Hydrogen exchange and protein folding

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Amide hydrogen-deuterium exchange is a sensitive probe of the structure, stability and dynamics of proteins. The significant increase in the number of small, model proteins that have been studied has allowed a better understanding of the structural fluctuations that lead to hydrogen exchange. Recent technical advances enable the methodology to be applied to the study of protein–protein interactions in much larger, more complex systems.

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Abbreviations

BPTI	bovine pancreatic trypsin inhibitor
CI2	chymotrypsin inhibitor 2
DHFR	dihydrofolate reductase
GdmCl	guanidinium chloride
HX	hydrogen exchange
OMTKY3	ovomucoid third domain

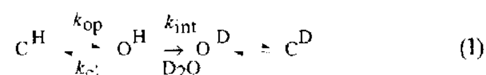
Introduction

Hydrogen exchange (HX) is a powerful technique that has been used for many years to study the structure, stability, folding, dynamics and binding of proteins. The pioneering work on HX focused on a small number of proteins. In the past few years, this has been extended to include a significant number of small, model proteins, many of which lack the disulphide cross-links or prosthetic groups that can complicate the analysis. The combination of HX and other folding studies of these systems has improved our understanding of the conformational dynamics of proteins at equilibrium. In this review, we concentrate on HX at equilibrium. We do not have room to discuss kinetic studies of protein folding, nor HX in denatured states.

Theory of the hydrogen exchange mechanism

Hydrogen exchange mechanism

The commonly accepted model is the two-step model of Linderstrøm–Lang [1], refined by Hvidt and Nielsen [2], and described as follows



$$C^H \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} O^H \underset{D_2O}{\overset{k_{int}}{\rightarrow}} O^D \rightleftharpoons C^D \quad (1)$$

H and D denote protonated and deuterated forms. C denotes the 'closed', exchange incompetent form, and O the 'open', exchange competent form. K_{op} is the rate constant for the opening step, k_{cl} is the rate constant for the closing or reproduction step. k_{int} is the rate constant for the chemical exchange reaction, which will depend on the protein primary sequence, pH and temperature. Under folding conditions, $k_{cl}>>K_{op}$, so that the observed rate constant for exchange, k_{ex}, can be defined as

$$k_{ex} = \frac{k_{op} \cdot k_{int}}{k_{cl} + k_{int}} \quad (2)$$

The two-process model

In the generally accepted two-process model, originally proposed by Woodward and co-workers [3,4], protected amide protons can exchange through relatively rare global unfolding events, or through fluctuations of the native state. The nature of the native state fluctuations are still unresolved, but most investigators assume the two-step, Linderstrøm-Lang model when analysing both global and native-state exchange kinetics.

EX1 and EX2 kinetics

From equation 2, there are two limiting conditions for exchange. If the open species equilibrates rapidly with C, so that the closing step, k_{cl}, is faster than the exchange step k_{int}, the the chemical exchange is the rate-limiting step, and k_{ex} reduces to

$$k_{ex} = K_{op} \cdot k_{int} \quad (3)$$

where K_{op} is the equilibrium constant for the opening reaction. This is known as the EX2 limit. The apparent free energy of exchange, ΔG_{ex}^{app}, can be calculated assuming k_{int}, which depends on pH, temperature and position in the protein, can be accurately inferred from peptide studies [5,6].

$$\Delta G_{ex}^{app} = -RT \ln K_{op} \quad (4)$$

where R is the gas constant and T is the temperature in Kelvin. Exchange under EX2 conditions, therefore, can be used to determine the apparent free energy of the underlying structural opening reaction.

In the other limiting case, EX1, the chemical exchange step is much faster than the rate constant for reprotection and so the opening step becomes rate-limiting:

$$k_{ex} = k_{op} \quad (5)$$

EX1 and EX2 exchange mechanisms can be distinguished from the pH dependence of exchange, from the decay of

nuclear Overhauser enhancement signals, or using mass spectrometry (see, for example, [7–9]).

