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ARTICLES

Rapid Analysis of Protein Structure and Dynamics by Hydrogen/Deuterium Exchange Mass Spectrometry

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An automated approach for the rapid analysis of protein structure has been developed and used to study acid-induced conformational changes in human growth hormone. The labeling approach involves hydrogen/deuterium exchange (H/D-Ex) of protein backbone amide hydrogens with rapid and sensitive detection by mass spectrometry (MS). Briefly, the protein is incubated for defined intervals in a deuterated environment. After rapid quenching of the exchange reaction, the partially deuterated protein is enzymatically digested and the resulting peptide fragments are analyzed by liquid chromatography mass spectrometry (LC-MS). The deuterium buildup curve measured for each fragment yields an average amide exchange rate that reflects the environment of the peptide in the intact protein. Additional analyses allow mapping of the free energy of folding on localized segments along the protein sequence affording unique dynamic and structural information. While amide H/D-Ex coupled with MS is recognized as a powerful technique for studying protein structure and protein–ligand interactions, it has remained a labor-intensive task. The improvements in the amide H/D-Ex methodology described here include solid phase proteolysis, automated liquid handling and sample preparation, and integrated data reduction software that together improve sequence coverage and resolution, while achieving a sample throughput nearly 10-fold higher than the commonly used manual methods.

KEY WORDS: hydrogen/deuterium exchange, mass spectrometry, protein structure, protein dynamics, laboratory automation, therapeutic proteins, human growth hormone.

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INTRODUCTION

Current State of Protein Structure Analysis

Proteins are the primary targets for most drug discovery efforts. In addition, proteins themselves have become increasingly important as therapeutic agents. Whether the protein is used as a small molecule drug target or protein therapeutic, preservation of the protein's 3-dimensional structure is critical to drug development, and methods to improve the understanding of protein structure are increasingly important for the pharmaceutical industry. While many biophysical methods measure characteristic protein properties, most techniques, such as circular dichroism, differential scanning calorimetry and ultra centrifugation, provide rather global information. Only X-ray crystallography and nuclear magnetic resonance (NMR) can give localized, high-resolution structural information on proteins. The utility of both crystallography and NMR is well recognized and substantial efforts to further develop and utilize these technologies are underway. Both techniques, however, have limitations in applicability and throughput. For crystallography, crystallization remains the major obstacle

and certain proteins, including many membrane proteins and intrinsically disordered proteins, are inherently noncrystallizable. Even with state-of-the-art high-field magnets, selective labeling methods, and new pulse sequences, some proteins are too large for analysis by NMR. In addition, for both techniques, sample proteins can be studied only under a limited set of conditions, such as in the solid state for crystallography and at high concentrations for NMR. Therefore, the ability of mass spectrometry (MS)-based hydrogen/deuterium exchange (H/D-Ex) methods to examine protein structure and dynamics in a nearly unlimited set of solution conditions and protein concentrations fills an existing gap in protein analysis methods.

Brief History of H/D-Ex

In the early 1950s, Linderstrøm-Lang and colleagues described the use of H/D-Ex for the study of protein properties.[1] The use of proteolysis to increase the resolution of structural information obtained through hydrogen exchange was pioneered by Rosa and Richards[2] and extended by Englander.[3] In these earliest experiments, the tritium-exchanged proteins were digested by a protease, the resultant peptide fragments separated by high-performance liquid chromatography (HPLC), and the level of tritium incorporation measured. In the early 1990s, Zhang and Smith described a methodology in which H/D-Ex reactions were followed by steps involving proteolysis, HPLC separation, and mass spectrometric analysis.[4] In this approach, medium-resolution information could be obtained by measuring the deuterium incorporation within each proteolytically generated peptide fragment. Since then, analogous methods have been applied to study protein structure,[4,5] protein dynamics,[6–8] protein–ligand interactions,[9,10] and protein–protein interactions.[11–14]

The dramatic advances in protein MS coupled with incremental improvements in various steps within the experimental H/D-Ex procedures have made this technology a powerful means to detect H/D-Ex phenomena. While the H/D-Ex technology has proven useful, the experimental steps remain labor-intensive and discontinuous, resulting in low sample throughput and relatively poor resolution. To facilitate incorporation of the unique insights offered by the H/D-Ex approach into modern drug discovery, we sought to simultaneously increase sample throughput and increase the resolution of data output to near single amide.

In this article, enhancements that significantly streamline the H/D-Ex approach are presented. The improvements include solid phase proteolysis, automated liquid handling, and development of data reduction software. As a result, we have an integrated system for rapid, comprehensive, and high-resolution analysis of protein structure,[15] protein–ligand interactions,[16] and protein–protein interactions.[17] As detailed here, application of the technique to study the pH-dependent dynamics of human growth hormone (hGH) showed complete sequence coverage which enabled monitoring of the backbone amide exchange dynamics throughout the entire protein at both pH 7.0 and pH 2.6.

