochloride (GdmCl) and acetone were purchased from EMD Chemicals, Inc. (Gibbstown, NJ). Deuterated GdmCl was prepared by dissolving the GdmCl purchased from EMD Chemicals, Inc. in D_2O and lyophilizing the sample to dryness. Four dissolution/lyophilization cycles were used to maximize the deuterium content of the GdmCl. Immobilized porcine trypsin and immobilized bovine chymotrypsin (sequencing grade) were purchased from Princeton Separation (Adelphia, NJ). Dithiothreitol (DTT), iodoacetamide (IDA) and acetonitrile (ACN) were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid (FA) was purchased from Thermo Scientific (Rockford, IL) and the 10k filter units were purchased from Milipore (Billerica, MA).

Stroma free hemoglobin (SFH) was obtained by precipitation by ammonium sulfate followed by a step where the endogenous phosphates are removed and then the protein is purified by FPLC following a previously published method.[16] Hp, predominantly of the 1-1 phenotype, was purchased from Sigma-Chemical (St. Louis, MO) and was 99% pure. For complex formation, Hb was mixed with Hp in a 1:1 ratio (protein concentration). The complex was further purified using a BioSep 3000 column from Phenomenex (Torrence, CA) using 50 mM potassium phosphate buffer (pH 7.4). Spectroelectrochemistry and MALDI-MS were used to verify the Hb-Hp complex formation as described previously.[17]

Thermodynamic Analysis of Model Protein Systems

Histidine H/D exchange analyses on Rnase A were initiated on a 1 mM stock solution of fully protonated protein prepared in H_2O. A 10 μL aliquot of the stock solution was diluted into 90 μL of deuterated 20 mM sodium phosphate buffer (pD 7.4) containing 20 mM sodium acetate, 100 mM NaCl, and the following GdmCl concentrations: 0.0, 0.6, 1.2, 1.7, 2.2, 2.5, 3.0, 3.2, 4.3, and 5.3 M. The samples were incubated for 5 days at 37 °C. The H/D exchange reactions in each buffer were quenched by addition of 10 μL of FA before addition of 1 mL of acetone to precipitate protein in each denaturant-containing buffer. The protein precipitation step was allowed to procceed overnight at −20 °C before the samples were centrifuged at 20,000 × g for 30 min to pellet the insoluble protein. The resulting protein pellets were dissolved in 50 μL of 0.1 M ammonium bicarbonate (AmBic) buffer (pH 8) containing 0.1% SDS. The samples were reduced with 10 mM DTT at 80 °C for 15 min, and alkylated with 20 mM IDA in the dark for 30 min. The protein was digested with a combination of 0.5 μg immobilized trypsin and 0.5 μg immobilized chymotrypsin for 1 hr each at 37 °C and gentle shaking. The samples were centrifuged and the immobilized proteases were separated from the supernatant, which was adjusted to pH 2–3 with 10% TFA. The SDS in each supernatant was removed using SDS removal tips (PolyLC, Columbia, MD) according to the manufacturer's protocol. The resulting samples were subjected to LC-MS/MS analyses as described in the Supporting Information.

Histidine H/D exchange analyses on myoglobin, BCAII, Hb, and the Hb-Hp complex were initiated on 0.5 to 1 mM stock solutions of these proteins that were prepared in H_2O. In each case a 10 μL aliquot of stock solution was diluted into 90 μL of deuterated 50 mM MES buffer (pD 7.4) containing 50 mM NaCl and the following GdmCl concentrations: 0, 0.7, 1.2, 1.8, 2.2, 2.4, 2.7, 2.9, 3.2, 3.6 and 4.2 M (for myoglobin and BCAII), and 0, 1.4, 1.8, 1.9, 2.2, 2.4, 2.9, 3.4, 4.0, 4.5, 5.3 M (for Hb and Hb-Hp). The samples were incubated at 37 °C for either 5 or 11 days. After the specified H/D exchange time, a 50 μL aliquot of each H/D exchange reaction was removed and diluted into 350 μL H_2O. Each sample was added to a 10 k filter unit and centrifuged for 15 min at 14,000 × g. Two 200 μL aliquots of 1 mM Tris buffer (pH 8.5) containing 8 M Urea (referred to as UA solution) were sequentially added to each filter unit and then centrifuged for 15 min each at 14,000 × g. Aliquots of 100

μL of UA containing 20 mM TCEP•HCl or 40 mM MMTS were subsequently added to reduce and alkylate the protein samples in the filter units for 1 hr at RT. Three 100 μL aliquots of UA solution were sequentially added to the filter units and centrifuged for 15 min each at 14,000 × g. The protein in each filter unit was reconstituted in 120 μL of 50 mM AmBic buffer (pH 8.0) before the sample were digested in the filter unit with ~ 2 μg of trypsin for 12–15 hrs and with 1 μg of chymotrypsin for 1 hr at room temperature. The resulting samples were subjected to LC-MS/MS analyses as described in the Supporting Information.