EX1 and EX2 kinetics in detail

According to the Linderstrøm-Lang model [1,2] the exchange mechanism will depend on the relative rates of closing and exchange, k_{cl} and k_{int}. Early studies demonstrated that under native conditions exchange is usually in the EX2 limit [10], whereas EX1 kinetics were only observed under unfolding conditions or at extremes of temperature or pH [11]. It is now easier to distinguish the mechanism unequivocally, and it has become clear that EX2 kinetics are not universal, even under relatively benign conditions [7,12]. Small increases in temperature, pH or denaturant concentration, or mutation or binding of chaperones, can induce a change in mechanism [13,14,15•,16,17•].

The implications of these observations are several. The exchange mechanism cannot be assumed, it should be determined explicitly, under the most destabilising experimental conditions. Measurements of the apparent free energy of exchange, ΔG_{ex}^{app}, are only meaningful in EX2 conditions. Because exchange may occur by global unfolding, for which k_{cl} may be relatively small, or by native state fluctuations, which are presumably more rapid, both EX1 and EX2 kinetics may be observed in the same protein, as for barnase [14,15•]. The use of mutants to distinguish global and native-state exchange is important in making this distinction. The situation may become more complex as the protein is destabilised and global exchange becomes dominant [15•,18–20]. At some point, protons may be exchanging through both native state fluctuations, by an EX2 mechanism, and through global unfolding in an EX1 regime [21]. To allow for this, it has been proposed that a general two-process model be adopted to analyse the kinetics of HX [16,22•].

Data obtained in both EX2 and EX1 conditions have been combined to determine the kinetic parameters k_{op} and k_{cl} for the opening reaction of the globally exchanging residues in the small protein turkey ovomucoid third domain (OMTKY3) [23•]. k_{op} can be determined with some accuracy, but the determination of k_{cl} depends on the correct estimation of k_{int}. Values of k_{op} equal to the unfolding rate constant were obtained, supporting a global unfolding model. The relatively wide range of values of k_{cl} obtained correlate with ΔG_{ex}^{app}. The heterogeneity in k_{cl} may reflect some early refolding events or may simply reflect the uncertainty in k_{int} (used to determine both k_{cl} and ΔG_{ex}^{app}). It would be interesting to see this extended to study the rate constants for local fluctuations. However, as the pH is raised sufficiently for EX1 conditions to apply to rapid native-state fluctuations, the protein may become destabilised, in which case, native-state exchange will give way to global exchange. It is possible, however, to put a lower limit on the rate of native-state fluctuations by comparison with estimated values of k_{int} under EX2 conditions.

Hydrogen exchange and mutations

Gross changes in protein stability on mutation are easy to assess by standard methods. Since HX is a sensitive, site-specific probe of local stability, a comparison of HX in wild type and mutants can distinguish local and global effects of mutation [24–30]. Apart from the mutated residues and their immediate neighbours, it can be assumed, providing there is no gross structural change, that k_{int} will be the same for all residues in the wild type and the mutant, and so the change in the free energy of exchange, $\Delta\Delta G_{ex}$, can be determined thus:

$$\Delta\Delta G_{ex} = -RT\ln\frac{k_{ex}^{wt}}{k_{ex}^{mut}} \qquad (6)$$

where k_{ex}^{wt} and k_{ex}^{mut} are the exchange rates of wild-type and mutant, respectively. The value for $\Delta\Delta G_{ex}$ of globally exchanging residues gives an accurate measurement of the global effect of mutation. Mutation has little or no effect on the rates of native-state exchange. Local stabilising and destabilising effects can be inferred from changes in local exchange behaviour. It is possible to detect remote effects that may not be inferred from structural data [26]. In a similar way, the local and global effects of a change in oxidation state [31–33], or of ligand binding (see below) can be investigated.

Mutation has proved a valuable tool for distinguishing global and native-state exchange [14,25,34,35]. Many residues exchange with a significant ΔG_{ex}^{app}, yet have identical exchange rates in wild type and mutants with a wide range of stabilities. These residues are clearly exchanging by fluctuations of the native state that do not lead to global unfolding.

Hydrogen exchange and determination of global stability

Under EX2 conditions, there is often a subset of amide protons that exchange on a very slow timescale of months or even years. According to the two-process model [3,4] these protons only exchange when the protein fully unfolds. The open state is fully unstructured so the values of k_{int} from model peptides are a good measure of the rate of chemical exchange [5,6]. HX measurement can therefore provide information about the global transition under native conditions in which the unfolded state is populated at extremely low levels, and is an excellent way of obtaining thermodynamic parameters for the unfolding process — with the caveat that the effects of ^2H_2O on stability need to be explicitly determined (see [36–38] and references therein). The problems encountered in classic chemical or thermal denaturation experiments are avoided — the energetics of unfolding must be measured in the transition region, because under native conditions the population of the unfolded state is too small to be

detected and extrapolated to water, and assumptions about the nature of this extrapolation have to be made [39–43]. Further, under the extreme conditions used, proteins may exhibit some irreversibility or oligomerisation.