THEORY

Exchangeable Hydrogens

Protein hydrogen atoms can be grouped according to their H/D-Ex behavior. The first group of hydrogens exhibit very rapid exchange, and they include hydrogens from side chains containing –OH, –SH, $–NH_2$, –COOH, and $–CONH_2$ groups and hydrogens from the amino and carboxy termini. The exchange rates between these side chains and solvent are too fast for the real-time measurement of H/D-Ex. The second group includes carbon-bound aliphatic and aromatic hydrogens that do not participate in standard exchange reactions. These hydrogens will undergo isotope substitution only following activation by chemical treatment, such as reaction with hydroxyl radicals.[18] The third group includes hydrogens arising from the amide linkages between amino acids of the protein polypeptide chain. In a folded protein, backbone amide H/D-Ex rates are highly variable and can range over eight orders of magnitude.[7] Because rates of backbone H/D-Ex reflect the unique local environment of each amino acid in the 3-dimensional structure, H/D-Ex studies constitute a sensitive and unique method for protein structure analysis. As many main chain amide hydrogens in protein structures exchange over times ranging from seconds to days, the exchange kinetics can be readily followed in real-time by stable isotope labeling.

Amide Hydrogen Exchange

Amide hydrogens can be exchanged with solvent hydrogen through either acid, base, or water-catalyzed reactions.[19]

$$k_{ch} = k_H [H^+] + k_{OH} [OH^-] + k_{H_20} \quad (1)$$

At low pH, the acid-catalyzed reaction dominates, while the rates of the base-catalyzed reactions increase at higher pH values. The water-catalyzed reaction is independent of pH. Given the temperature dependence of exchange rates, the slowest exchange rates at room temperature are observed at about pH 2.7. Much higher exchange rates are observed near neutral pH where the amide hydrogen exchange reaction is mostly base-catalyzed.

"Intrinsic" Amide Hydrogen Exchange Rates

Hydrogen exchange rates along the polypeptide backbone exhibit nearest neighbor effects. Using NMR approaches, Englander and co-workers have accumulated experimental data that allow estimation of the "intrinsic" exchange rates for backbone amides within a random coil polypeptide.[19] The "intrinsic" exchange rates reflect protein sequence and experimental conditions such as pH, temperature, and hydrogen isotope. According to these calculations and experimental observations, backbone amide hydrogens within a random coil polypeptide at room temperature and neutral pH typically exchange within 10 to 1000 msec.

Amide Hydrogen Exchange Rates in Native Protein Structure

The exchange rates of backbone amide hydrogens often dramatically change after the polypeptide has adopted a folded 3-dimensional structure. Participation in hydrogen bonding,[20] distance from the protein surface,[5] and flexibility of the peptide chain[21] are several factors known to affect H/D-Ex rates. The retardation in exchange rate is heavily dependent on the protein structure and dynamics, and is termed the "protection factor." Protection factors as great as 10^8 have been observed, and in practical terms, this means that some amide hydrogens exchange with half-life times of years at room temperature and neutral pH.

Amide Hydrogen as a Thermodynamic Sensor of Protein Stability

Formalisms to relate the observed rates of amide H/D-Ex to thermodynamic stabilization of proteins have been developed.[3] Amide hydrogens of proteins in the native, folded state are proposed to exchange according to the following equation:

$$\text{closed} \underset{k_{cl}}{\overset{k_{op}}{\rightleftarrows}} \text{open} \xrightarrow{k_{ch}} \text{exchanged} \qquad (2)$$

where k_{op} is the rate of "opening," k_{cl} is the rate of "closing," and k_{ch} is the "intrinsic" chemical exchange rate as discussed above. In this scheme, the observable exchange rate is given by

$$k_{ex} = k_{op} \bullet k_{ch} / (k_{cl} + k_{ch}) \qquad (3)$$

For most proteins at or below neutral pH, amide H/D-Ex occurs by an EX2 mechanism,[23] where $k_{cl} > k_{ch}$ and Eq. 3 becomes

$$k_{ex} = k_{op} \bullet k_{ch} / k_{cl} = K_{op} \bullet k_{ch} \qquad (4)$$

The ratio of the observed amide H/D-Ex rate of the native conformation (k_{ex}) to that of a random coil (k_{ch}) represents the unfolding equilibrium constant (K_{op}), which is related to the free energy change upon unfolding (ΔG_{op}) by Eq. 5.

$$\Delta G_{op} = -RT \ln(k_{ex} / k_{ch}) = -RT \ln(K_{op}) \qquad (5)$$

The faster the exchange rate is, the less stable the folded state is. While the formalisms outlined above were initially developed for the study of protein folding on a global scale, more recently these analyses have been used to examine the intrinsic dynamic behavior of proteins and to quantitate the stability of localized regions within the protein structure. Using this extension, each amide hydrogen in the polypeptide can be viewed as a sensor of the thermodynamic stability of localized regions within the protein structure.

Localizing Perturbations in the Protein Structure

One of the most informative applications of the approach outlined above stems from the ability to locate regions of the protein that are perturbed following alteration of the protein's physical and chemical environment. For example, the method can be applied to understand both global and localized structural perturbations of ligand binding to the target protein. Coupling H/D-Ex with MS affords a rapid method for profiling ligand binding and protein structural perturbations induced by changes in physical environment such as variations in pH and temperature. H/D-Ex MS can aid

the optimization of refolding chemistry and formulation of protein therapeutics by determining effects of solution excipients and results of long-term stability studies. Here, we describe the use of H/D-Ex MS to monitor acid-induced perturbations to the solution structure of hGH.

METHOD OVERVIEW

On-Exchange Step

The first step of H/D-Ex is incubation of the analyte protein in a deuterated environment (Figure 1). Typically deuterated buffer is added directly to the lyophilized or concentrated protein sample and there are few restrictions on reaction conditions. As a result, H/D-Ex behavior can be studied as a function of protein and buffer concentrations, buffer composition, solution pH, presence of ligands, or presence of excipients. In a typical H/D-Ex assay, the difference in H/D-Ex properties of a protein under at least two different conditions is studied. To follow the deuterium buildup of individual amide hydrogens or sets of hydrogens, several on-exchange time points are sampled for each condition. Between 6 and 10 on-exchange incubation time points ranging from 10 to 300,000 sec are analyzed.

