Hydrogen exchange studies of protein structure
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Hydrogen exchange techniques, with their residue-level specificity, exquisite sensitivity, and adaptability to many solution conditions, are becoming essential to the study of protein stability, folding and dynamics. Recent studies have elucidated the structures of intermediates formed transiently during protein folding and rare partially folded ensembles present at equilibrium. Analysis of hydrogen exchange mechanisms has revealed protein stability and kinetics at the level of individual residues.

Introduction
Peptide amide hydrogens readily exchange with solvent hydrogens through a process referred to as amide hydrogen exchange. Several aspects of this phenomenon make it extremely useful for studying proteins: hydrogen exchange can be studied under many conditions, detected in a residue-specific manner, and it is exquisitely sensitive to rare conformational changes. Hydrogen exchange measurements have developed into powerful tools for studying protein structure, stability and dynamics. They have been used to examine the native state, partially folded equilibrium intermediates, kinetic folding intermediates, and association reactions. Recent studies have focused on specific mechanisms of hydrogen exchange and the extraction of kinetic and thermodynamic information from exchange rates. There are many excellent reviews of hydrogen exchange theory and methods [1-5,6*]. Here, we present an overview of several recent applications of hydrogen exchange to the study of protein conformations.

Basics of hydrogen exchange
It is well-known that the amide hydrogens (NH) of a peptide or protein will exchange with solvent hydrogens at a characteristic rate [7]. This exchange is catalyzed by H\(^+\) and OH\(^-\); and exchange rates in model peptides are minimal around pH 3 and increase as the pH is raised or lowered. The intrinsic exchange rate (\(k_{\text{obs}}\)) of an NH also depends on other environmental factors, including temperature, hydrogen isotope, steric blocking, and inductive effects from neighboring side chains [8,9]. These intrinsic exchange rates have been determined for each of the 20 amino acids under various conditions using model peptides [8-10].

Protein structure has a profound effect on amide hydrogen exchange rates. When a protein is structured in the vicinity of an NH, the NH exchanges more slowly than it would in an unstructured peptide. This slowing of exchange is termed protection and is reported as a protection factor, \(P = k_{\text{obs}}/k_{\text{in}}\) (where \(k_{\text{obs}}\) is the observed exchange rate and \(k_{\text{in}}\) is the expected rate for an unfolded peptide, see above). While the exact mechanism of protection is still unclear, this slowing is thought to arise from hydrogen bonding, low solvent accessibility and steric blocking.

The protection factors of a protein can be determined using a typical hydrogen exchange experiment: monitoring exchange of protonated protein in D\(_2\)O over time. Some NHs, typically those in solvent-accessible or flexible regions of the protein, will exchange too quickly to detect. NHs in highly structured regions will exchange slower than those in solvent accessible regions. In many proteins, the most slowly exchanging amide protons have protection factors \(P > 10^6-10^7\) and may require months to years to exchange completely.

Several methods can be used to detect hydrogen exchange events. Early experiments used tritium and scintillation counting to detect the bulk exchange characteristics of the protein [7]. In the past decade, developments in protein NMR have allowed residue-specific resolution of amide hydrogen exchange. Protons are exchanged with deuterons and the proton occupancy of the individual amide sites is monitored by two-dimensional NMR experiments [11]. The main limitations of NMR detection are the necessity for NMR assignments and relatively large amounts of protein. More recently, mass spectrometry methods have also been used to detect hydrogen exchange (reviewed in [12*]). While NMR allows the determination of an exchange rate for an individual amide site, mass spectroscopy can follow populations of proteins with different exchange characteristics and hence, different masses. In this respect, mass spectrometry is ideal for distinguishing two-state folding kinetics [13*] from obligatory folding intermediates [14*]. Two-state folding shows two populations separable by mass: the unfolded population and the native population. In the case of an obligatory folding intermediate, a third population with intermediate mass is detected, with a lag in the formation of the native state.

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Abbreviations
DHF R dihydrofolate reductase
\(k_{\text{in}}\) intrinsic exchange rate
\(k_{\text{obs}}\) observed exchange rate
NH amide hydrogen
\(\Delta G_{\text{HX}}\) free energy of the opening reaction
Hydrogen exchange as a probe of protein structure
Since structure hinders hydrogen exchange, measurements of protection factors are often used as a method to infer structure. Because hydrogen bonding is a source of protection, areas of secondary structure can be identified using this technique. Individual NH exchange rates are often measured during an NMR structure determination to confirm secondary structure assignments and to provide H-bond constraints during a structure calculation.