For many proteins, the value of ΔG_{ex} corresponds well to the free energy of unfolding, ΔG_u determined using spectroscopic probes or calorimetry [12,14,35,44,45,46•]. In bovine pancreatic trypsin inhibitor (BPTI) [47], RNase A [18,48], yeast iso-1-cytochrome *c* [33], protein G [49,50] and OMTKY3 [12], $\Delta G_{ex} > \Delta G_u$. Some have invoked a supra-unfolded open state, which lies at a higher energy than the unfolded state that is accessed in conventional denaturation experiments. Alternatively, conventional denaturation experiments may give incorrect values of ΔG_u because of the problems listed above [35,36,46•,50,51•], or the ΔG_{ex} may be invalid because of a shift to the EX1 limit [12]. In summary, HX may provide the most accurate measure of the free energy of unfolding under physiological conditions.

Another common observation is a rather wide range of values of ΔG_{ex} for the slowest exchanging residues. Studies of mutant proteins are invaluable because they have allowed us to determine, unequivocally, which protons exchange by global unfolding [14,25,34,35].

Nonglobal exchange and local stability

What is the nature of the opening reactions that allow HX to occur on faster timescales than global unfolding? To what extent is HX a measure of stability at these sites? As well as a lower free energy of exchange, these opening reactions are characterised by having a low activation enthalpy [52,53]. Two structural models have been proposed. In the local unfolding model, exchange takes place through small conformational fluctuations, ranging from the breakage of a single hydrogen bond to the unfolding of segments of structure [54,55]. k_{op} and k_{cl} represent local, cooperative opening and closing fluctuations, and K_{op} reflects local stability. Significantly, it is assumed that the open state will always be completely unstructured, so that values of k_{int} from peptide studies can be used [5,6]. An alternative mechanism involves the penetration of water molecules and catalyst ions via small amplitude, noncooperative motions, resulting in the formation of channels to the protein interior [4]. In this case, k_{int} is not the same as the exchange rate from peptide studies. Both models invoke small movements of the protein structure, and therefore must reflect some features of local stability because they require the breaking and making of interactions within the protein. The local unfolding model views the chemical exchange reaction as occurring in bulk solvent whereas in the penetration model it occurs inside the protein (for a recent review of evidence for the two models, see [56]).

Do the observed exchange rates point to a penetration model rather than a local unfolding model? An α helix should provide a good test because it is a single segment of structure. There are two common observations. The first is a periodicity of ΔG_{ex}^{app} [13,14,35,57,58], with sites on a solvent-exposed face exhibiting a smaller ΔG_{ex}^{app} than those that face the interior of the protein. The second is decreasing protection from the centre to the ends [33,59]. This has been pointed to as evidence for the penetration model, because according to the local unfolding model, adjacent protons in the same region of substructure would be expected to undergo the same local unfolding event and therefore should exhibit the same ΔG_{ex}^{app} [58]. A more realistic physical model is that a unit of structure such as an α helix does undergo cooperative fluctuations; however, a variation in ΔG_{ex}^{app} would be expected because the fluctuations may not expose all the sites equally to solvent [60•]. Thus, the values of k_{int} may not be the same as those in an unstructured peptide, and the variation in ΔG_{ex}^{app} may simply reflect the variation in true values of k_{int}. In this case, the different degrees of protection along an α helix does not reflect different, nonconcerted unfolding events, but rather differences in solvent accessibility. It is clear also that stability can be conferred on a site not just by hydrogen bonding, but also by packing (burial) [58,61]. Central residues in an α helix often pack against, and are buried by, other core residues. Nevertheless, native state HX rates do, qualitatively, reflect local stability, as is clear from the local effects of mutation [24–26,29] and from comparative studies of similar structural elements in the same protein [59]. It seems to us that native-state exchange is best described by an ensemble of fluctuations, involving both cooperative local unfolding and penetration of solvent [56].