Quenching/Denaturation Step

After a defined incubation period in a deuterated environment, the protein mixture is directly added to an acidic solution (~ pH 2.5) containing protein denaturants that is maintained near 0°C (Figure 1). This step serves two purposes: first, to quench the exchange reaction and slow undesirable back exchange to hydrogen; and second, to mildly denature the analyte protein to facilitate digestion by acid proteases. All subsequent procedures are conducted under the "quench conditions" to minimize the loss of incorporated deuterium.

Progressive Proteolysis

To localize the deuterium positions after the on-exchange reaction, the analyte protein is digested into a collection of peptides (Figure 1). Because of the low pH conditions in which the protein and peptide samples are held, standard proteases such as trypsin and Lys-C are unsuitable. The acid-stable protease, pepsin, is widely used in H/D-Ex studies. Other acid-stable endoproteinases and carboxypeptidases can be employed to achieve higher sequence coverage and amide resolution. Digestion times usually range from 10 to 120 sec.

HPLC Separation of Peptide Fragments

For a protein of about 50 kDa, more than one hundred peptides are typically generated using pepsin. Prior to mass analysis, the peptides are separated using HPLC to minimize mass overlap and suppression of peptide ionization in the electrospray source

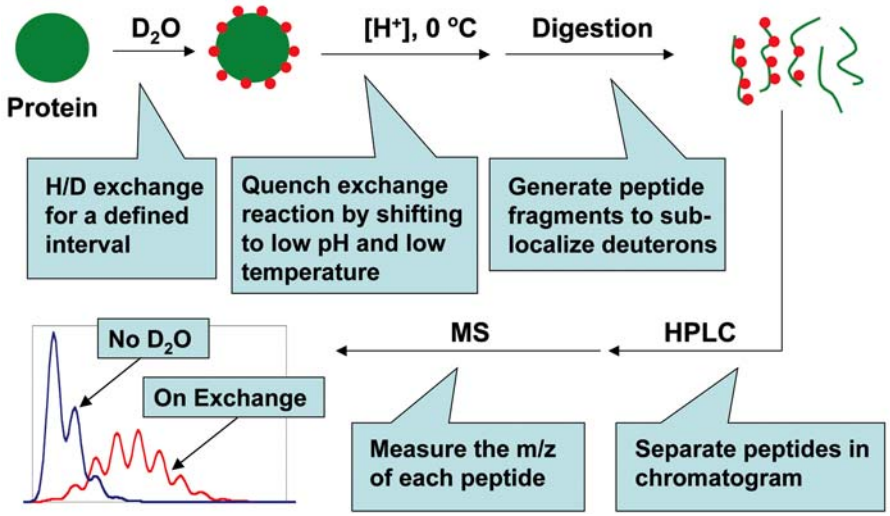

FIGURE 1

H/D-Ex coupled with proteolysis and MS overview.

of the mass spectrometer. However, the length in time of the HPLC gradient needs to be appropriate to minimize the loss of deuterium through back exchange with solvent.

Optimization of Digestion and Separation Conditions

Prior to conducting H/D-Ex experiments, the digestion and separation conditions for each analyte protein are optimized. The parameters that are optimized include type and size of protease column(s), type and concentration of denaturants, absence or presence of reducing reagents such as Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), flow rate through protease column(s), and LC gradient parameters. The primary goal of the optimization procedure is to obtain as complete sequence coverage as possible. To achieve near single amide resolution, it is important to generate many overlapping peptides.[13,15]

Sequence Identification of Proteolytic Fragments

To quickly identify peptides from each digestion condition employed, spectral data were acquired in data-dependent MS/MS mode with dynamic exclusion. The MS/MS data set was then analyzed employing the Sequest program (ThermoFinnigan, San Jose, CA) to identify the amino acid sequence for each parent peptide ion. This tentative peptide identification was verified by visual confirmation of the parent ion charge state and the exact mass for each peptide in the MS data set acquired in profile mode.

Mass Spectrometry

The HPLC eluent is introduced directly into an electrospray ionization mass spectrometer (ESI-MS). The direct coupling of proteolysis, peptide separation, and mass analysis eliminates the need for intermediate storage of deuterated peptides, a step commonly described by others. Both ion-trap and quadrupole time of flight (QqTOF) instruments are currently used for mass analysis.

Automated Sample Handling

A fully automated system for acquiring H/D-Ex MS data starting with a stock solution of the non-deuterated protein has been developed (Figure 2). This system performs the entire operation described in Figure 1, and

