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EX1 Hydrogen Exchange and Protein Folding†

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ABSTRACT: Slow amide hydrogen exchange is an increasingly popular tool for investigating structure and function in proteins. The kinetic model for slow hydrogen exchange has two limits, called EX2 and EX1, wherein the thermodynamics and kinetics of protein motions, respectively, are reported by the exchange data. While many laboratories have demonstrated that EX2 exchange can indeed provide accurate results regarding the thermodynamics of protein stability, the potential of EX1 exchange to follow the kinetics of protein unfolding and folding is only beginning to be realized. EX1 hydrogen exchange has advantages over more traditional folding experiments: it provides single-residue resolution, as well as whole-molecule information, the latter of which can be interpreted in terms of the cooperativity of unfolding. However, key questions remain regarding the interpretation of EX1 hydrogen exchange.

Hydrogen exchange is a popular and powerful technique for studying protein folding, as well as other types of motions in proteins. Phenomenologically, it is the replacement of a labile hydrogen—one bonded to a nitrogen, oxygen, or sulfur atom—for a solvent hydrogen. While the exchangeable hydrogens in a protein include both backbone amide hydrogens and many side chain hydrogens, most studies focus on the backbone amide hydrogens because these are the slowest to exchange and thus the most easily detected by experiment.

For a labile hydrogen to exchange with solvent hydrogen, the labile hydrogen probably needs to be both free from intramolecular hydrogen bonding and exposed to the solvent (*1*−*3*). Therefore, hydrogen-bonded secondary structure and burial within the interior of a protein will prevent hydrogen exchange, a phenomenon called protection. A protected amide hydrogen cannot exchange until molecular motions ranging from local fluctuations in conformation to global unfolding open the labile hydrogen to the solvent and break intramolecular hydrogen bonds. Thus, the exchange rates of amide hydrogens that are protected from exchange in native proteins provide details about the motions that open these hydrogens to exchange.

Thanks to the efforts of many laboratories, a consensus has emerged over the last 10 years or so that hydrogen exchange can be used to determine the thermodynamics of protein stability and possibly other conformational changes as well. While some caution is still advised, the results for most globular proteins are consistent with this trend. More recently, hydrogen exchange has emerged as a tool for investigating the kinetics of protein folding and other conformational changes under native conditions. The use of hydrogen exchange in native proteins to study the kinetics of protein motions is the focus of this article.

A MODEL FOR SLOW HYDROGEN EXCHANGE

The most popular model for hydrogen exchange of native proteins dissolved in D_2O is shown here (*4*):

$$NH_{cl} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} NH_{op} \xrightarrow{k_{rc}} ND$$

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According to this model, an exchange-labile hydrogen that is slow to exchange most often resides in the closed state, from which exchange cannot occur. Occasionally, the protein experiences a motion that brings the amide hydrogen to an open state from which exchange may occur. The opening motion proceeds with the rate constant k_{op} and the closing motion with the rate constant k_{cl}. From the open form, exchange can occur with the rate constant k_{rc}, determined from model compound studies (*5*). At pH 4 and above, the chemistry of exchange is base-catalyzed, so k_{rc} increases with increasing pH (*6*). Most experiments are performed using a vast excess of D_2O so that exchange is irreversible. From the model proposed by Hvidt (*4*), the observed rate constant for exchange, k_{ex}, can be expressed as

$$k_{ex} = \frac{k_{op}k_{rc}}{k_{cl} + k_{rc}} \quad (1)$$

with the assumption that $k_{op} \ll k_{cl}$, which is reasonable for a native protein.

