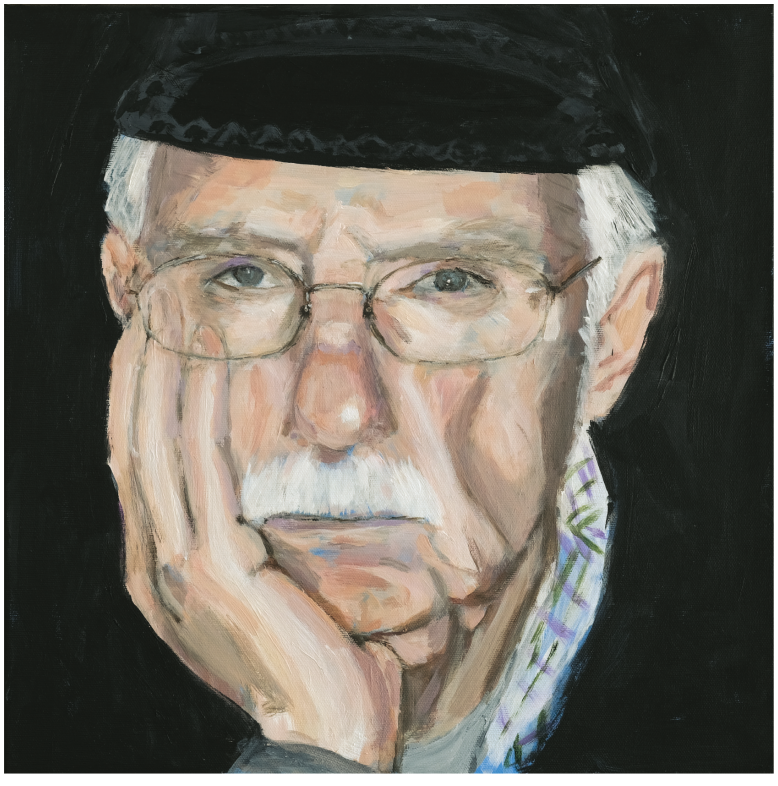

S. Walter Englander

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HX and Me: Understanding Allostery, Folding, and Protein Machines

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Abstract

My accidental encounter with protein hydrogen exchange (HX) at its very beginning and its continued development through my scientific career have led us to a series of advances in HX measurement, interpretation, and cutting edge biophysical applications. After some thoughts about how life brought me there, I take the opportunity to reflect on our early studies of allosteric structure and energy change in hemoglobin, the still-current protein folding problem, and our most recent forward-looking studies on protein machines.

Contents

INTRODUCTION

As a child, all things science were fascinating to me, and I felt that scientists must be like gods. Atypically, I never had a chemistry set or built a ham radio. Instead, I found inspiration in the night sky. I can still see it, pitch black in those days, with thousands of shining stars, each one a whole other world. And how small was I.

In real life, my childlike attempts to think like a scientist had to compete with the intense religiosity of my father and our toxic home environment, reinforced by the Depression, widespread antisemitism, and the sudden cessation of a flood of mail from family left behind in Nazi-occupied Hungary. I was attracted by the intellectualism and the humanism of Judaism but oppressed by the doctrinal demands that I felt compelled to obey. That mindset lasted until I entered graduate school and was able to construct a counter-narrative. If God exists, and he knows that the worst depredations of man against man spring from blind faith, leading the world's most civilized people to foster the Nazi horrors, then he would judge blind faith, including believing in him, as the worst possible sin. I found this reasoning to be marvelously liberating. It may also help to explain my lifelong devotion to the sanctity of truth, religious in its intensity, and therefore my knee-jerk iconoclasm. In an age of disinformation, I believe the mantra "how do you know that" should be taught starting from kindergarten.

I was shy and timid as a child, and that lasted well into my adult years. It later turned out that I love to talk about and argue about science, but I've never learned how to do social chitchat. The fact that I happened to be good at sports—football and softball in season—helped to keep me somewhat balanced. I eventually attended the University of Maryland and got through college by working summers as a busboy in a Long Island hotel, where a cousin ran the dining room and where the well-to-do New York machers parked their wives for the summer. It was a great time. I learned all of those wonderful Hungarian curse words, had my first real girlfriend, spent every afternoon on the beach, and made a lot of money in tips. It did spell the end of my softball career—except for our annual departmental retreat later at Penn, faculty vs students, which we systematically lost (**Figure 1**).

At Maryland, I discovered physics and math and did well, but I had never had to study and never learned how to do it until I finally had to in my third year. My mental IQ saved me, but when it comes to practical world things, I am hopeless. I attribute all of that to a 2,000-year collaborative experiment in applied evolution in which the external world kept the Jews separate while the community rewarded scholars with the best wives and lifelong funding.

As graduation approached, I applied to 10 graduate schools in biophysics, got accepted by all but the University of Pennsylvania, ironically, and went to the University of Pittsburgh, the only one that offered me financial support, unlike nowadays. I worked on tobacco mosaic virus RNA,

Figure 1

Faculty 2, students 11.

lysogeny in lambda phage, analytical ultracentrifugation, and electron microscopy; met my future wife and lab colleague Joan Jenkins; got married (1953); and was drafted into the post-Korean-War army. Apparently my father managed to sway the draft board in a vain attempt to separate me from my shicksa girlfriend.