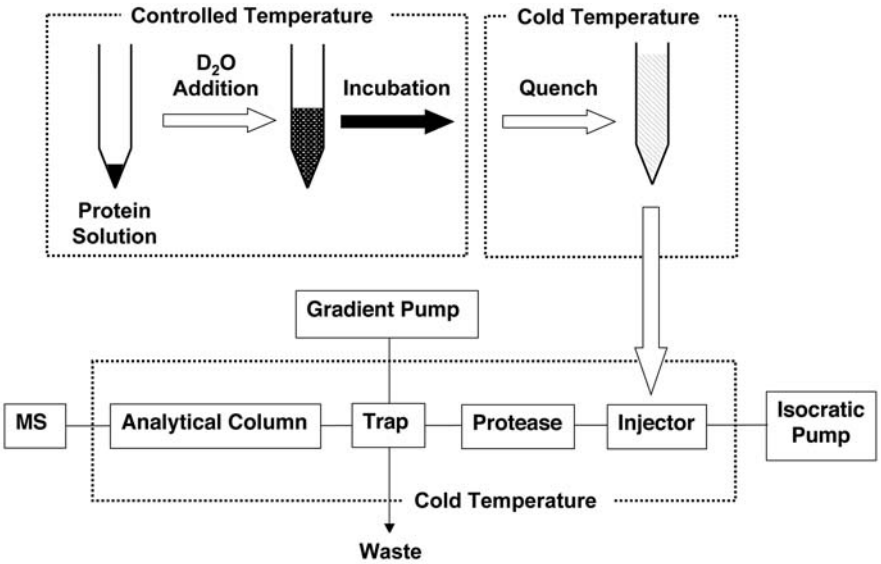

FIGURE 2

Automation flow diagram and temperature control. The entire process is automated. A liquid handler mixes a protein solution with a deuterated buffer to initiate on-exchange, and adds a quench buffer after a programmed period of incubation. The quenched mixture is then loaded into the injector. Using an isocratic pump, the exchanged sample then flows from the injector through the protease column to a C18 trap. After a valve switch, a gradient is used to elute peptides from the trap via the C18 analytical column into the MS.

more than 100 experiments were run continuously without human intervention. This system can be dissected into two parts: the liquid mixing part and the protein processing part. The liquid mixing part, shown at the top of Figure 2, consists of two liquid handlers and two temperature-controlled units, one held at room temperature and the other set near 0°C. To initiate on-exchange, a small amount of protein solution is mixed with a selected deuterated buffer within the syringe and the syringe plunger moved up and down several times to mix the two solutions. The mixture is incubated for a programmed period of time near room temperature. The exchange mixture is then added to a quench solution near 0°C. After quenching the exchange reaction, the entire sample is injected onto the protein processing part which includes injection loops, protease column(s), a trap column, an analytical column, three electronically controllable valves, and isocratic and gradient pumps. The injector, columns, and valves reside in a low-temperature chamber to minimize the loss of deuterium by back exchange (bottom half of Figure 2). The quenched protein solution is pumped in series through a column containing the immobilized protease and a trap to capture the peptide fragments. Extra pumping at this step can eliminate any undesirable polar additives, such as salts and denaturants. The gradient pump is activated following digestion of the protein on the protease column(s), and the peptides captured on the analytical column are eluted directly into the mass spectrometer.

Data Extraction

A software system capable of extracting and cataloging the large number of data points obtained during each experiment has been developed in collaboration with Sierra Analytics (Modesto, CA). First, the software imports the Sequest results described above and registers the molecular weight, charge state, and retention time of each peptide. Second, after importing the LC-MS results of an exchanged sample, the software finds each registered peptide peak in the LC-MS data file. Finally, the software selects the appropriate retention time range and *m/z* range to calculate the centroid value for each peptide. The automated data analysis system streamlines most of the data handling steps that are currently done manually and results in a significant increase in overall efficiency. In addition, automated data analysis reduces the potential for errors associated with manual handling of large data sets. The savings in time can be illustrated by a typical set of experiments where each exchange time point yielded about 100 peptide fragments, and 10 LC-MS experiments were performed (1 nondeuterated, 1 fully deuterated and 8 exchange experiments). In these experiments, 1000 peptides (= 100 × 10) needed to be analyzed. The experimental data are tracked in a 2-dimensional spreadsheet indexed by LC retention time and *m/z* peaks from the mass spectrometer. Determination of the peptide identity, calculation of the average molecular weight, and determination of percent deuteration level (number of deuterium atoms measured divided by the maximum number of deuterium observable) are operations usually performed by separate programs. Assuming the relevant data for each peak could be manually extracted in 2 min, complete analysis of the data would require about 33 h. In contrast, the same data were analyzed in less than an hour with our software running on a standard PC.

Informatics

The overall information repository that integrates in-house data with outside databases is shown in Figure 3. The system oversees data organization and archiving, and facilitates data interpretation. For the protein under investigation, standard bioinformatics processes of search and alignment are performed and homologues are obtained. The qualifying sequences are cross-referenced in a number of available databases, and the features of the proteins (such as domains, glycosylation sites, and disulfides) are extracted and processed into an in-house database that is developing towards BioDAS compliance (www.biodas.org). Additional features are obtained by running prediction tools such as those available from ExPASy (www.expasy.org) or from the EMBOSS suite (www.hgmp.mrc.ac.uk/Software/EMBOSS/). The 3-dimensional models from the PDB database are also collated.

Data Display

Several generic programs have also been implemented to facilitate the visualization of H/D-Ex data. H/D-Ex data can be displayed as a "stacked" bar chart or "color map" that is aligned with the primary protein sequence. The features within our repository can also be compiled in the same view (see Figure 3). Any or all of these sources can also be dynamically projected onto a 3-dimensional model of the protein structure (Figure 3).

Data Interpretation

For calculation of free energies of stabilization, intrinsic exchange rates are taken from reference[19] and the free energy change can be calculated using Eq. 5. While the utility of H/D-Ex for protein analysis was suggested more than 50 years ago, physical interpretations of H/D-Ex results are not fully established. For example, although involvement in hydrogen bonding, distance from the protein surface and flexibility of the polypeptide chain influence the rate of amide hydrogen exchange, there have been few attempts to translate quantitatively these factors into exchange rates.[20] It was envisioned that high throughput capability for generating reliable H/D-Ex data would result in a database useful for correlating the H/D-Ex data and various physical parameters.