In 1968, Rosenberg and Chakravarti concluded that hydrogen exchange for bovine carbonic anhydrase was best explained by two distinct types of motions, unfolding and local motions (*7*). Hilton and Woodward then demonstrated that exchange in basic pancreatic trypsin inhibitor (BPTI)[1] results from two processes, low-energy local motions and high-energy global unfolding (*8*, *9*). This concept of a two-process model for exchange was nicely substantiated by studies of the denaturant dependence of exchange in native ribonuclease A (*10*). This study, in turn, inspired Qian and co-workers to present a general quantitative framework for the two-process model for hydrogen exchange and its denaturant dependence (*11*). Shortly thereafter, Bai and colleagues demonstrated that exchange of several residues in cytochrome *c* is dominated by local motions at low denaturant concentrations and global unfolding at high denaturant concentrations, suggesting that multiple motions can indeed contribute to exchange of a given amide hydrogen (*12*). When multiple fluctuations lead to opening of the same amide hydrogen to exchange, then each fluctuation contributes to the observed exchange rate for that amide hydrogen. The observed k_{ex} in this situation is a sum of each individual k_{ex} for all contributing fluctuations.

EX1 AND EX2 LIMITS FOR HYDROGEN EXCHANGE

Once an amide hydrogen reaches the open state, the balance between closing without exchange and moving forward with exchange dictates the two limits, EX1 and EX2, demonstrated in Figure 1. When $k_{rc} \ll k_{cl}$, eq 1 simplifies to $k_{ex} = k_{rc}(k_{op}/k_{cl})$. In most studies, k_{rc} depends on hydroxide ion concentration, so this expression represents an apparent bimolecular reaction and as such is termed the EX2 limit of hydrogen exchange. The ratio k_{op}/k_{cl} is equivalent to the equilibrium constant for opening, K_{op}. Since k_{rc} can be calculated from model compound data, exchange rates from the EX2 limit can be used to determine K_{op}, if the model is correct. The apparent free energy of opening or exchange, ΔG_{HX}, is expressed as $-RT \ln K_{op}$ and can be calculated with data from EX2 exchange. Thus, hydrogen exchange in the EX2 limit provides thermodynamic data.

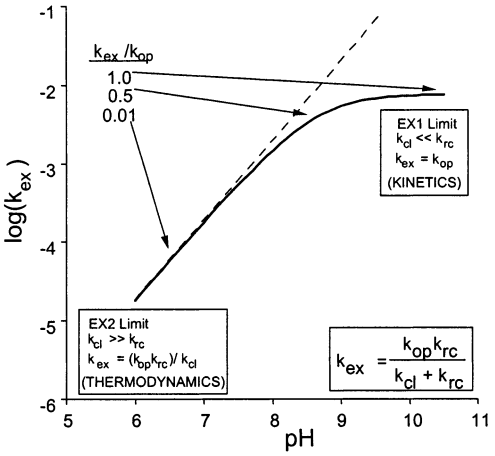

FIGURE 1: Predicted pH dependence of k_{ex} and the switch between EX2 and EX1 hydrogen exchange. This simulation was performed using eq 1 and values of k_{op} and k_{cl} of 0.01 and 20 s^{-1}, respectively. At pH 4, the value of k_{rc} used was 0.0075 s^{-1}. At low pH, when $k_{rc} \ll k_{cl}$, EX2 hydrogen exchange occurs. Under these conditions, k_{ex} increases with increasing pH and provides thermodynamic data. EX1 exchange occurs at high pH, where $k_{rc} \gg k_{cl}$. The value of k_{ex} remains constant with increasing pH and provides kinetic data (adapted from ref *34*).

When $k_{rc} \gg k_{cl}$, eq 1 simplifies to $k_{ex} = k_{op}$, an apparent unimolecular reaction in which exchange always occurs from the open state before the amide proton closes to exchange, leaving the exchange rate unaffected by the rate of catalysis. This situation is termed the EX1 limit of hydrogen exchange. Since the chemistry of hydrogen exchange is base-catalyzed at pH > 4, k_{rc} tends to be much slower than k_{cl} below neutral pH. Thus, EX2 exchange generally occurs at low pH. However, k_{rc} increases with increasing pH, and in some cases, this increase is sufficient to make $k_{rc} \gg k_{cl}$ such that the EX1 limit of hydrogen exchange is reached. For example, typical values of k_{cl} for the slowest exchanging amide hydrogens in ubiquitin in the presence of subdenaturing concentrations of denaturant are about 50 s^{-1}. At 15 °C, the average k_{rc} value for these amide hydrogens is about 0.01 s^{-1} at pH 5 and 100 s^{-1} at pH 9 (*13*). At pH 5, the amide hydrogens are thus well within the EX2 limit of hydrogen exchange. However, at pH 9, $k_{rc} \gg k_{cl}$, putting the exchange in the EX1 limit. In 1979, Hilton and Woodward were the first to observe EX1 hydrogen exchange (*8*). EX1 exchange was observed again shortly thereafter by Wedin in 1982 (*14*) and Roder in 1985 (*15*).