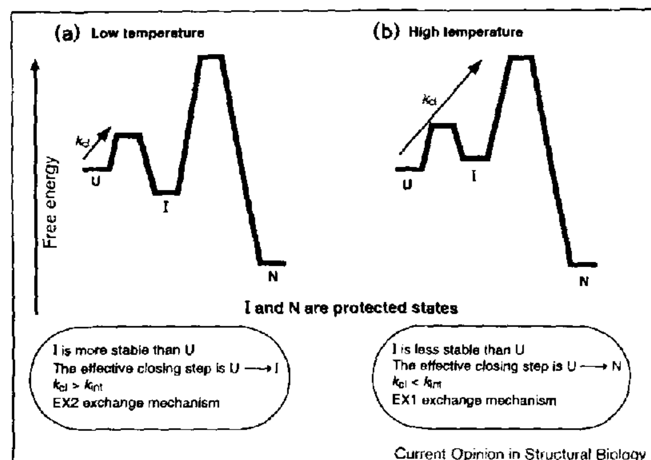
Theoretical studies based on the local unfolding model predict protection factors that agree well with those observed in a number of proteins [62,63]. It would be interesting to see these studies extended to barnase and chymotrypsin inhibitor 2 (CI2) for which there are extensive site-specific data from HX, folding, structural and peptide studies.

Equilibrium studies and protein folding pathways

HX allows us to observe states that are populated at very low concentrations at equilibrium that cannot be observed directly by standard methods. Partly structured, stable folding intermediates should fall into this category. In barnase, there is a folding intermediate, I, that has a stability of ~3 kcal mol^{-1} relative to the unfolded state [64,65]. We expected, by the comparison of mutants with different stabilities of I, to be able to distinguish protons that exchanged from the intermediate [34]: we were unable to do so [14]. Protons either exchanged from the native state or from the globally unfolded state. No protons had exchange kinetics that could be simply correlated with the stability of I. The presence of I, however, can be inferred from the observed changes in exchange mechanism on mutation or on increasing the temperature

(Figure 1; P Dalby, J Clarke, AR Fersht, unpublished data).

Figure 1



Effect of temperature on the exchange mechanism in barnase. For the globally exchanging residues, which are protected in both the intermediate (I) and the native state (N), there is a relatively abrupt switch from EX2 to EX1 kinetics as the temperature is raised from 33 to 37°C at p^2H 7.8 [14]. At both temperatures, the rate constant for folding, $k_{I-N} < k_{int}$. **(a)** At lower temperatures, the effective reprotection step is the conversion of the fully unfolded state (U) to I, which is very rapid. **(b)** As the free energy of I becomes higher than U at higher temperatures, there is, in effect, a change in the closing step to the complete refolding reaction, U to N. This leads to an abrupt change in the closing rate constant and a switch to EX1 kinetics. In destabilised mutants, this switch occurs at lower temperatures, but in stabilised mutants, EX2 kinetics are maintained at 37°C.

Englander and co-workers [19,66•] carried out similar experiments on cytochrome *c*, using guanidinium chloride (GdmCl) to destabilise the protein rather than mutation. It had previously been reasoned that partially unfolded structures might be destabilised to different extents by denaturant [18]. Partially unfolded forms were identified with subglobal values of ΔG_{ex}^{app} and *m*, the dependence of ΔG_{ex}^{app} on denaturant. These could be ranked in order of apparent free energies. Similar results were obtained with RNase H [44]. It is suggested that because these structures resemble kinetic folding intermediates identified by HX pulse labelling and molten globule states occupied under extreme conditions, they may represent sequential folding intermediates — thus a folding pathway might be elucidated by HX at equilibrium [19,44,66•]. It is very important to note, however, that equilibrium measurements alone cannot be used to infer kinetic pathways [15•,60•].

