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Mechanisms and uses of hydrogen exchange

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Abstract

Recent work has largely completed our understanding of the hydrogen-exchange chemistry of unstructured proteins and nucleic acids. Some of the high-energy structural fluctuations that determine the hydrogen-exchange behavior of native macromolecules have been explained; others remain elusive. A growing number of applications are exploiting hydrogen-exchange behavior to study difficult molecular systems and elicit otherwise inaccessible information on protein structure, dynamics and energetics.

Introduction

Hydrogens on main-chain and side-chain groups distributed throughout all protein and nucleic acid molecules are in continual exchange with the hydrogen atoms of the solvent. Hydrogen-exchange measurements are non-perturbing and can provide site-resolved information on macromolecular properties and their behavior. Protein hydrogen-exchange studies were initiated by Linderstrøm-Lang and the Carlsberg group in the mid-1950s [1,2], and following his untimely death were continued by a small group of devotees. The recent resurgence of interest in hydrogen-exchange studies is attributable to new methodologies, especially NMR and more recently mass spectrometry, and new applications which use hydrogen-exchange labeling to examine difficult biophysical systems. These advances have been paralleled by increases in understanding of the chemical and physical fundamentals of the hydrogen-exchange process itself. (For broad background reviews of this subject, see [2–4].)

Chemistry of hydrogen exchange

The exchangeable hydrogens of proteins include the main-chain peptide group NH and the side-chain protons bound to N, O and S atoms of polar groups. In nucleic acids, the ring imino NHs and the exocyclic amino group (NH_2) protons show facile exchange. Carbon-bound hydrogens do not exchange easily.

Although polar-group hydrogens are labile, they are covalently bound and exchange with solvent hydrogens only as a result of distinct chemical reactions. The underlying proton-transfer steps depend on the acid-donating and base-accepting propensities of the donor and acceptor groups [3,5]. Because of their extreme pKs, peptide group NHs are catalyzed only by H^+ and OH^- ions (in water) so that a log(rate) versus pH curve is V-shaped with a minimum rate occurring between pH 2 to 3, where halftimes average >1 h at 0°C. Nearest neighbor side chains can impose inductive (electron-withdrawing) effects [6,7] that shift the V-shaped rate curve along the pH axis, and steric blocking effects [7] that decrease both acid and base catalyzed rates. The exchange of protein side-chain hydrogens [7–9] and nucleotide

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hydrogens [3,10–12] is similarly catalyzed by H^+ and OH^-, and some groups with moderate pKs may also be catalyzed by weak acids and bases, such as buffer salts [3,5].

In brief, exchange rates depend on pH and temperature [7], the hydrogen isotopes involved [13], buffer salts [3], and added solvents [14,15]. For peptide-group NHs, the important factors have now been accurately calibrated [7,13,14] in small-molecule models, and the calibrations have been validated in unfolded proteins [16,17]. Nucleotide hydrogen exchange (HX) obeys the same chemical principles, but leads to exchange on a time scale no longer than seconds [10–12,18].

Thus it is now possible to accurately predict HX rates for random-coil polypeptides and polynucleotides under any ambient conditions. The manipulation of HX chemistry represents an important variable in any attempt to understand the structural dynamics that determine HX behavior and also for designing experiments that use HX in the many applications now available.

Structural physics

Structural effects can slow protein and nucleic acid HX by large protection factors (P), measured relative to the baseline rate determined by intrinsic chemical exchange. In proteins, P may well reach 10^{10}. Nucleic-acid duplex structure imposes relatively small protection factors, but tertiary folding may cause greater slowing [19]. Protection from HX effectively results from physical blocking of the chemical steps, and almost always involves H bonding. The exchange of protected hydrogens occurs through transient high-energy structural fluctuations that relieve the steric block by physically separating the blocking (H-bonded) groups. These transient conformations have been suggested to involve breakage of a single H bond [1,2], unfolding reactions [20–22], or penetration of the ionic catalysts through the macromolecule [4,23].