Thermodynamic Analyses of Proteins in a Yeast Cell Lysate

The yeast cell lysates, which typically contained ~10 mg/ml of total protein, were prepared as previously described using a cyclophilin A (YDR155C) overexpression strain that was purchased from Open Biosystems, Huntsville, Al, USA.[13] More detailed information about the yeast cell lysate preparation is provided in the Supporting Information. In the first yeast cell lysate experiment, 10 μl of the yeast cell lysate, which contained about 100 μg of total protein, was diluted into 90 μL of deuterated 20 mM phosphate buffers (pD 7.4), containing the following GdmCl concentrations: 0.5, 0.8, 1.1, 1.6, 2.0, 2.9, 2.5, 3.6, 4.0, or 4.5 M. The protein samples in each buffer were subject to the same histidine H/D exchange analysis as described above for Rnase A except that protease digestion step was allowed to proceed for 6 hr and it only involved one protease, trypsin, with ratio of 1:20 enzyme to substrate ratio.

In the second yeast cell lysate experiment, the yeast cell lysate was divided into two 135 μl fractions, with each fraction containing ~ 1350 μg of total protein. A 15 μL aliquot of a 1 mM $ZnCl_2$ solution was added to one fraction and 15 μl of H_2O was added to the second fraction. Both fractions were incubated at room temperature for 15 min, before 10 μL aliquots of each fraction were diluted into 90 μL of deuterated MES buffers (pD 7.4) containing 50 mM NaCl and the following GdmCl concentrations: 0.0, 1.2, 1.4, 1.9, 2.2, 2.4, 2.7, 2.9, 3.2, 4.2, and 5.3 M. The protein samples in each buffer were subject to the same histidine H/D exchange analysis as described above for Rnase A except that after the acetone precipitation, the protein pellets were dissolved in freshly prepared 50 mM Ambic buffer (pH 8.0) containing 6 M urea before they were reduced with 10 mM DTT at 80 °C for 15 min, alkyated with 20 mM IDA for 30 min in the dark, diluted 5-fold with 50 mM Ambic buffer (pH 8.0), and digested with 1:20 trypsin for ~ 6 hrs. The protease digested samples were analyzed by LC-MS/MS as described in the Supporting Information.

Histidine HDX Data Analysis

The deuterium content of histidine-containing peptides was determined by calculating the weighted average mass of the five most abundant isotopologues observed for each peptide in the mass spectra obtained in the LC-MS/MS analyses. This weighted average mass value was used to calculate a $\Delta Mass_{wt,av}$ value of a given histidine-containing peptide at each denaturant concentration by subtracting the theoretical weighted averaged mass value expected for the fully protonated peptide, which was also calculated using the five most abundant isotopologues expected for each peptide. The $\Delta Mass_{wt,av}$ value was then plotted as a function of the denaturant concentration. The data in the ΔMasses versus [GdmCl] plots were fit to a standard sigmoidal equation, equation (1), using Sigma Plot in order to evaluate the concentration of denaturant at the transition midpoint of the resulting sigmoidal curve (i.e., the $C_{1/2}$ value).

$$y = y_o + \frac{a}{1 + e^{-\left(\frac{x - x_o}{b}\right)}} \quad (1)$$

In equation (1), x was the [GdmCl], y was the ΔMass value and x_o was the $C_{1/2}$ value. Ultimately, the $C_{1/2}$ value was used to calculate a folding free energy according to equation (2).

$$\Delta G_f = -mC^{1/2}_{SUPREX} - RT\left[\ln\left(\frac{\frac{k_\phi t}{0.693} - 1}{\frac{n^n}{2^n}[P]^{n-1}}\right)\right] \quad (2)$$

In equation (2), which was previously been reported for the analysis of SUPREX data,[8] ΔG_f is the folding free energy of the protein, k_ϕ is first order rate constant of the slow H/D exchange reaction at the C_2 position on an unprotected histidine imidazole side chain, m is $\delta\Delta G_f/\delta[GDmCl]$, T is the temperature, R is the ideal gas constant, t is the H/D exchange time, and [P] is the protein concentration expressed in n-mer equivalents. In all the ΔG_f value calculations described here using equation (2), T was 310 K, k_ϕ was set at a value of 0.288 day^{-1} (based on the data in reference [14]), and n was 1 with exception of the Hb analyses in which n was 2 (as the Hb tetramer has been shown to dissociate into to α/β dimers in other GdmCl-induced equilibrium unfolding experiments[18]). In the Rnase A and myoglobin analyses, previously determined m-values of 3.1[19] and 3.71[20] kcal/(mol M) (respectively) where used in equation (2) for the ΔG_f calculations. In the transition midpoint analysis method used to analyze the Hb and Hb-Hp data, the $C_{1/2}$ values obtained at the different H/D exchange times were fit to equation (2) using a linear least squares analysis in which the y-intercept and slope of the best-fit line were taken as the ΔG_f value and m-value, respectively.

K_d Value Determination

The K_d value of the Hb-Hp complex was calculated using equation (3):

$$K_d = \frac{4L_{total}e^{-\frac{\Delta\Delta G_f}{NRT}} - 4P_{total}(e^{-\frac{\Delta\Delta G_f}{NRT}} - 1)}{\left(2e^{-\frac{\Delta\Delta G_f}{NRT}} - 1\right)^2 - 1} \quad (3)$$

In equation (3), the derivation of which has been previously described,[9] L_{total} is the concentration of ligand and P_{total} is the concentration of protein, N is the number of independent binding sites, R is the gas constant, T is the temperature in Kelvin, and $\Delta\Delta G_f$ is the binding free energy. The binding free energy was calculated from the ΔG_f values obtained for Hb in the absence and in the presence of Hp.