HYDROGEN EXCHANGE AND PROTEIN FOLDING

Results from many laboratories demonstrate that hydrogen exchange can monitor protein motions, including unfolding, under native conditions. Native proteins sample a very large number of conformations, including the unfolded state, in accord with their Boltzmann populations (*16*). For example, unfolding rates and free energies of unfolding for turkey

[1] Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; MS, mass spectrometry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; OMTKY3, turkey ovomucoid third domain.

ovomucoid third domain (OMTKY3) and ubiquitin have been accurately characterized by native-state hydrogen exchange (*13*, *17*), demonstrating the usefulness of exchange for studying protein folding and unfolding under native conditions. In particular, the EX1 limit can give rate constants for folding and unfolding if the amide hydrogen being studied exchanges only through the globally unfolded conformation. If the amide hydrogen were to open through other motions, which presumably would occur more frequently than unfolding, then the other motions would dominate exchange and obscure the contribution of unfolding to exchange (*18*).

The denaturant dependence of an amide hydrogen's rate of exchange under EX2 conditions is the basis for interpreting exchange in terms of three types of protein motions: global unfolding, local fluctuations, and subglobal motions (*19*). The premise for this interpretation is that sensitivity to denaturant is proportional to the amplitude of the motion (*20*). Of all of the possible protein motions, global unfolding thus shows the greatest denaturant dependence because it is the largest amplitude motion. At high denaturant concentrations, all amide hydrogens exchange through global unfolding with a strong denaturant dependence. Amide hydrogens with a strong denaturant dependence even at very low denaturant concentrations exchange through global unfolding under all conditions. Those with no significant denaturant dependence at low concentrations exchange through local fluctuations under native conditions. The third class is characterized by moderate denaturant dependence at low concentrations of denaturant. Under native conditions, these exchange through a relatively large-scale subglobal motion that nevertheless does not completely unfold the protein.

Classification of residues into folding groups using denaturant dependence of EX2 exchange is a common technique (*10*, *12*, *21*, *22*). Generally, residues are assigned to a group if their denaturant dependences of exchange share similar slopes and magnitudes. Hoang and co-workers recently took a major step toward experimentally confirming the validity of such groupings; for cytochrome *c*, EX1 exchange data demonstrate that the distribution of opening rates agrees with the folding pathway derived from denaturant dependences (*23*).

Results from a variety of laboratories strongly suggest that the subset of most slowly exchanging amide hydrogens in native protein, often called the slow-exchange core, exchanges only upon unfolding. For example, several studies show that ΔG_{HX} for the slow-exchange core is approximately equal to ΔG_u, the free energy of unfolding measured by macroscopic methods (*10*, *12*, *13*, *17*, *24*−*26*). Furthermore, the values of k_{op} and k_{cl} for the slow-exchange core of ubiquitin accurately reflect the rate constants of unfolding and folding of the protein (*13*, *17*). In rare instances, all amide hydrogens of a protein can exchange without global unfolding, as is the case for the SH3 domain of α-spectrin (*27*). Nevertheless, results for most proteins suggest that k_{op} and k_{cl} values from the slow-exchange core correspond to the rate constants for unfolding and folding, k_u and k_f.