The kinetic and thermodynamic relationships that connect structural unfolding reactions with measured HX rates were described by Linderstrøm-Lang and colleagues [1,2], and have been multiply repeated in the subsequent literature. If refolding of the transient structural opening is fast compared with the intrinsic chemical exchange rate [6,7,13], exchange will be a second-order reaction with a rate proportional to the concentration of catalyst. This situation is known as the EX2 limit [2]. The measured exchange rate divided by the intrinsic chemical rate then yields the opening equilibrium constant (1/P) from which one can obtain the equilibrium free energy and other thermodynamic parameters of the operative opening reactions. The EX1 limit [2] is reached when reclosing is slower than chemical exchange. The exchange rate is then equal to the opening rate, from which activation parameters of the operative structural opening can be obtained.

A great deal of effort has been expended to distinguish EX1 and EX2 behavior and thus obtain kinetic or equilibrium parameters for structural opening reactions. One might expect no dependence on pH for EX1 exchange rates and a factor of 10 per pH unit for EX2 exchange rates, but a pH dependence of the opening reaction itself can obscure both of these criteria. EX2 behavior can be confirmed directly for any single condition simply by comparing the opening parameters calculated for two or more protons that have different intrinsic chemical rates [24•]. Concerted EX2 exchange will give the same computed protection factor (equilibrium constant) for all protons exposed by the same opening when their intrinsic rate factors [7,13] are accounted for. In the special case where two NHs in close proximity exchange at the same rate through the same EX1 opening, the amplitude of the NH-NH nuclear Overhauser effect decreases linearly with the degree of H to D exchange [25].

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Protein HX almost always achieves the EX2 limit. Exchange that does not fully satisfy the EX2 limit has been suggested for some examples of local unfolding [26], although this depends on the assumption that opening reactions are pH-independent. Data obtained recently show that even global unfolding reactions often reach the EX2 limit [27•,28,29•,30]. A deduction significant for studies of protein folding is that re-protection, possibly by global refolding, can occur within 50 ms. Indeed, local refolding can occur on a sub-microsecond time scale [31,32]. EX1 exchange has been seen in some special cases, when exchange is attributable to one way unfolding above the melting transition [33], or to transient global unfolding in destabilized proteins [25,29•]. Exchange in duplex nucleic acids generally satisfies the EX2 limit, except at very high catalyst concentrations [10].

Levels of structural unfolding

Recent work has shown that some protein hydrogens exchange with solvent only through transient whole-molecule unfolding, some exchange through sizeable but still subglobal unfoldings, and others exchange through very local fluctuations. These multiple mechanisms have undoubtedly confused a great deal of earlier work designed to understand the structural determinants of HX behavior.

The globally unfolded state is populated only at very low levels under native conditions, as dictated by the Boltzmann relationship. Nevertheless, global unfolding can govern the exchange of hydrogens that are otherwise very highly protected. Exchange through transient global unfolding has now been demonstrated for cytochrome (cyt) *c* [27•], ribonuclease A [27•,34], staphylococcal nuclease [28], barnase [29•], protein GB2 [35], OMTKY3 [30] and the G domain of protein A (Y Bai, P Wright, personal communication). In such cases, HX measurements of the most protected NHs can be used to obtain equilibrium thermodynamic parameters for the global unfolding reaction (e.g. $\Delta G = RT\ln P$) at conditions far below the melting transition, avoiding the problems commonly encountered in classic melting experiments.

A hitherto unsuspected HX mode that involves the cooperative unfolding of large but still subglobal segments of structure has been found in experiments with cyt *c* [24•]. Some cyt *c* hydrogens can exchange only through these large (or even larger, e.g. global unfolding) unfolding reactions. Other NHs within each cooperatively unfolding segment can exchange more rapidly, through smaller opening reactions, but these NHs are entrained by the larger unfolding reactions when the large unfoldings are selectively promoted by low concentrations of denaturant or mildly increased temperatures.