Hydrogen exchange as an indirect probe of protein structure
Protein conformations not amenable to direct crystallographic or NMR studies can be probed indirectly with hydrogen exchange. The hydrogen exchange reaction is carried out under the desired conditions (low pH, bound to ligands, etc.) and then the protein is rapidly returned to its native state to quench exchange (Figure 1a). NMR is then carried out on the native conformation. NHs that do not exchange significantly before or during NMR data collection can be used indirectly as specific probes of protein structure. Since these amide protons show essentially no exchange during the detection step, they report on the degree of protection under the specified conditions.

Equilibrium molten globules
An excellent example of this quenching technique is the structural studies on flexible, partially folded proteins termed molten globules. Many proteins populate molten globule-like ensembles under mildly denaturing conditions, including low pH, high salt and low denaturant concentrations [15,16]. The quenched hydrogen exchange technique has been used to determine the structured regions in several of these molten globule and other partially folded states. These include apomyoglobin [17], cytochrome c [18-20], a-lactalbumin [21-23], b-lactoglobulin [24], equine and hen egg white lysozyme [25,26],

Figure 1
(a) Quenched hydrogen exchange experiment
Resuspend protein in D_2O under desired conditions
Allow exchange for various amounts of time
Quench HX (return to native conditions)
Detect HX pattern in native state
NMR Spectrum

(b) Pulse-labeling hydrogen exchange
Dilute into H_2O at low pH to initiate refolding for time, t
Short HX pulse at high pH
Quench HX at low pH (complete refolding)
Detect HX pattern in native state
NMR Spectrum

Summary of (a) the quenched and (b) the pulse labeling hydrogen exchange methods.
monellin [27], ribonuclease A [28,29], ribonuclease HI [30*], tendamistat [31*], and ubiquitin [32].

These hydrogen exchange studies reveal that molten globules generally retain structure in a core region, whereas the rest of the protein is largely disordered. For example, α-lactalbumin, a protein comprised of an α-helical domain and a β-sheet domain, populates a molten globule conformation at pH 2. A recent hydrogen exchange study on human α-lactalbumin [33] shows that part of the helical domain (namely helices B and C) is protected in the acid state, while the β-sheet domain is unstructured, consistent with studies on other α-lactalbumins [21,34]. Interestingly, the most protected region in the acid state of human α-lactalbumin (helix B) does not correspond to the most protected region in the native state (helix C). In the case of another acid state molten globule, that of ribonuclease HI, the most stable regions of the native state (helices A and D, and β-strand 4) are also the most stable regions of the acid state [30*,35**].

Protein complexes
Hydrogen exchange can also be carried out on large protein complexes, with later separation of the individual components for detection. Paterson et al. [36] used this method to determine the binding epitope of a cytochrome c antibody complex. The interactions between the chaperonins GroEL and SecB and several protein substrates have been investigated using hydrogen exchange [37–42,43*,44*,45,46]. These chaperonins are thought to aid folding by binding to misfolded or aggregated proteins and then releasing them, giving them another opportunity to fold correctly. It was unclear whether these chaperonins bind completely unfolded conformations, or if they might accommodate partially-folded conformations as well. One of these studies investigated the structure of a folding intermediate of the protein dihydrofolate reductase (DHFR) bound to GroEL [44*]. The DHFR–GroEL complex was subjected to exchange conditions for various lengths of time, and then hydrogen exchange was quenched by simultaneous dissociation from the chaperonin and folding to the native state. NMR analysis showed that the central β-sheet of DHFR is protected in the GroEL bound intermediate, suggesting that the native topology is preserved in the complex.

Transient kinetic intermediates
Indirect hydrogen exchange techniques have also been used to probe the acquisition of structure during the protein folding process. Two types of exchange experiment have been used to detect short-lived kinetic intermediates: the competition experiment and the pulse-labeling experiment. In the competition experiment, folding and exchange are initiated simultaneously, setting up a competition between exchange of an amide site and the acquisition of protection as structure is formed [47]. Conditions are chosen so that exchange of an NH site will be rapid unless the NH becomes structured in an early kinetic intermediate. Once refolding is completed, the residues protected early in folding can be detected using NMR on the native state [48,49]. Technically, the competition experiment is relatively simple, as it requires only a single mixing step. The major limitation of this technique, however, is that only the earliest intermediates are detected, and information on the later stages of folding cannot be obtained. The competition experiment was recently used to detect the structured regions in an early kinetic folding intermediate of ribonuclease HI [50]. The central α-helix of ribonuclease HI, helix A, shows significant protection early in folding.