Studying protein folding and unfolding through hydrogen exchange has several advantages over other methods. Some of these advantages are due to the ability to perform an experiment without the protein spending a significant portion of time in the unfolded form; in contrast, refolding experiments require the protein to be denatured prior to folding. Extended periods in denaturing conditions can lead proteins to aggregate, which may result in artifacts in refolding experiments (*28*). Additionally, proline isomerization can occur if the protein is exposed to unfolding conditions. Proline is the only amino acid for which both the cis and trans backbone configurations are energetically accessible. In the folded structure, proline is locked into one of the two configurations, but when the protein is unfolded, the cis and trans forms are in slow equilibrium with each other. If a proline is in the nonnative configuration when refolding is initiated, then the folding rate will also reflect the isomerization of the proline back to its native configuration (*19*). This is not an issue in native state hydrogen exchange studies because the protein is not allowed to linger in the unfolded state (*13*, *28*, *29*). Data from both RNase A and ubiquitin support this claim, because k_{cl} is faster than k_f in both proteins due to proline isomerization in the macroscopic folding experiments (*13*, *29*).

The use of hydrogen exchange to study protein folding has advantages beyond maintaining the protein in the native state. For instance, under favorable circumstances both the thermodynamics and kinetics of folding and unfolding are observed by simply monitoring the pH dependence of k_{ex}, rather than needing to perform multiple sets of experiments (*13*, *17*, *28*, *30*, *31*). Also, no optical probes such as fluorophores are required for hydrogen exchange, so there is no need to specially engineer proteins, which may lead to other problems (*28*). Finally, states that are not trapped by kinetic barriers are easily studied with exchange, unlike traditional folding experiments, which require the protein to populate a state to detect it (*28*).

Perhaps the greatest advantage of using hydrogen exchange to study folding and unfolding stems from the techniques used for detection. NMR allows exchange to be followed at individual residues, especially when multidimensional experiments are performed (13, *27*, *32*). Moreover, monitoring hydrogen exchange by mass spectrometry (MS) yields data at the whole-molecule level and thus is useful in studying correlated motions. Additionally, MS can be coupled with proteolysis to narrow exchange down to a few residues, as was done to characterize the folding intermediates of an amyloidogenic mutant of human lysozyme (*33*). NMR and MS thus provide complementary information on exchange and are particularly useful in combination (*34*).

There are drawbacks to studying folding and unfolding with hydrogen exchange. One of the most prominent is the uncertainty in the determination of k_{rc}. Model compound data are obtained from studies with small polypeptides. The applicability of these data to amide hydrogens in the context of an entire protein has been called into question, especially concerning the solvent accessibility of the open amide hydrogen in a partially structured protein compared to an unfolded polypeptide (*2*, *35*, *36*). Several studies of hydrogen exchange in denatured proteins have found good agreement between predicted and experimental k_{rc} values (*37*, *38*). Proper determination of k_{op} does not require an accurate value of k_{rc}, since $k_{ex} = k_{op}$ in the EX1 limit. However, determination of k_{cl} requires knowledge of both k_{rc} and k_{op} (*17*).

Therefore, k_{op} can be determined much more accurately than can k_{cl}, making hydrogen exchange more directly and validly applied to unfolding than to folding.

THE MOLECULAR MECHANISM OF SLOW HYDROGEN EXCHANGE

The detailed molecular mechanism of hydrogen exchange in proteins is not well understood, leading to further ambiguities in the application of exchange to the study of protein folding. There has been much debate over the magnitude of motion required for exchange to occur. Woodward and co-workers argue that small amplitude motions can allow solvent molecules to enter the protein structure. These can then catalyze exchange without the amide hydrogen ever encountering bulk solvent (*8*, *9*). Others suggest that exchange occurs through larger amplitude motions such as local reversible unfolding (*2*, *15*). In a thorough review, Dempsey suggests that both are correct, each representing limits in a continuum of structural fluctuations leading to exchange (*39*). For exchange to occur, the amide hydrogen probably cannot be involved in an intramolecular hydrogen bond, but the distance between hydrogen-bonded atoms at which a hydrogen bond is broken is unknown. Without understanding the constraints on hydrogen bonds, it is impossible to discern the amplitude of the motion required for exchange to occur at a protected amide hydrogen.

Some insight regarding the minimum motions needed to disrupt protein hydrogen bonds may be found in an interesting study by Sundaralingam and Sekharudu (*40*). They evaluated protein crystal structures to determine the types of motion required for a water molecule to completely disrupt a hydrogen bond. Their results provide a nice hypothesis regarding the fluctuations required to break a hydrogen bond. In the end, molecular dynamics simulations are probably the best technique to address this question, and several investigators are starting to approach the subject (*41*−*46*).