APPLICATION OF H/D-EX TO hGH

Importance of hGH

Human growth hormone is a 191-amino acid polypeptide of approximately 22 kDa that is released in a pulsatile fashion from anterior pituitary somatotrope cells. The hormone modulates the growth hormone receptor, and its release is stimulated by growth hormone–releasing hormone (GHRH) and ghrelin, and antagonized by somatostatin. The hormone functions to promote linear growth during adolescence and modulate many physiological functions after completion of linear growth. Since 1984, somatropin (recombinant DNA-derived hGH) therapy has been applied in the treatment of growth hormone-deficient children to promote linear growth. Growth hormone deficiency in adults has been implicated in abnormalities in body composition, increased risk of cardiovascular disease, increased visceral adiposity which can lead to increased insulin resistance, decreased muscle mass, and decreased bone mass. However, growth hormone replacement therapy in adults has been limited due to safety concerns. Because of the potential clinical benefit to patients with adult-onset growth hormone deficiency, much attention has been focused on devising treatment protocols that maximize benefits while minimizing risks that may result from prolonged excessive growth hormone exposure.

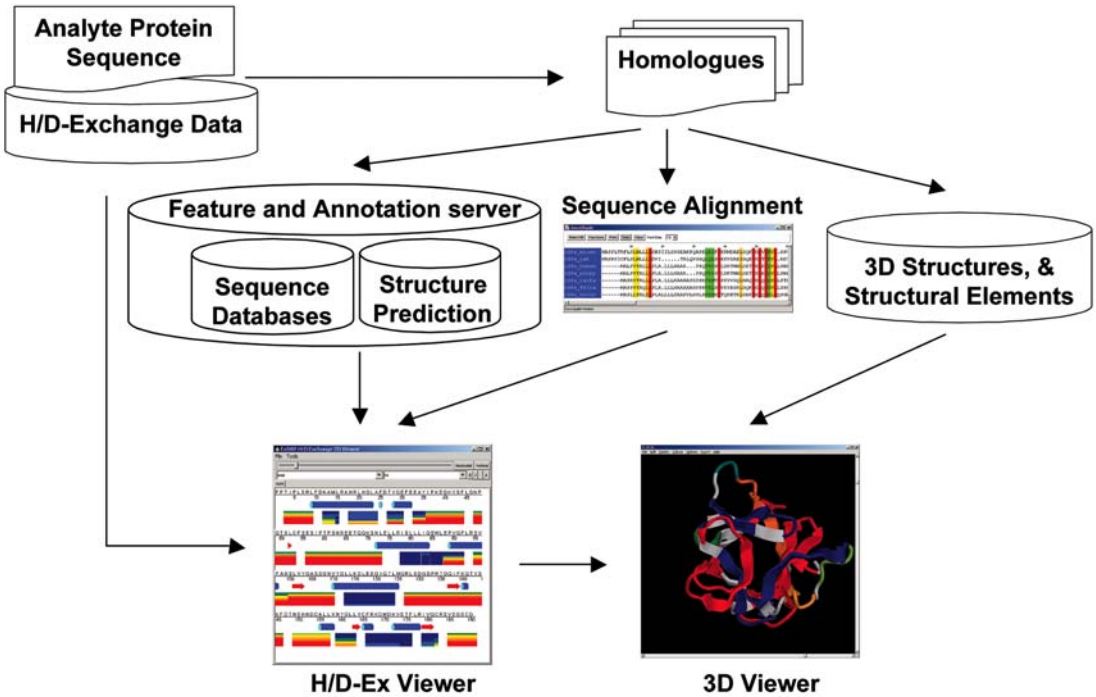

FIGURE 3

Data integration system. The integration of experimental information into public and proprietary databases is indicated by *arrows*.

pH-Dependent Folding of hGH

Human growth hormone is known to exhibit distinct conformations at acidic and neutral pH.[23] While the native state is populated at neutral pH, an alternative, less stable conformation predominates at acidic pH. Although the molecular conformations at acid and neutral pH share virtually identical extents of secondary structure, differences in the tertiary structure have been observed. The less stable acidic conformation is also implicated as the intermediate for undesirable aggregation.[23] To characterize these two conformations, NMR studies using ^{15}N relaxation parameters and amide H/D-Ex rates were determined.[24] Here, pH-dependent H/D-Ex MS experiments were conducted to independently test the existence of two conformations and to evaluate the two H/D-Ex detection techniques.

Digestion of hGH

Before performing the automated H/D-Ex experiments, digestion conditions for hGH were optimized to generate a set of peptide fragments that covered the entire sequence. Parameters that were optimized included type and concentration of denaturants, concentration of TCEP, and flow rate through the protease column. In addition, the H/D-Ex quench solution was optimized and it was found that one part of exchanged protein solution combined with one and a half parts of an 8-M urea, 1-M TCEP solution, afforded optimal coverage of the hGH sequence. Using the optimized conditions, 51 peptides, covering 100% of the sequence, were identified and analyzed (Figure 4). Twenty-five peptides, still covering 99% of the sequence (shown as solid lines in Figure 4), were used in the subsequent H/D-Ex analyses.