In contrast, the pulse-labeling experiment can be used to characterize kinetic intermediates populated at any time during the folding process [51,52]. Basically, very short (msec) ‘pulses’ of hydrogen exchange are applied at different times in the folding process, to detect the amount of structure acquired (Figure 1b). An initial mixing reaction dilutes deuterated, unfolded protein to conditions where refolding is initiated, but exchange is slow. After a certain (adjustable) refolding time, the protein is subjected to a short, high pH pulse, where exchange of the unprotected NHs is very fast. NHs protected by structure within the folding time do not exchange during the pulse. After the short pulse, the pH is dropped to slow exchange and complete refolding. The native protein can then be analyzed by NMR or mass spectrometry. Several modifications of this general experiment can resolve important details of the folding process. For instance, by varying the intensity of the labeling pulse (usually by varying the pulse pH), the existence of multiple folding pathways can be determined [53,54]. Gladwin and Evans [55*] recently combined aspects of the competition and pulse-labeling experiments to detect protection in the first few milliseconds of folding of hen lysozyme.

Since the original experiments of Baldwin and Udgaonkar [51] and Roden et al. [52], the folding of about 17 proteins has been studied by the pulse-labeling hydrogen exchange technique: acyl-coenzyme A binding protein [56]; apomyoglobin [57]; apoplastocyanin [58]; barnase [59]; cytochrome c [52]; dihydrofolate reductase [60]; interleukin-1β [14*,61], α-lactalbumin [62]; hen [63–65], human [66] and T4 lysozyme [67]; staphylococcal nuclease [68]; the B1 immunoglobulin domain of streptococcal protein G [69]; ribonuclease A [51,54,70]; ribonuclease HI [71*]; ribonuclease T1 [72]; and ubiquitin [73]. Recent experiments, first on apomyoglobin [57] and then ribonuclease HI [71*], have shown that the structures of the early kinetic intermediates resemble the equilibrium acid molten globules. These partially folded conformations show protection in the same secondary structural elements. These results indicate that molten globules can be good models of transient kinetic folding intermediates.

Each of these quenching techniques relies on trapping the isotopic label in the native conformation, which limits the
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probes available for analysis to those amide protons that are very slowly exchanging in the native state (see above). This biases the models derived from these data to only include structured regions of the native conformation; any non-native interactions present under the exchange conditions will not be detectable. To circumvent this difficulty, Roder and co-workers [74] have recently quenched the hydrogen exchange reaction in conditions unbiased by the native structure, namely, organic solvent. While organic solvent effectively quenches exchange, the NMR spectra of proteins under such denaturing conditions are difficult to assign.

Interpreting hydrogen exchange mechanisms

As hydrogen exchange has become a common tool for analysis of protein structure, more sophisticated means of analyzing and interpreting hydrogen exchange data have been developed. The basis of these interpretations is the underlying mechanism of protein hydrogen exchange itself. A protected amide hydrogen is ‘closed’ to exchange, and becomes accessible to exchange through an ‘opening’ event. Exchange with solvent is then assumed to occur from this ‘open’ state at the exchange rate for an unstructured peptide \( k_{\text{in}} \) [8].

\[
\text{Close} \quad \frac{k_{\text{op}}}{k_{\text{cl}}} \quad \text{Open} \quad \frac{k_{\text{in}}}{k_{\text{ex}}} \quad \text{Exchanged}
\]

where \( k_{\text{op}} \) and \( k_{\text{cl}} \) are the rate of the opening and the closing reaction, respectively.

There are two limiting cases in the above scheme [7]. The first case, called EX1, occurs when \( k_{\text{in}} > k_{\text{cl}} \). Under EX1 conditions, the observed hydrogen exchange rate, \( k_{\text{obs}} \), reflects the opening reaction \( k_{\text{obs}} = k_{\text{op}} \). In the second limiting case, called EX2, where \( k_{\text{cl}} > k_{\text{in}} \), the observed hydrogen exchange rate reflects the equilibrium constant between the closed and open states \( k_{\text{obs}} = k_{\text{op}} k_{\text{in}} \). Thus, depending on the exchange mechanism, the observed hydrogen exchange kinetics can report on the kinetics or thermodynamics of the opening event.

Mayo and Baldwin [75] initially proposed studying hydrogen exchange in low levels of denaturant (where >99% of the molecules are in the native conformation) to determine if denaturation induces exchange by binding to partially folded or globally unfolded exchange intermediates. Shortly thereafter, Bai et al. [76] and Qian et al. [77] showed that these studies could distinguish two mechanisms of ‘opening’: unfolding events and local fluctuations (small, local perturbations of the native conformation). NHs whose exchange rates show a denaturant dependence are thought to exchange through unfolding events, whereas those that are denaturant-independent exchange through local fluctuations. Under the conditions of the experiment (EX2 exchange) the observed hydrogen exchange rates can be related to the free energy of the opening reaction \( \Delta G_{\text{HX}} \) of a particular NH. By studying the exchange behavior of cytochrome c, Englander and co-workers [78] used this idea to identify the globally unfolded and three novel partially unfolded conformations, all in equilibrium with the native conformation. In the case of a second protein, ribonuclease H, the most stable amide sites correlate remarkably well with the protected NHs in the kinetic intermediate (Figure 2) [35**,50,71**]. It appears that, for ribonuclease H, the most thermodynamically stable regions of protein fold first, supporting a hierarchical folding model.