IDENTIFYING EX1 EXCHANGE

To use EX1 hydrogen exchange in the study of protein folding, identification of the EX1 limit must be unambiguous (*47*, *48*). If the EX2 limit is treated as if it is in EX1 exchange, then k_{op} will appear to increase with increasing pH. Similarly, if the EX1 limit is misidentified as EX2 exchange, apparent superprotection, where the exchange core appears more stable than the folded protein (*49*), will result at increasing pH (*48*, *50*).

Several methods are used to distinguish between the EX1 and EX2 limits of hydrogen exchange. Perhaps the best is the dependence of k_{ex} on pH. In favorable cases, a plot of log k_{ex} versus pH for a given amide hydrogen is diagnostic of the two limits (Figure 1). Under EX2 conditions, $k_{ex} = k_{rc}(k_{op}/k_{cl})$, where k_{rc} is the only factor that should change with pH. Because the chemistry of exchange is primarily base-catalyzed, k_{rc} increases by 1 order of magnitude with every pH unit increase. Ideally, in the EX2 limit of exchange, log k_{ex} therefore increases with pH with a slope of one. In the EX1 limit of exchange, k_{ex} is simply equivalent to k_{op}, which should remain constant with changing pH as long as k_{op} and k_{cl} are independent of pH. EX1 exchange is thus manifested in a pH-independent plateau of k_{ex} at the value of k_{op} (Figure 1). When data are available for both EX1 and EX2 exchange limits, these data can be fit to determine the values of k_{op} and k_{cl}.

Two experimental designs are common for such an analysis. The most frequent type involves determining k_{ex} as a function of pH (*9*, *13*, *15*, *17*, *30*). However, one may also hold the time of exchange constant and vary the pH to obtain similar results (*23*, *30*, *31*). There are caveats involved in using the pH dependence of k_{ex} to distinguish EX1 and EX2 exchange. In principle, the stability of the protein must be known across the entire pH range used for exchange. If k_{op} and k_{cl} change over the pH range studied, neither pH dependence pattern described above will be conclusive by itself. For example, if the protein is destabilized at high pH, then k_{op} may increase with increasing pH. In this case, the high pH plateau may not be observable, or it may be observed over a very narrow range of pH, possibly leading to the interpretation that EX2-type exchange becomes prevalent again at higher pH (*8*, *9*, *15*, *36*). Alternatively, while a plateau at high pH most likely results from a switch to the EX1 limit of hydrogen exchange, it may in fact be due to stabilization of the protein at higher pH (*31*). If the protein is stabilized at higher pH, then K_{op} will decrease. In some cases, this decrease may be enough to balance the continual increase of k_{rc} with increasing pH. Such a balance will lead to a near constant value of k_{ex} in the absence of EX1 exchange.

As an alternative to determining the pH dependence of k_{ex} over a broad range of pH, a comparison of k_{ex} values at two different pH values is often used to distinguish between EX1 and EX2 exchange (*29*, *32*, *50*). If k_{rc} increases approximately 1 order of magnitude per each unit increase in pH, then exchange is considered to be in the EX2 limit. If no increase in k_{ex} is observed, then exchange is classified as EX1. A concern with this approach is the use of only two data points. The Koide lab recently used a modified version of a two-pH test for EX1 exchange that decreases the possible errors (*28*). They take advantage of the widely varying values of k_{rc} within a protein at one pH; k_{rc} for each amide hydrogen can differ by 2−3 orders of magnitude. Thus, they assign residues into groups sharing dynamic characteristics and plot k_{ex} for two or three pH values vs k_{rc} rather than pH. This provides a much greater spread of the data along the *x*-axis. However, the information is not strictly residue-specific, and the accuracy of the interpretation relies upon the proper assignment of dynamic groups. Wagner and Wüthrich used a similar analysis in 1984 to assign residues of BPTI into such groups (*51*).