During chromatographic separation of the peptide pool, deuterium atoms incorporated within the first two amides of each peptide are rapidly lost through back exchange with solvent hydrogens.[19] Consequently, H/D-Ex MS cannot follow the deuterium buildup of those amide hydrogens. Loss of deuterium buildup information for the first two residues of peptide fragments often creates gaps in the H/D-Ex plot, even though those residues are covered in the peptide map. In the experiments described here, H/D-Ex MS followed 149 amide hydrogens in hGH out of 183 nonproline residues (81%).

H/D-Ex of hGH at pH 2.6 and 7.0

The H/D-Ex results of hGH are summarized in Figure 5. Each block represents a peptide fragment and consists of eight rows that represent eight on-exchange time points. The deuteration level at each time point is color-coded according to the diagram shown at the top right. Peptides with slowly exchanging amide hydrogens are represented by blue bars, while red

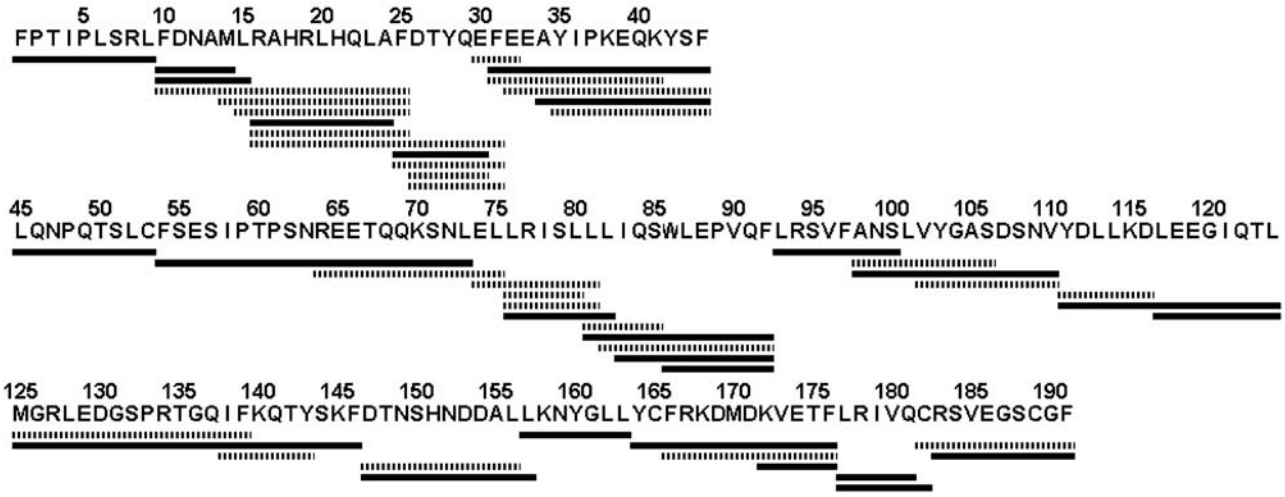

FIGURE 4

Sequence coverage of hGH. *Black lines* indicate pepsin-generated peptide fragments. The entire sequence was covered by 51 peptides. Twenty-five peptides used in the study are shown as *solid lines* and the other peptides identified and analyzed but not used in this study are shown as *dashed lines*.

bars represent peptides that contain rapidly exchanging amide hydrogens. Blocks representing on-exchange at pH 7.0 are on the top row of Figure 5, while blocks representing on-exchange at pH 2.6 are shown on the bottom row. Light blue cylinders above the sequence indicate the helices identified from the X-ray crystal structure of hGH (1HGU).

There are four regions in hGH for which amide H/D exchange rates are very slow at both pH conditions tested. These regions include amino acids 15–35, 78–87, 113–124, and 159–182, and correspond to the helices hGH involved in the helix bundle, a structural fold frequently found in protein hormones and other signaling proteins. The hGH helix bundle contains four nearly parallel α-helices. Adjacent helices have antiparallel polypeptide chain sense and the helices are interconnected by long regions of extended polypeptide that traverse the length of the helix bundle.

High levels of deuterium incorporation are observed for the loop regions of hGH, so that at the later time points, greater than 90% deuteration is observed at both pH 7.0 and 2.6. The initial slow rate of exchange for loops observed at pH 2.6 is likely a reflection of the slower intrinsic rate of exchange at acidic pH rather than a pH-dependent difference in the loop dynamics.

The pattern of deuterium buildup within the hGH helices changes as a function of pH. At neutral pH, helix B and the central part of helix D are resistant to exchange, while amides within helix C show some deuterium incorporation. Alterations at low pH include significantly less deuteration at the amino terminal end helix D and throughout C helix, and decreases in exchange at the amino terminal of helix A. On the other hand, slightly more deuteration at the helix A C-terminal is observed at low pH. Clearly, packing within the helix bundle of hGH has changed, and the alterations are consistent with the known versatility of this common structural motif in which the sites of closest interaction among the helices can change while simultaneously preserving the helix secondary structure elements.[25]

Overall the H/D-Ex results for hGH correlate with the existence of secondary structure, with the helical regions of high local secondary structure showing lower rates of exchange.