The effect of GdmCl on the exchange behaviour of RNase A [16,18], barnase [15•], CI2 [51•] and protein L [46•] have also been examined. In RNase A, barnase and protein L, an increase in GdmCl concentration results in a change to EX1 kinetics for the globally exchanging protons. In protein L, the pH was manipulated to maintain EX2 over the whole denaturant range [46•]. In neither CI2 nor protein L are partially unfolded states observed. Data from other proteins have also been interpreted in terms of a mixture of global and native-state exchange only [20,67,68]. In both barnase and CI2, where mutants allow native-state exchange to be assigned with certainty, a number of nonglobally exchanging residues have a significant *m* value, yet these are not associated with productive unfolding fluctuations. It is possible that a mixture of EX1 and EX2 kinetics, in conditions where a mixture of global and native-state exchange occurs, could result in misinterpretation of ΔG_{ex}^{app} and *m* values measured by GdmCl dependence of exchange [15•,22•]. These investigations demonstrate that care has to be taken with these experiments; however, if folding intermediates with significant kinetic barriers between them do exist at equilibrium, then one might expect to see the results observed with cytochrome *c* and RNase H. It will be interesting to follow this story.

In barnase and CI2 we observe no relationship between the established folding pathways [69,70] and exchange behaviour at equilibrium [34,51•,60•], whereas such a relationship has been observed for other proteins [19,25,44,66•,71,72]. This may reflect, in part, the experimental techniques used to measure folding. Kinetic studies, using techniques such as stopped-flow circular dichroism and protein engineering, can detect the formation of secondary structure that is not detected by HX. This emphasises the importance of using a number of probes to measure folding pathways [73].

Protein–protein interactions

The residue-specific resolution of HX has been used with great effect in recent years to look at protein–protein and protein–ligand interactions, and has revealed that binding can affect the stability and dynamics of a protein at sites that are very distant from the site of interaction [49,74–81]. An alternative to monitoring by NMR is electrospray mass spectrometry [7,82], which has the unique ability to resolve the relative populations of differently labelled states. Three detailed studies of protein–chaperonin interactions are highlighted below.

The conformational state of dihydrofolate reductase (DHFR) that binds to GroEL was probed by mass spectrometry at different stages of its ATP-driven folding reaction [83•]. DHFR is bound in a partly folded state. After a round of ATP hydrolysis, DHFR rebinds to GroEL in a state with a very similar HX-labelling pattern. The proposed model is that DHFR folds gradually on GroEL, and at each stage of the ATP cycle the chaperone allows minor adjustments of the DHFR structure to be made to allow it to complete folding properly. NMR was also used to provide more detail about the DHFR–GroEL complex [84], and showed that protection was greatest for residues in the central β-sheet of the native protein.

β-lactamase binds to GroEL only at an elevated temperature: this slows the rate of exchange, affording it protection similar to that observed for the protein in the absence of chaperone at lower temperature [85]. These results indicate that the state that is bound is highly protected. The authors suggest that elevated temperature enhances the 'breathing' motions of the protein that expose surfaces that bind to the chaperone.

The resolution of NMR is highlighted in a study of barnase and the chaperones GroEL and SecB [17•,86]. Two important observations were made. Firstly, chaperone binding accelerates the exchange only of those residues that exchange by global unfolding of the native protein. Secondly, a shift from EX2 to EX1 was observed at the globally exchanging sites. When combined with kinetic folding data that show that the highest affinity state is the protected folding intermediate, the results suggest that GroEL and SecB correct misfolding by using protein–protein binding energy to denature a bound, partly folded or misfolded polypeptide to its fully unfolded state, thus allowing it to refold properly. HX analysis of the cyclophilin–GroEL complex also revealed that GroEL can catalyse global unfolding of the bound substrate [87].

Conclusions

Although it is becoming clear that HX can be a reliable method for determining the global stability of a protein, and this may be invaluable for systems that are not amenable to the standard methods of chemical or thermal denaturation, the nature of the structural fluctuations other than global unfolding that lead to exchange is still very much unresolved. The exchange mechanism need not be assumed, but can now be determined at a residue-specific level. Moreover, the range of papers published in the past two years on much larger, more complex systems demonstrates the capacity for HX to investigate questions of dynamic stability. It is probable that the use of other techniques, notably mutagenesis, alongside HX will allow us to fully understand and exploit the technique. Technical advances, including the introduction of mass spectrometry and relaxation methods that can measure very rapid exchange, will allow us to realise the potential of HX measurement as conceptualised 40 years ago by Linderstrøm-Lang.

Note added in proof

The unpublished work by P Dalby, J Clarke and AR Fersht mentioned in the text is now in press [88].

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