RESULTS AND DISCUSSION

Histidine HDX Protocol

The protocol developed here (Figure 1) involves dilution of a protein sample into a series of deuterated buffers containing increasing concentrations of a chemical denaturant (e.g., GdmCl). The protein samples in each deuterated buffer are allowed to undergo H/D exchange at 37 °C and pD 7.4 for the same amount of time. The H/D exchange time (t in equation (2)) should be at least 5 days, which is equivalent to ~2.5 half-lives of the H/D exchange reaction of a C_2 proton in the imidazole side-chain of an unprotected histidine residue[14] (a detailed discussion about H/D exchange time selection is included in the Supporting Information). In the H/D exchange reactions all the labile hydrogens in a protein are subject to exchange including the relatively slow exchanging C_2 protons in the imidazole side chain of histidine residues, the fast exchanging amide hydrogens, and the even faster exchanging side-chain hydrogen atoms bonded to nitrogen, oxygen and sulfur. Ultimately,

the H/D exchange reactions are quenched by acidifying the solution and lowering temperature. The protein samples in the denaturant containing-buffers are each subjected to a desalting step in which the denaturant and the D_2O are removed using either spin columns, acetone precipitation, or TCA precipitation.

After the desalting step the protein samples are reduced, alkylated, and digested with a proteolytic enzyme (e.g., trypsin) according to standard for mass spectrometry-based proteomic protocols. During these sample handling steps, the deuterons incorporated into the peptide backbone and the amino acid side chains are nearly all exchanged back to protons, whereas the large majority of deuterons that were exchanged into the C_2 position of the imidazole side chain of histidine residues are not back-exchanged to protons because of the longer half-life required for back-exchange. The resulting peptides are subjected to a mass spectrometric analysis in which the peptides are sequenced to identify histidine-containing peptides and the mass spectral data is used to determine a $\Delta Mass_{wt,av}$ value for each histidine-containing peptide at each denaturant concentration in which the H/D exchange reaction was performed on the intact protein. Ultimately, the $\Delta Mass_{wt,av}$ values obtained for a given histidine-containing peptide are plotted as a function of the denaturant concentration, and the data are used to calculate a protein folding free energy as described in the **Experimental** section.

Analysis of Two-State Folding Systems

Rnase A, which contains four histidine residues (His-12, His-48, His-105, and His-119), was initially analyzed using the above protocol. Based on the results of earlier histidine H/D exchange studies of Rnase A in which the time course of histidine H/D exchange was studied,[14] His-105 and His-119 in Rnase A are solvent exposed, His-12 is partially protected, and His-48 is buried in the hydrophobic core of Rnase A's native three-dimensional structure. The slow histidine H/D exchange data obtained here for one of the detected His-48 containing peptides is shown in Figure 2. As expected for a globally protected histidine residue, there was a clear denaturant dependence to the H/D exchange behavior of His-48 (Figure 2A). A His-12 containing peptide showed a similar denaturant dependence to its H/D exchange behavior but the curve had smaller amplitude (see Figure SI-1 in the Supporting Information). Visual inspection of the isotopologue distributions obtained here for two other Rnase A peptides, Rnase A(105–115) and Rnase A(106–120) covering His-105 and His-119, indicated that these histidine residues were each ~50% deuterated and that the extent of deuteration was unchanged with denaturant concentration. Such H/D exchange behavior is expected for peptides containing these histidine residues, which were from solvent exposed regions of protein structure (see discussion of expected deuteration levels in Supporting Information).

In the histidine H/D exchange analysis of myoglobin, peptides covering 9 of the 11 histidine residues in myoglobin's primary amino acid sequence were identified in the mass spectral readout. These 9 histidine residues included 2 that are globally protected (His-24 and His-64), 5 that are partially protected (His-36, His-81, His-82, His-113, His-116), and 2 that are exposed (His-48 and His-119), based on an analysis of myoglobin's three-dimensional structure.[21] The data obtained on peptides containing the two protected histidine residues, His-24 and His-64, showed a clear denaturant dependence to their H/D exchange behavior and yielded $C_{1/2}$ values that were 1.7 M and 1.6 M [GdmCl], respectively (see Figure SI-2A and SI-2B in the Supporting Information). The data obtained on peptides containing a partially protected histidine residue could also be fit to a sigmoidal curve, however, the amplitude of the curve was small (Figure SI-2C in Supporting Information). Peptides containing an exposed histidine residue (e.g., His-119) were essentially all exchanged at each denaturant concentration (see e.g., Figure SI-2D in Supporting Information).

The histidine H/D exchange protocol described here utilizes a peptide readout. However, the $C_{1/2}$ values generated for the histidine-containing peptides detected in the mass spectral readout are representative of the protein folding unit from which they come. In the case of a two-state folding globular protein such as Rnase A and myoglobin, each protein molecule is considered to be a single folding unit,[20] and therefore the $C_{1/2}$ values derived from different globally-protected histidine-containing peptides in these proteins are expected to be similar and to yield folding free energies that are comparable to those determined for the intact protein using other techniques. Indeed, the $C_{1/2}$ value measured here for the RnaseA(47–62) peptide can be used in equation (2) to calculate a ΔG_f value of −6.9 kcalmol^{-1} for Rnase A, which is within 30% of that previously determined[19] for this protein (see Table 1). The similar $C_{1/2}$ values obtained from the two different globally protected histidine-containing peptides in the myoglobin analysis (see Table 1) are consistent with a two state folding mechanism. The ΔG_f values calculated using equation (2) and these $C_{1/2}$ values are also similar and within 20% of that previously determined[20] for myoglobin (see Table 1).