**Figure 2**

Comparison of the stability of the native state and the structure of the kinetic intermediate of ribonuclease H. (a) Stability of the native state determined by hydrogen exchange in low levels of denaturant [35**]. Regions that unfold with an average free energy \( \Delta G_{\text{uLF}} \) of 7.4 kcal/mol are shown in dark grey; regions that unfold with an average \( \Delta G_{\text{uLF}} \) of 8.7 kcal/mol are shown in light grey; and regions that unfold with an average \( \Delta G_{\text{uLF}} \) of 10 kcal/mol are shown in black. (b) Protection in the kinetic intermediate [71**]. Unprotected amide protons (NHs) are shown in dark grey, slightly protected NHs are shown in light grey, and well protected NHs are shown in black. Adapted from [71**] with permission.

Mutagenesis is another way to differentiate NHs that exchange through global unfolding from those that exchange through local fluctuations. A mutation that alters the stability of the protein \( \Delta G_{\text{uLF}} = \Delta G_{\text{uLF(mutant)}} - \Delta G_{\text{uLF(wildtype)}} \), where \( \Delta G_{\text{uLF}} \) is the free energy of the unfolding reaction) should have the same effect on the hydrogen exchange kinetics \( \Delta G_{\text{HX}} = \Delta G_{\text{HX(mutant)}} - \Delta G_{\text{HX(wildtype)}} \) of NHs that exchange through global unfolding: \( \Delta G_{\text{uLF}} = \Delta G_{\text{HX}} \) for these NHs. Those NHs that exchange through local fluctuations of the native conformation should show no correlation between their hydrogen exchange behavior and the global stability of the protein. Mutational analysis studies of barnase [79–81] and chymotrypsin inhibitor 2 [82*] show that the NHs that exchange through global unfolding do not directly correlate with the kinetic intermediate or transition state structures of these proteins, as has been proposed [83].
Recent work by Arrington and Robertson [85*] has taken advantage of the switch from EX2 to EX1 kinetics with increasing pH in their studies of turkey ovomucoid third domain (OMTKY3). Under EX2 conditions, the observed hydrogen exchange rate is related to the ratio of \(k_{diss}/k_{on}\). Under EX1 conditions, \(k_{diss}\) is related to \(k_{on}\). Thus, by measuring the exchange kinetics of the NHs that exchange through global unfolding over both exchange regimes, Arrington and Robertson were able to determine \(k_{diss}\) and \(k_{on}\). These rate constants describe the unfolding and refolding rate constants for the protein, which in this case are on the order of microseconds — too fast to measure by conventional stopped-flow methods.

Conclusions

The versatility of amide hydrogen exchange has allowed a glimpse at structures and conformations not usually accessible to structural biologists: transient intermediates, rare conformations and large complexes. Future hydrogen exchange studies will no doubt expand the information gained from these experiments, hopefully illuminating the details of subtle conformational changes in proteins (such as local fluctuations) and their role in protein folding and stability.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


A complete and current review of the development of hydrogen exchange methodology with emphasis on the contributions of Lindstrom-Lang. It also includes a thorough description of the native state hydrogen exchange technique.


An example of the use of the quenched hydrogen exchange technique to probe the structure of the molten globule of ribonuclease H. Structure in the molten globule is located predominantly in a central, helical region of the protein.


An example of the use of quenched hydrogen exchange to detect structure in a partially folded state induced by organic solvent. Although the trifluoroethanol state of tendamistat is not amenable to structure determination by direct NMR or crystallography, native-like topology in the \( \beta \)-sheet region was detected using hydrogen exchange.


Hydrogen exchange in low levels of denaturant was used to detect rare unfolding events in the native state of ribonuclease H. Despite the apparent two-state behaviour of this protein, two partially folded conformations were identified. These partially folded conformations resemble the kinetic folding intermediate and the acid-state molten globule.


The structure of human dihydrofolate reductase bound to GroEL was probed using hydrogen exchange and mass spectrometry. The bound protein showed some protection, indicating that it had stable structure in a small region.


A kinetic intermediate of human dihydrofolate reductase was bound to GroEL and studied with pulse-labeling hydrogen exchange and NMR. Well protected amide hydrogens were observed in the central \( \beta \)-sheet.


The combination of the competition and pulse-labeling experiments allowed the authors to study the first few milliseconds of folding for hen lysozyme and ubiquitin.


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The switch from EX2 to EX1 exchange was used to determine $k_{on}$ and $k_{off}$ (i.e. folding kinetics) for the well protected amide hydrogens in turkey ovomucoid third domain.