This kind of HX behavior was found for four structural units, composed of α-helices and Ω-loops, that together account for the cyt *c* molecule. The identity of each unfolding unit can be revealed by the identity of the hydrogens that the unfolding entrains. The free energy of each unfolded state and its other thermodynamic parameters can be obtained from the measured HX rates and their dependence on temperature. The factors that determine the cooperative subglobal unfolding units seem fundamentally important for understanding protein design and cooperativity, but remain poorly defined.

Global and subglobal unfoldings transiently produce random-chain conformations that fully expose main-chain NHs and release side-chain constraints. For global unfolding, this seems clear from the agreement found between values for the free energy of unfolding measured by HX and values separately measured in classical melting experiments [27•,28,29•, 30,34,35]. The random-coil conformation can be documented by the equivalence of unfolding equilibrium constants calculated for NHs with disparate intrinsic chemical-rate factors, as in the EX2 test noted before [24•]. Deviations from equivalence will identify residual blocking in the open state.

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For local protein fluctuations, which determine the exchange of many protein hydrogens, both the unfolded conformation and the native-state determinants are currently obscure. Some local fluctuations appear to expose only one hydrogen at a time, others involve four to eight concerted hydrogens [36]. The large HX slowing factors often seen for local fluctuations point to surprisingly large free energies. It seems possible that residual blocking in locally unfolded states may be a serious issue [7,22,27•], even if not in larger unfoldings. Nevertheless, free-energy changes measured by some local unfolding reactions have been found to match externally measured values [37,38].

In duplex nucleic acids, exchange appears generally to depend on local base-pair scissoring motions [10]. The behavior of nucleic-acid hydrogens protected by tertiary folded structure remains to be documented.

In summary, new advances include the ability to distinguish global, subglobal and local unfolding events. These measurements reveal parts of the otherwise invisible manifold of conformationally excited partially unfolded states. The measurement of whole-molecule unfolding can provide global thermodynamic parameters directly at native conditions. The cooperative subglobal unfolding illuminates the multi-state nature of protein structure and may define kinetic folding intermediates [24•]. The ability to distinguish these different classes of unfolding will be essential to understanding the still enigmatic local fluctuations. In this effort, due consideration should be given to the possible role of large steric blocking effects in the partially unfolded state.

Applications

Hydrogen exchange can be measured at an unresolved level by tritium counting and by various spectroscopic methods (infrared [39,40], Raman [41], UV absorbance [42], NMR), at intermediate resolution by tritium exchange labeling together with fragment-separation methods [14,43,44], and at the ultimate resolution of individual protons by NMR methods and neutron crystallography [21]. Recent applications of mass spectrometry also hold promise [45–50,51•]. These methods utilize all combinations of H-H, H-D and H-T exchange. Straightforward HX measurements are now widely used to investigate structure and dynamics in proteins, to obtain site-resolved information on bond energies [38,52•,53], and as an aid in NMR assignments.

The use of HX labeling approaches yields special advantages. Here, differences in HX rate are used to selectively place exchangeable label on sites of interest under conditions best suited for the experiment. The label is then locked in place by exploiting the chemical and physical determinants described above, and the H-D exchange pattern is read out under conditions optimized for the analysis [36,54].

HX labeling has been used to define the secondary structure present in partially folded equilibrium forms. The protein is exposed to H-D exchange labeling for increasing periods of time, and the timed samples are then returned to native conditions where the H-D pattern is trapped and can be analyzed by high-resolution NMR. This approach has been used to study proteins under molten-globule conditions [55–63], in organic solvents [64–66], and in the lyophilized state [67]. Such forms are not accessible to the usual crystallographic or NMR approaches except in special cases [68,69].