Analysis of a Non-Two-State Folding System

BCAII, which contains a total of 11 histidine residues in its primary amino acid sequence and is a known non-two-state folding protein,[22] was also analyzed using the protocol described above. Peptides containing 9 of the 11 histidine residue in BCAII were detected in the LC-MS/MS readout, and 5 of these 9 histidine residues (i.e., His-93, His-95, His-106, His-118 and His-121) were found to be from globally protected regions in BCAII's native three-dimensional structure, based on the histidine H/D exchange behavior of peptides containing these residues (Figure 3). Histidine-containing peptides containing these 5 histidine residues yielded sigmoidal curves with two clear transitions (Figure 3), with $C_{1/2}$ values of 1.4 M and 3 M. These results are consistent with the presence of a folding intermediate that is stabilized in ~2 M GdmCl. The presence of such an intermediate has been suggested in other chemical-denaturant induced equilibrium unfolding/refolding studies using intrinsic fluorescence spectroscopy.[22, 23]

Analysis of a Protein-Protein Interaction

An important application of the histidine H/D exchange protocol developed here is the detection and quantitation of protein-ligand binding interactions. In order to test the ability of the protocol to detect and quantify a protein-ligand binding interaction, the known protein-protein interaction between Hb and Hp was analyzed.[16] A Hb sample and a sample of the Hb-Hp complex were each subject to the histidine H/D exchange protocol described here using H/D exchange times of both 5 and 11 days. In these analyses a total of 9 hemoglobin peptides containing 6 of the 10 histidine residues in the α chain of hemoglobin and 5 of the 9 histidine residues in the β chain of hemoglobin were identified in the LC-MS/MS readout (see Table SI-1 in the Supporting Information).

The $\Delta Mass_{wt,av}$ values generated for 7 of the 9 histidine-containing peptides in Hb were all similar (i.e., ~0.6 Da) and did not change with denaturant concentration (data not shown) in either the Hb or Hb-Hp analyses, suggesting that the histidine residues in these seven peptides (see Table SI-1 in Supporting Information) were solvent exposed in hemoglobin's three-dimensional structure. The $\Delta Mass_{wt,av}$ values recorded for 2 of the 9 histidine-containing peptides detected, Hb(116–126) of sequence TPAVHASLDKF from the α chain and Hb(83–95) of sequence GTFATLSELHCDK from the β chain, showed a denaturant dependence (see Figure 4) and yielded similar $C_{1/2}$ values (see Table 1) at similar H/D exchange times. These results suggest that they belong to the same folding unit, even though they were derived from different subunits in the hemoglobin complex. These data are consistent with hemoglobin's three-dimensional structure, which has the histidine residues in these peptides buried in the heme pocket.[24]

Hb is a tetramer in solution composed of two α chains and two β chains with different amino acid sequences but similar 3-D structures.[24] The GdmCl-induced equilibrium unfolding reaction is known to be biphasic with the Hb tetramer dissociating to two dimers, each containing an α and β chain, before each of the α/β dimers unfold at high denaturant concentration (> 5 M).[18] The $C_{1/2}$ values recorded for the two globally protected histidine-containing peptides in our H/D exchange experiments on Hb are consistent with that expected for the tetramer-dimer transition reported earlier.[18] It is also noteworthy that there is a significant difference (i.e., ~ 0.4–0.6 M) between the $C_{1/2}$ values obtained for the Hb-Hp complex at 5 and 11 days. A similar, but smaller shift of ~0.3 M was observed for Hb at 5 and 11 days for the Hb-Hp complex. As we have previously described for the analysis of SUPREX data, such $C_{1/2}$ value shifts as a function of H/D exchange time can be used to evaluate ΔG_f and *m*-values using the transition midpoint analysis method.[8] The ΔG_f and *m*-values derived for the Hb peptides in the presence and absence of Hp using the transition midpoint analysis method are summarized in Table 1.

The $C_{1/2}$ values obtained for Hb in the presence and absence of Hp, indicate that the Hb α/β dimer is stabilized in the Hb-Hp complex. If the ΔG_f values obtained for the two peptides in the Hb analysis are averaged and the ΔG_f values obtained for the two peptides in the Hb-Hp analysis are averaged, then the resulting average ΔG_f values, −8.2 and −11.5 kcal/mol (respectively), can be used to quantify this increased stability (i.e., calculate an average binding free energy of −3.3 kcal/mol). This binding free energy (i.e., $\Delta\Delta G_f$ value) can be used in equation (3) to generate a K_d value of 0.24 nM for the Hb-Hp complex. This K_d value is approximately 10-fold lower than that previously reported using surface plasmon resonance spectroscopy (SPR).[17] The weaker binding affinity measured in the SPR experiment may be a result of the protein immobilization that was necessary in the SPR experiment. It is also possible that the difference may be a result of inaccuracies in our *m*-value assignments, which were obtained by a linear extrapolation involving only 2 data points. Unfortunately, the use of more data points in the linear extrapolation would require the use of impractically long H/D exchange times (e.g., an estimated 3–6 weeks would be required to shift the $C_{1/2}$ values 0.5 M lower than the 11 day $C_{1/2}$ values recorded here) (see Supporting Information).