An indirect method of detecting EX1 exchange is to look for correlated exchange, exchange in which multiple amide hydrogens exchange in the same conformational fluctuation. If the motion leading to exchange is highly correlated, then multiple residues will open to exchange simultaneously. If exchange is in the EX1 limit, then all amide hydrogens that open will undergo exchange. In this case, a correlated motion leads to correlated exchange of multiple hydrogens. However, if exchange is in the EX2 limit, an amide hydrogen will open

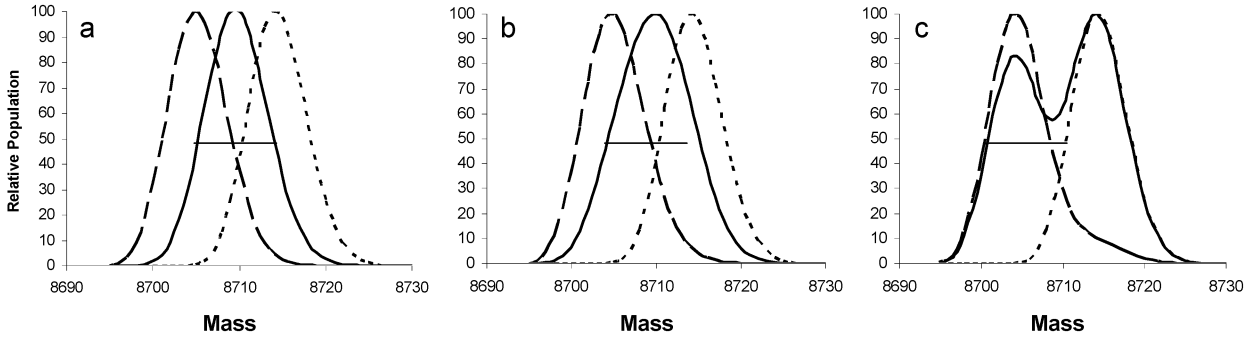

FIGURE 2: Effect of EX1 hydrogen exchange through correlated motions on mass spectral peak shapes. Simulated peak shapes at the start (dashed lines), $t_{1/2}$ (solid lines), and completion (dotted lines) of hydrogen exchange of 10 amide hydrogens are shown for varying numbers of residues involved in a correlated motion. The thin lines across the $t_{1/2}$ peaks at half-height are all identical lengths and illustrate the differences in peak widths. Panel a shows a case with no correlated motions. The peak shape at $t_{1/2}$ is nearly binomial. Panel b shows a case with five residues exchanged through a correlated motion. The peak shape at $t_{1/2}$ is significantly broadened. In panel c, all 10 residues exchange through a correlated motion. At $t_{1/2}$, two peaks exist, representing the entirely protonated species at low mass and the entirely deuterated species at high mass.

and close many times before exchanging. Therefore, correlated motions will not lead to correlated exchange.

Detection of correlated exchange is most often achieved through hydrogen exchange followed by MS (Figure 2) (*34*, *52*−*56*). Exchange of a proton for a deuteron is manifested in a one unit mass increase in the protein mass. Peak shapes in mass spectra are binomial, reflecting the naturally occurring distribution of isotopes. If exchange is uncorrelated, then peak shapes throughout exchange will remain essentially binomial: the peaks will simply increase in mass as protons are replaced with deuterons. However, if exchange is correlated, then the peaks will become broader, especially near the $t_{1/2}$ of exchange. Each peak may even split into two peaks, one protonated, representing the molecules that have not exchanged, and one deuterated, representing the molecules that have undergone the correlated exchange event. In one of the first applications of this technique, Miranker and colleagues found evidence for parallel refolding pathways in lysozyme (*55*).

Detection of correlated exchange can also be accomplished by monitoring nuclear Overhauser effects (NOEs) between exchanging hydrogens by NMR (*15*, *57*). The NOE is seen when magnetization is transferred from an irradiated proton to a nearby proton, generally within about 5 Å, and provides a measure of through-space proximity between protons. If exchange is uncorrelated, then the NOE will be diminished with increasing time of exchange. In contrast, the NOE remains constant throughout correlated exchange. The NOE data for BPTI suggest that the slow-exchange core exchanges through a correlated motion (*15*). This technique is somewhat limited in that it can only give information on protons separated by very short distances.