Localized Free Energy Changes in hGH at Two pH Values

The sampling of deuterium buildup at time points ranging from 30 to 100,000 sec allows estimation of the

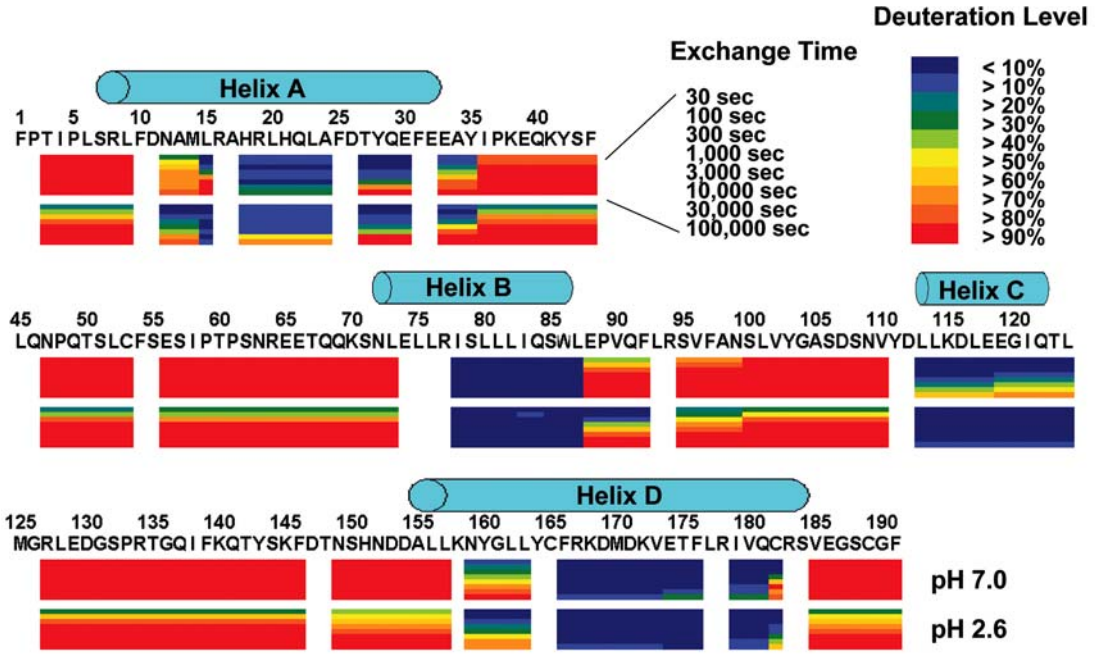

FIGURE 5

H/D-Ex results of hGH at pH 7.0 and 2.6. Each block represents a pepsin-generated peptide. Each block has eight time points, and the level of deuterium incorporation is indicated by colors that vary according to the legend at the top right. *Upper* and *lower blocks* show the results at pH 7.0 and 2.6, respectively.

free energy change upon folding using Eq. 5. Because this analysis eliminates effects arising from differences in intrinsic exchange rates, the approach is especially useful for the analysis of pH-dependent structural changes. Figures 6a and 6b show the localized free energy changes for hGH determined by H/D-Ex at pH 7.0 and 2.6, respectively. Regions of high stabilization are colored blue, and regions of lesser stabilization are colored red. Regions not followed by the technique are indicated by gray. The data clearly show that the overall hGH structure is significantly more stable at neutral pH. At pH 7.0, interactions between helix B and the central portions of helices A and D were sufficiently stable so that several peptide fragments derived from this region showed no deuteration even after incubation for 100,000 sec. It was estimated that free energy changes upon folding in these regions is greater than 7 kcal/mol/residue at pH 7.0. Overall, the loop regions are the least stable.

Regions of greatest stabilization in the helix bundle shift as a function of pH (Figures 6a and 6b). As noted above, at neutral pH regions of high stability are located at the central portion of the helix bundle. At pH 2.6, the region of highest stability in the structure has markedly shifted so that most of the stabilizing interactions are located near the end of the bundle containing the N- and C-termini. In contrast to the stabilization energies of larger than 7 kcal/mol/residue at pH 7.0, stabilization energies approach only 5 kcal/mol/residue at pH 2.6.

Comparison of H/D-Ex Results by MS and NMR

Similar pH-dependent changes in tertiary structure were observed in the H/D-Ex results obtained by MS and NMR. For example, significant protection factors were observed throughout the helix bundle at neutral pH by NMR[24] and by MS (Figures 6a and 6b). Significant protection from exchange was observed only near the N- and C-terminus end of the bundle at acidic pH by both analytical methods. Although the exchange rates for only 22 and 69 backbone amides could be followed by NMR at pH 7.0 and 2.7, respec-