Analysis of Proteins in a Yeast Cell Lysate

In order to investigate the scope of the histidine H/D exchange protocol described here, the protocol was applied to the analysis of the proteins in a yeast cell lysate. In this analysis a total of 780 unique peptides from 250 different proteins were identified in the LC-MS/MS analysis, and 93 of the 780 peptides were histidine-containing peptides. It was possible to extract $\Delta Mass_{wt,av}$ values for 50 of these histidine-containing peptides. In the case of the other 43 histidine-containing peptides it was difficult to extract meaningful ΔMass values as the ion signals for the isotopologues from these peptides were relatively low and/or not well-resolved from other peptides in the mass spectral analyses.

Out of the 50 histidine-containing peptides that were successfully analyzed, 10 histidine-containing peptides from 6 different proteins had $\Delta Mass_{wt,av}$ values with a denaturant dependence. The $C_{1/2}$ values of the peptides ranged from 0.5 to 1.5 M (see Table SI-2). The results obtained on the four histidine-containing peptides from 3-phospho-glycerate kinase (3PGK) are noteworthy. The three histidine-containing peptides that all came from the N-terminal domain of the protein all had a $C_{1/2}$ value of 1.5 M (see Table SI-2); and the one histidine-containing peptide that cames from the C-terminal domain of the protein had a $C_{1/2}$ value of 0.5 M (see Table SI-2). This is in good agreement with the results of previous protein folding studies on 3PGK that revealed this protein has two different functional domains that fold independently from each other. [25]

Superoxide Dismutase 1 (SOD-1) was one of the proteins that yielded a histidine-containing peptide with denaturant dependent $\Delta Mass_{wt,av}$ values in the yeast cell lysate analysis (see Table SI-2). SOD-1 is a Cu-Zn binding protein. Cu^{2+} is known to strongly affect the stability of protein in vivo but much less is known about the importance of Zn^{2+} on protein stability in vivo.[26] It is noteworthy that the ΔMass versus [GdmCl] plots generated here for SOD-1 yielded a $C_{1/2}$ value similar to that previously reported for the apo form of the protein using a spectroscopic readout.[27] As part of this work we investigated the impact of increasing the Zn^{2+} concentration on the stability of this protein in the context of the whole cell lysate. The results show a $C_{1/2}$ value shift of approximately 0.4 M GdmCl (Figure 5) indicating the SOD-1 was stabilized in the presence of the Zn metal ion in this "*ex vivo*" experiment. In this case the calculation of a K_d value is not possible because a slow histidine H/D analysis of the apo form was not obtained (i.e., the endogenous levels of zinc that were present in the initial cell lysate sample was not known). Nonetheless, our results on SOD can be used to qualitatively identify SOD-1 as a Zn-binding protein.

CONCLUSION

The slow histidine H/D exchange protocol outlined here is complementary to other chemical modification and mass spectrometry-based protocols that have been recently described for use in LC-MS/MS based bottom-up proteomics platforms[10, 13] as it provides a new amino acid probe for characterizing the global and subglobal unfolding reactions of proteins and protein-ligand complexes in these experiments. While the results of our cell lysate analysis suggest that a large fraction of the histidine residues in proteins are solvent exposed and not useful for the described protocol, it is noteworthy that many metallo-binding proteins[28] (such as the myglobin, BCA II Hb, and SOD-1 proteins analyzed in this work) do indeed have buried histidine residues that are useful for the described protocol. Thus, the described methodology is likely to be most broadly useful for the analysis of ligand binding interactions involving metallo-proteins and enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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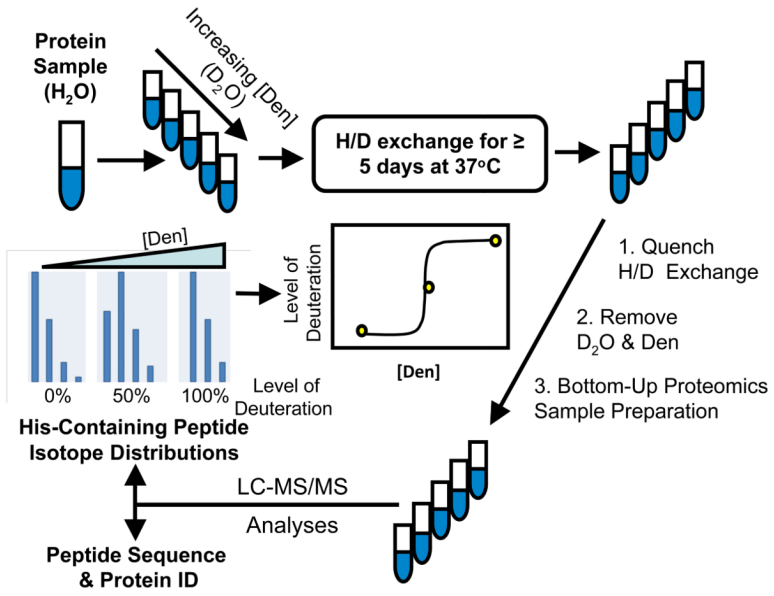

Figure 1.
Schematic representation of the slow histidine H/D exchange protocol developed here.