A similar HX labeling approach has been used to study interactions and dynamics at high resolution in complexes that are otherwise too large for NMR analysis [70]. Hydrogen/deuterium-exchange labeling is performed in the complex, the components are separated, and then one is separately analyzed for the H-D pattern generated while in the complex. Recent work has studied the cyt *c*–cyt *c* peroxidase complex [71,72], protein–antibody

interactions [35,70,73,74], enzyme–inhibitor complexes [75,76], and the interaction of proteins with the massive GroEL chaperonin [51•,77,78], with hydroxyapatite [79], with micelles [80], and in the crystalline state [81,82]. The observation in some of these studies of long-range HX changes that are remote from the apparent interaction surface may profit from reinterpretation in terms of the larger-scale unfolding reactions noted before. Some recent studies of large complexes by direct HX measurement in the complex are noteworthy (calmodulin–target peptide [83•]; DNA–repressor [84]).

A most striking application is the structural elaboration of kinetic folding intermediates that exist for <1 s. In fast-mixing experiments, the HX pulse-labeling experiment [85,86] can label transient intermediates in a structure-sensitive way with better than 10 ms time resolution using H-D exchange. At various time points during the ~1 second period during which a protein is refolding, a H-D exchange labeling pulse is imposed by suddenly raising the pH to 9 in order to label peptide NH sites that are not yet H-bonded. (At pH 9.5 and 10°C, the exchange halftime is ~1 ms.) The structure of intermediate forms that may become transiently populated at various times during the kinetic folding sequence can then be inferred by high-resolution analysis of the H-D labeling pattern trapped in the refolded native protein. This experiment has now been performed successfully for about a dozen different proteins [54,87–90].

The capability to discern folding intermediates may be greatly enhanced by the native-state HX experiment noted above, which can define sizeable but still subglobal structural unfolding units [24•]. Results available suggest that the high-energy partially unfolded forms (PUFs) seen in an equilibrium sense by native-state HX may also represent the kinetic intermediates that determine folding and unfolding pathways. For example, in cyt *c*, the highest energy PUF seen in the native-state experiment [24•] corresponds to the kinetic intermediate defined by HX pulse labeling [86]; both structures retain intact N- and C-terminal helices. However, an intermediate detected in kinetic-folding experiments with barnase [91] was not separately discerned by native-state HX [29•].

All of the HX applications just noted use a kinetic labeling mode in which hydrogen-isotope label is selectively emplaced by virtue of simple differences in HX rates. A more complex functional labeling method causes the hydrogen-isotope label to be placed selectively at functionally important sites by virtue of the change in HX rate that those sites experience in the functional transition being studied. For example, in experiments on the allosteric transition in hemoglobin (Hb) [36] and on protein structure change in active muscle [92], tritium label was exchanged-in for limited times in the fast-exchanging form (oxy-Hb; activated muscle), then locked into the slow-exchanging form (deoxy-Hb; rigor muscle), and exchanged-out for increasing times. This protocol produces samples with label positioned at allosterically active sites only. The functionally interesting sites can then be identified, and the sensitivity and energetic contribution of the individual sites to allosteric state, effectors, and other modifications can be separately measured. These methods are especially pertinent for proteins that are too large for NMR analysis, and have used instead a tritium-labeling and fragment-separation analysis [43,44]. The addition of on-line mass spectrometry promises to enhance the resolution obtainable [45].

HX labeling approaches have not yet been applied to nucleic-acid studies, where the intrinsic chemical rates are relatively fast, and slowing due to secondary structure is much less effective than in proteins. These factors make it difficult to lock in and later analyze H-D exchange patterns using high-resolution methods. The largely unexplored role of tertiary-structure interactions in more effectively slowing nucleic-acid HX may hold promise in this regard.

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Conclusions

Increases in our knowledge of and our ability to control the chemical and physical determinants of macromolecular HX processes has led to applications that can analyze systems and acquire information not generally accessible by other means. Recent work has shown how detailed information on protein structure, dynamics and energetics can be obtained for massive complexes, poorly structured equilibrium intermediates, short-lived kinetic intermediates, allosteric systems, and the high-energy partially unfolded forms that constitute the excited-state manifold of protein and nucleic-acid molecules. These HX approaches now provide an unparalleled opportunity to probe the energetic and dynamic foundations of macromolecular structure and structure–function relationships.

Abbreviations

cyt	cytochrome
D	deuterium
HX	hydrogen exchange

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•• of outstanding interest

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