A possible problem with relying on correlated exchange to distinguish EX1 from EX2 exchange is that it works only if correlated motions are indeed responsible for exchange. For example, only four of the thirteen hydrogens in the slow exchange core of OMTKY3 show correlated exchange in MS studies (*34*, *52*). However, the pH-dependence of k_{ex} for most of the other nine core residues demonstrates significant EX1 character (*17*). We hypothesize that a variety of uncorrelated or partially correlated motions give rise to exchange at these residues.

In general, observation of correlated exchange is good evidence for EX1 exchange but observation of uncorrelated exchange does not prove that exchange is in the EX2 limit. An additional challenge is that multiple MS peaks do not necessarily mean that exchange is in the EX1 limit. For example, a native protein may sample multiple conformations that are slowly interconverting and each of these could have distinct exchange properties, giving rise to two peaks in the MS experiments. Overall, identification of EX1 exchange is best done using multiple tests.

To determine values for k_{op} and k_{cl}, EX1 exchange must be reached. However, achieving EX1 exchange may be challenging for many proteins under normal conditions. In these cases, methods to selectively decrease k_{cl} relative to k_{rc} and thus push the system toward the EX1 limit can be employed. Three techniques are commonly used to reach the EX1 limit of exchange. Raising the pH, as described above, can raise k_{rc} sufficiently to overtake k_{cl} (*30*, *47*, *54*). Additionally, including low levels of denaturant may decrease k_{cl} enough to reach EX1 without significantly populating the unfolded form (*13*, *47*, *54*). Finally, increasing the temperature can also favor EX1 exchange (*47*, *53*).

EX1 EXCHANGE AND CORRELATED MOTIONS IN PROTEINS

Hydrogen exchange provides a unique opportunity to study correlated motions in proteins. For example, of the 13 slowest exchanging amide hydrogens in OMTKY3, only four exchanged through a correlated motion (*34*, *52*). The conclusion was that OMTKY3 unfolds through a number of different unfolding reactions rather than one cooperative event. These results demonstrate the potential for using EX1 hydrogen exchange to address the cooperativity of unfolding at the level of hydrogen bonding.

As described above, correlated motions can be detected directly by hydrogen exchange in conjunction with NMR measurements of the NOE or with MS. MS analysis of hydrogen exchange provides information on correlated motions through the shapes of the peaks. Correlated motions lead to a divergence from binomial peak shapes (Figure 2), and simulations can be performed and compared to the

experimental data to determine the number of residues that participate in the correlated motion (*34*, *52*). Alternatively, NOE data can be used to study correlated motions, as described above (*15*, *57*).

In some cases, indirect approaches are used to interpret hydrogen exchange data in terms of correlated motions. For example, a frequently used method assigns residues into a correlated group when the energetics of exchange have both the same dependence on denaturant concentration and the same absolute values of ΔG_{HX} (*18*, *29*, *48*). This approach is highly informative and readily provides reasonable hypotheses regarding correlated motions, but the contribution of correlated motions is inferred, not demonstrated, in such studies.

Hydrogen exchange studied by MS can detect correlated motions under the EX1 exchange regime. This sort of experiment has thus far been applied principally to the amide hydrogens that exchange only upon global unfolding of proteins. Before beginning MS studies of correlated motions, investigation of exchange by NMR, if possible, helps to identify the residues involved in the slow exchange core. However, this can also be done, albeit with lower structural resolution, by MS and proteolytic fragmentation (see below), and this is probably the only approach that is routinely practical with very large proteins and complexes.

A typical $^1H/^2H$ exchange experiment will start with protein in D_2O at low pH, for example, pH 5, where exchange is very slow. Often, the $t_{1/2}$ of exchange under these conditions is on the order of days to weeks. A pH jump is then performed to bring the protein to a higher pH where exchange is faster and in the EX1 limit. From this solution, aliquots can be quenched in low pH, cleaned as necessary, and analyzed with mass spectrometry. Alternatively, MS can be performed in line with exchange to allow real-time detection of faster events (*54*). Once MS data have been obtained, an algorithm is available that allows the simulation of a predicted mass spectrum for varying numbers of residues involved in correlated motions and even allows for the inclusion of multiple correlated motions (*34*, *52*). These simulated spectra can then be compared to the experimental spectra to determine which model for correlated motions best matches the data.