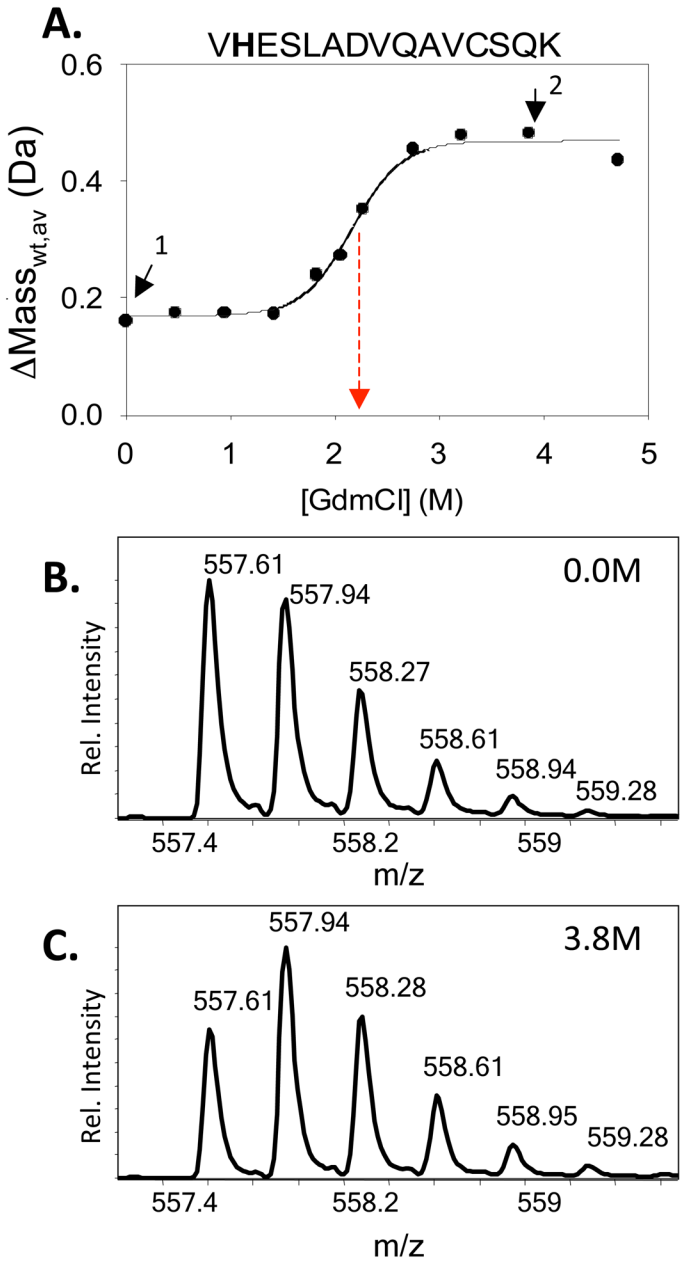

Figure 2.

Slow histidine H/D exchange data for Rnase A. Data obtained for a His-48-containing peptide of sequence, VHESLADVQAVCSQK, is shown in (A). The solid line represents the best fit of the data to equation (1), the dotted arrow indicates $C_{1/2}$ value, the arrows labeled "1" and "2" indicate the data points for which the raw mass spectral data is shown in (B) and (C), respectively.