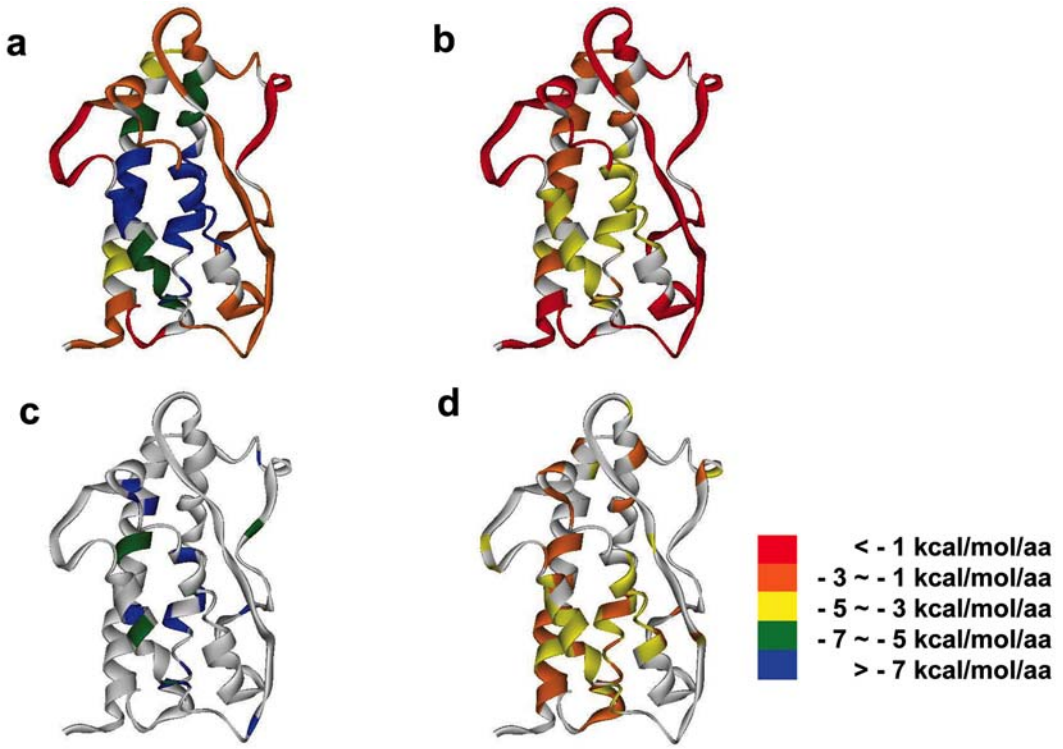

FIGURE 6

Free energy change upon folding of hGH as determined by MS and NMR. Folding free energies are mapped on the X-ray structure (1HGU) by colored segments according to the key at the lower right. *Gray* indicates the residue is not followed by the method used. **a:** Results for pH 7.0 by MS; **b:** results for pH 2.6 by MS; **c:** results for pH 7.0 by NMR; **d:** results for pH 2.7 by NMR.

tively, the stabilization free energies calculated from NMR and MS data are consistent for both samples. In addition, results from both H/D-Ex MS and H/D-Ex NMR experiments show that the acidic conformation of hGH is more flexible overall and significantly less stable.

Comparison of H/D-Ex by MS and by NMR as Structure Analysis Techniques

In the study of the pH-dependent hGH dynamics and stability, similar overall views of the protein structure were obtained using the MS and NMR H/D-Ex methods (Figure 6). Nevertheless, each method offers distinct advantages. An advantage of the H/D-Ex NMR method is the resolution that can be achieved, as NMR-accessible exchange rates are measured for single amides. In this example, the MS method averaged 6 amide resolution (149 amino acid residues monitored by 25 peptides). The advantage of the MS method is protein sequence coverage. For hGH, 149 amide hydrogens were followed with the MS method, while only a fraction (22 and 69 amide hydrogens at neutral and acidic pH, respectively) were followed by NMR. H/D-Ex MS is more widely applicable than H/D-Ex NMR, which requires singly or doubly labeled proteins that are highly soluble in the exchange medium. H/D-Ex MS can be used to analyze nonlabeled proteins at low micromolar concentrations. Differences in throughput are more difficult to assess because the experimental procedures are quite different. For reference, the automated H/D-Ex MS system described generated the data reported here within one week.

Experimental Procedures for hGH Studies

Human growth hormone was purchased from Bachem (King of Prussia, PA) and used without further purification. The experimental procedures are analogous to previously reported methods.[17] Briefly, 5 μL of hGH solution (180 μM) were added to 15 mL of D_2O solution (either 100 mM AcOH at pH 2.6 or 25 mM HEPES at pH 7.0) to start the exchange reaction. Exchange reactions were conducted for 30, 100, 300, 1000, 3000, 10,000, 30,000, and 100,000 sec. The exchange solution was then quenched by shifting the pH to ~2.5 and lowering the temperature by addition of 30 μL of cold 8 M urea, 1 M TCEP solution. The quenched solution was immediately passed through a small solid-phase pepsin column (353 μL bed volume, porcine pepsin from Sigma coupled to PerSeptive Biosystems 20AL support material per the manufacturer's instructions) placed in series with a C18 trap (Small Molecule Micro Trap, 20 μg, Michrom), to allow contemporaneous collection of the proteolytic products. Using a flow rate of 200 μL/min, the digestion and collection steps were completed within 2 min. Subsequently, the peptides loaded on C18 trap were eluted through C18 analytical column (Magic C18, 1.0 mm D × 50 mm, Michrom) with a linear gradient of 10% to 50% B over 30 min (solvent A was 0.05% trifluoroacetic acid in water, and B was 80% acetonitrile, 20% water, 0.01% trifluoroacetic acid). Mass spectrometric analyses were performed on a Finnigan LCQ mass spectrometer with capillary temperature at 200°C.

The average back exchange of these experiments was 24% (76% retained).

CONCLUSION

The application of amide H/D-Ex phenomenon to study proteins was first envisioned in 1950s. Although the potential has been recognized and increasing numbers of researchers use the technique, until now the method remained time consuming and labor intensive. The various improvements reported here result in a system that routinely affords comprehensive sequence coverage, high sample throughput, and high amide resolution. The utility of the system is demonstrated by the characterization of the therapeutic protein hGH. Other potential applications of this technology include design of protein constructs for X-ray crystallography and NMR, aid in protein structure prediction, demonstration of bio-equivalence of protein therapeutics, formulation optimization for protein therapeutics, and detection of binding mode and conformational changes associated with protein–small molecule and protein–protein interactions.

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