The MS experiment described above does not give residue-specific resolution. Ideally, identification of the residues involved in the slow exchange core is achieved by NMR studies. Alternatively, MS can be combined with proteolysis to narrow down the candidates for correlated motions to residues within specific fragments (*1*, *33*). Another approach is to use MS/MS fragmentation to form fragments analogous to those from proteolysis. This technique may lead to scrambling of protons and deuterons between fragments, rendering the data interpretations inaccurate. In some instances, it may be possible to alter mass spectral conditions to minimize scrambling (*58*). Additionally, MS itself does not appear to cause scrambling; only when fragmentation is used does it become a problem (*59*).

The use of the NOE to study correlated motions is technically challenging and the limitations are significant. In general, the amide hydrogens being studied must be within 5 Å of each other. It is possible now to observe NOEs between amide hydrogens up to 12 Å apart, but this approach requires either predeuteration of the protein or decoupling of protons located between the amide hydrogens being studied (*60*). On the other hand, NOE studies of correlated motions permit direct identification of the residues involved in the motions, a significant advantage over MS studies of such motions.

Hydrogen exchange in the EX1 limit provides rates of opening and closing that are analogous to the rates of unfolding and folding for the slow exchange core. If multiple motions lead to the exchange of a given amide hydrogen, then the most frequent motion will dominate exchange and obscure the slower motions. Unfolding is generally the slowest motion observed in hydrogen exchange studies of proteins, and thus the only amide hydrogens for which the exchange data can be accurately applied to folding are those that exchange only upon global unfolding of the protein. The local motions that lead to exchange of the other amide hydrogens may or may not be on the folding pathway and cannot be unambiguously assigned to folding intermediates (*48*). Similarly, the kinetic information gained from EX1 exchange may not translate to the order of events in unfolding (*61*), and EX2 exchange data by themselves cannot be used to determine the kinetic order of events (*35*). Combining kinetic and thermodynamic analysis of EX1 and EX2 hydrogen exchange can be quite informative, however. Yan and co-workers used such an analysis to eliminate possible intermediates in the folding of OspA and therefore discern a reasonable folding pathway (*28*). Similarly, kinetic hydrogen exchange data was used to verify folding intermediates proposed from equilibrium studies with cytochrome *c* (*23*).

An ongoing discussion in the literature addresses whether hydrogen exchange data is sufficient to describe protein folding pathways and transition states in protein folding. One question is whether the slow exchange core is the same as the folding core, that is, the residues that are the first to fold. Several proteins do show similar folding cores and slow exchange cores (*62*, *63*). However, the partially unfolded forms of barnase and chymotrypsin inhibitor 2 identified by hydrogen exchange data do not correspond to kinetic folding intermediates (*35*). Similarly, alternative interpretations of results for barnase from conventional folding studies (*64*, *65*) and exchange experiments (*65*−*68*) lead to different models for its folding pathway. Further studies should help us fully understand the application of hydrogen exchange to folding and whether it is more or less informative on intermediate formation than conventional studies.

Hydrogen exchange in the EX1 limit is uniquely suited to the study of protein dynamics and protein folding. Studying hydrogen exchange by NMR provides residue-specific data on dynamics, while MS studies can yield information on correlated motions. The application of hydrogen exchange to protein folding is still growing, making it crucial to understand both the advantages and the limitations of this technique. One key area for future research is the detailed molecular basis for slowing of hydrogen exchange by protein structure.

NOTE ADDED AFTER PRINT PUBLICATION

Noel D. Lazo was omitted from the list of authors in the version of this paper published 12/31/03 (ASAP) and in the January 27, 2004, issue (Vol. 43, No. 3, pp 587−594). The correct electronic version, which includes Lazo's current address, was published on the Web 02/27/04, and an Addition and Correction appears in the March 30, 2004, issue (Vol. 43, No. 12).

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