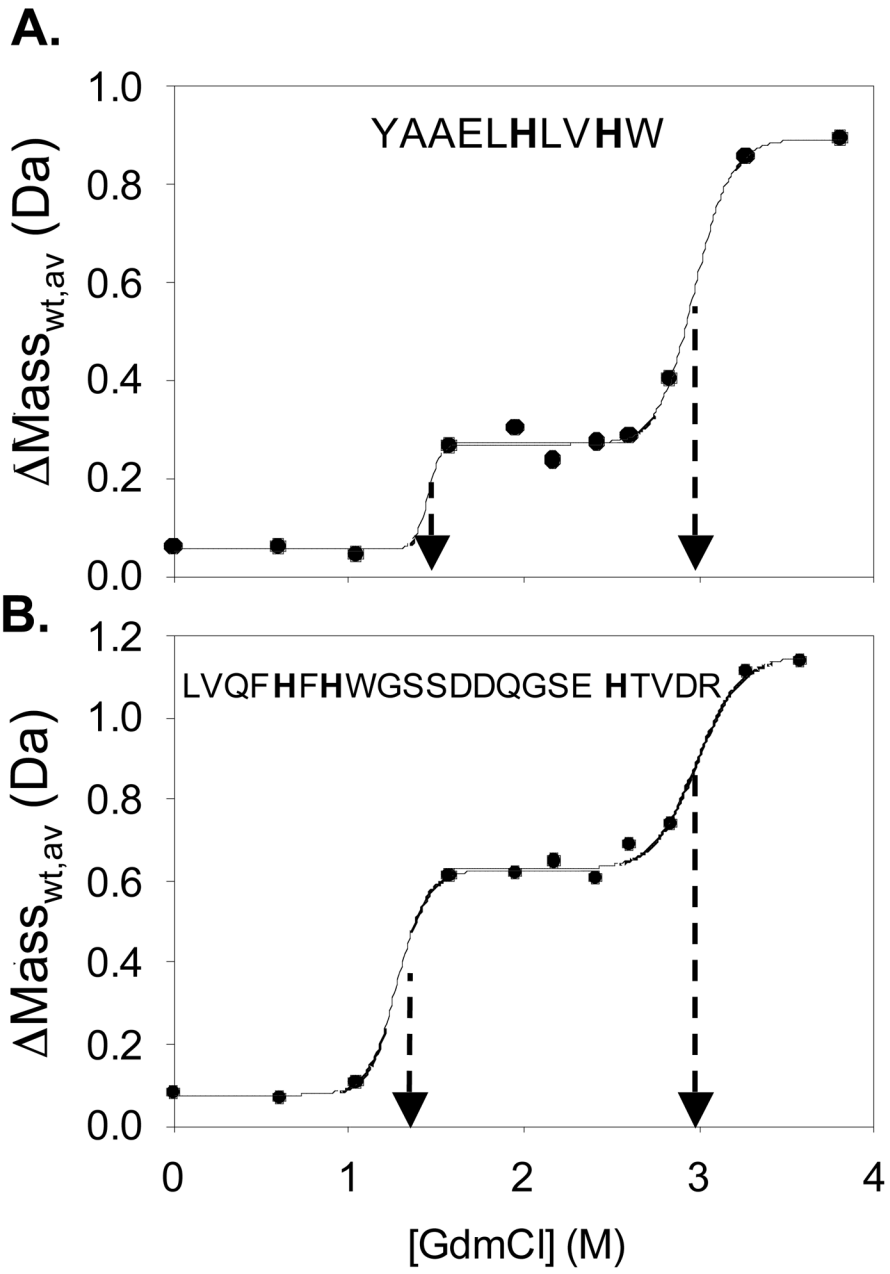

Figure 3.

Slow histidine H/D exchange data for BCA II. Data obtained on a peptide containing histidine residues, His-118 and His-121, is shown in (A) and data obtained for a peptide containing histidine residues, His-93, His-95 and His-96 is shown in (B). The dotted arrows indicate $C_{1/2}$ values. The solid lines represent the best fit of the data to equation (1), with the data in each transition being fit separately.

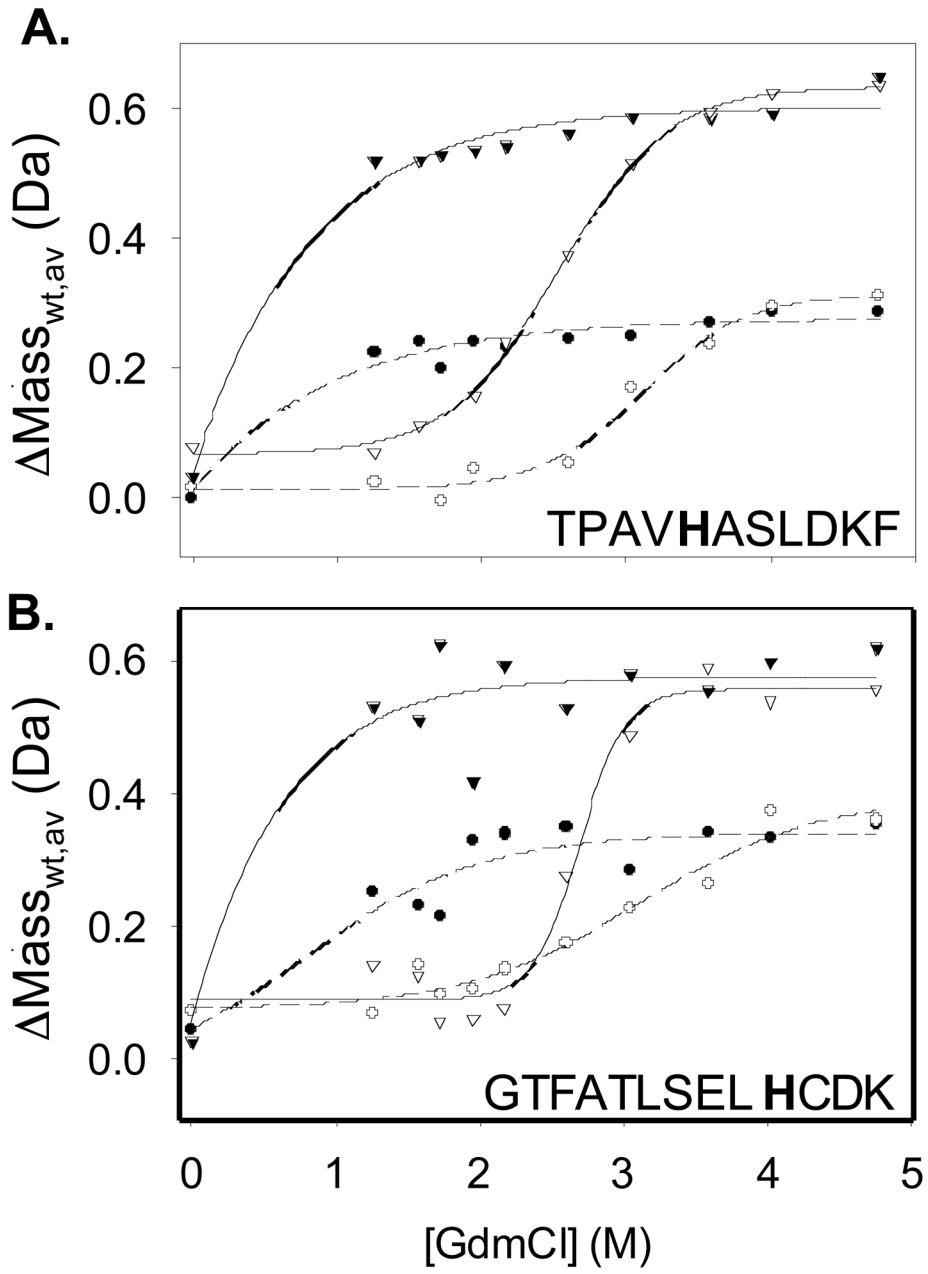

Figure 4.

Slow histidine H/D exchange data for Hb and the Hb-Hp complex. Data obtained on His-120 containing peptide from the α chain of Hb after 5 days (○ and ●) and 11 days (▽ and ▼) in the presence (○ and ▽) and absence (● and ▼) of Hp is shown in (A). Similar data obtained on a His-92 containing peptide from the β chain of Hb in the presence and absence of Hp is shown in (B). The lines represent best fit of each data set to equation (1).

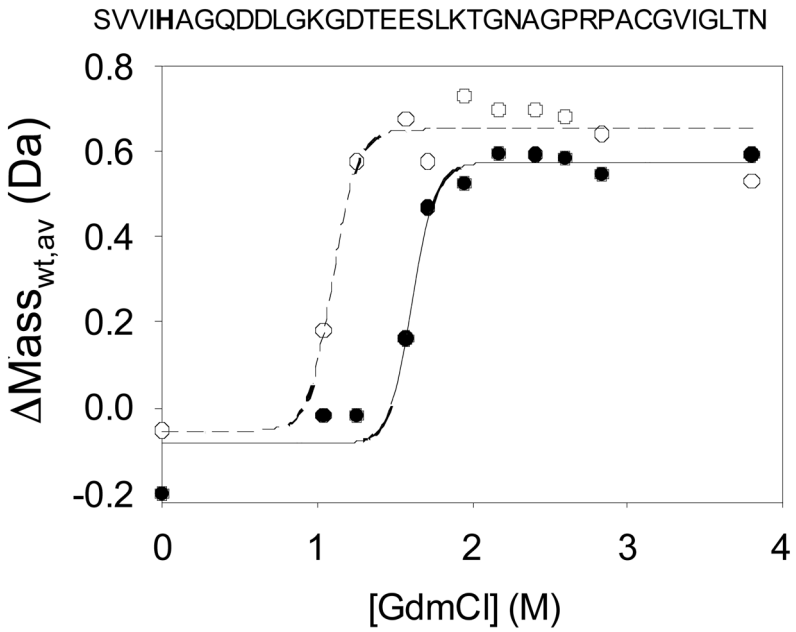

Figure 5.
Representative Histidine H/D exchange data obtained from a His-120 containing peptide of superoxide dismutase 1 (SOD-1) in the presence (● and solid line) and absence (○ and dotted line) of added Zn^{2+}.

Table 1

Thermodynamic Parameters obtained on model proteins. Values in parenthesis were previously determined by others using more conventional experimental approaches.

Protein Peptide	$C_{1/2}$ (M)	m (kcal $mol^{-1}M^{-1}$)	ΔG_f (kcal mol^{-1})
Rnase A			
VHESLADVQAVCSQK[a]	2.2 (2.99[c])	(3.1[c])	−6.9 (−9.2[c])
Myoglobin			
HGTVVLTALGGILK[a]	1.7 (1.63[d])	(3.71[d])	−6.4 (−6.0[d])
HGTVVLTALGGILK[b]	1.6 (1.63[d])	(3.71[d])	−6.7 (−6.0[d])
VEADIAGHGQEVL[a]	1.7 (1.63[d])	(3.71[d])	−6.4 (−6.0[d])
VEADIAGHGQEVL[b]	1.7 (1.63[d])	(3.71[d])	−7.1 (−6.0[d])
Hb			
TPAVHASLDKF[a]	0.7 (0.7[e])	2.43	−8.2 (−8.3[f])
TPAVHASLDKF[b]	0.4 (0.7[e])	2.43	−8.2 (−8.3[f])
GTFATLSELHCDK[a]	0.7 (0.7[e])	2.43	−8.2 (−8.3[f])
GTFATLSELHCDK[b]	0.4 (0.7[e])	2.43	−8.2 (−8.3[f])
Hb - Hp			
TPAVHASLDKF[a]	3.1 (NA)	1.28	−10.5 (NA)
TPAVHASLDKF[b]	2.5 (NA)	1.28	−10.5 (NA)
GTFATLSELHCDK[a]	3.1 (NA)	1.78	−12.0 (NA)
GTFATLSELHCDK[b]	2.7 (NA)	1.78	−12.0 (NA)

[a] 5 days exchange.

[b] 11 days exchange.

[c] Value from reference 19.

[d] Value from reference 20.

[e] Values from reference 18.

[f] Value from reference 29.

"NA" indicates value not available.