I had imagined that the army, marching to music and playing ball with the boys, would be fun. It was impressively bad. Among all of the other evils, it was Joe McCarthy days, and the FBI considered me a security risk. My graduate school professor had earlier worked with a professor who served years in prison for testifying that he didn't know the soon-to-be executed atomic spies, Ethel and Julius Rosenberg, when it was later shown that he had met them at a social event. Accordingly, I spent a year peeling potatoes, and much worse, and became the oldest unpromoted buck private in our unit. I eventually got security clearance by conning an FBI interviewer and was transferred to Dugway Proving Grounds in Utah, the top-secret center of US bacteriological and radiological warfare. I worked in the most secure area, entering each day with a temporary badge because the army had lost my papers and never really ascertained that I had indeed been cleared. It was a good period except that we were downwind from the Nevada Nuclear Test Site, which I blame for the death of our first son from leukemia two days before his sixth birthday.

On returning to civilian life, I went back to grad school, got my PhD, and joined Bill Harrington's lab in Chris Anfinsen's section at the US National Institutes of Health (NIH) for a first postdoc. At the time, Chris was slogging away on his Nobel effort on protein folding, which

Figure 2

Joan Jenkins Englander, lab tech extraordinaire.

may sound providential given my later interest, although it was only coincidental. There I met my to-be-lifelong friends Pete von Hippel, Marty Gellert, and Gary Felsenfeld, outstanding scientists all and all still productive into their 90s. It was Gary at the University of Pittsburgh who had earlier directed me to Bill Harrington's lab, where I first learned about proteins, working on ribonuclease denaturation and proteolysis, and then on myosin structure with Marty Gellert by multiangle light scattering to decide how many strands myosin has (29).

I then followed our collaborator, Pete von Hippel, for a seven-year experience at Dartmouth. Pete realized that I was good at thinking, not so much at doing, and had the great sense to hire my wife Joan to work with me. That insight led to a 40-year period during which Joan served as an astonishingly able lab scientist (**Figure 2**). She would come in in the morning, make her buffers, do the day's experiment, and not go home until she could look at the results, typically a three-day job. She also handled all of the lab business at which I was generally hopeless. We lost Joan after 46 years due to an unsuspected heart condition. Eight years later, my care and feeding were taken over by another good and talented woman, Carole Clarke, who among many other things painted the image at the beginning of this essay. It turns out we had grown up about two miles apart in Baltimore but didn't meet until very much later at Penn where Carole wrote speeches for the president and others.

I've always been very impressed by how kindly providence has treated me, in thrusting hydrogen exchange into my hands, which opened the door to precisely the kind of puzzles I like to wrestle with, and especially in bringing to my lab so many talented people who were happy

to join in doing what I love to do. My long-time colleague Leland Mayne has made inestimable contributions to our work over almost 40 years. He understands everything and can do anything. Josh Wand brought nuclear magnetic resonance (NMR) to our lab after teaching it to himself in visits to distant labs. He assigned the Cytochrome c (Cyt c) 2D NMR spectrum using a new strategy he developed and allowed us to justify the purchase of our own high-field magnet. He made all of our folding work possible. Bob Molday and Yawen Bai laid the groundwork that underlies all hydrogen exchange (HX) analysis. Yawen together with Tobin Sosnick developed the bases of our protein folding work. ZhongYuan Kan's experimental and computer skills made all of our HX mass spectrometry (MS) analysis work so effective. The expert work of Xiang Scott Ye on AAA+ protein machines now shows the way toward the future of HX MS studies. Many others, who I apologize for not naming, have contributed importantly to our progress.

In mapping out this brief autobiography, I have taken the opportunity to revisit and reflect on a few of our previous efforts. For readers with the courage to proceed, I hope this tour may provide some insights that benefit their own science.

MY INTRODUCTION TO HYDROGEN EXCHANGE

My scientific life has tracked HX methodology from its earliest days to its current frontline status. Although our research has touched on many fields, most of it flowed from an early experience that wholly by accident thrust me into contact with the hydrogen exchange phenomenon, just then pioneered by Kai U. Linderstrøm-Lang and his coworkers at the Carlsberg Labs (35, 49, 50) in Copenhagen. At the NIH, my mentors Bill Harrington and Pete von Hippel were interested in collagen structure, fashioned on the poly-L-proline II helix, which is remarkably stable despite the absence of any H-bonding. Could that be explained by water molecules cross-bridging between backbone carbonyls? I put together a method based on the labeling of water by radioactive tritium (T), which had become available due to then-current H-bomb development, together with the new Sephadex gel filtration capability. The idea was to tritium-label water molecules, perhaps bound to polyproline, then quickly separate the polymer from tritiated solvent by gel filtration and measure the hopefully still-bound radioactive tritium. Well of course it didn't work, but it turned out that the same method was able to measure protein H-T exchange.

The tritium-gel filtration method is artifact free and much simpler than the complex methods developed by Lang. For the next 25 years it supported HX studies by a growing community of devotees. I first tried it out on ribonuclease A. It worked wonderfully and directly measured the wide range of kinetic HX for all of RNAse's 124 residues. Those many hydrogens had to come from all over the protein, presumably including Pauling's α-helix and β-sheet structures and embodying the dynamic motions that allowed his protecting H-bonds to separate and reform as Lang had postulated (50). It took me a year to fully believe that I could actually measure molecules in this way. Pete von Hippel, who participated in all of the thinking and whose grants had funded the work, did not put his name on that groundbreaking paper (18). It does turn out that some scientists have elevated qualities. I've tried to follow Pete's lead.

The Dartmouth experience led to an interview at the University of Pennsylvania and my first public seminar about this backwater hydrogen exchange thing, which was totally boring and poorly delivered. Mildred Cohn saw something in it and so I was hired. I have worked there for 54 years. Grant funding was at first easy thanks to the explosion of government support for science due to Sputnik and Vannevar Bush, even though no one could appreciate the potential of HX at the time. I didn't either. Nevertheless, our funding has managed to last for all of that time, allowing me the freedom to follow my own interests and instincts, often different from what my grant applications proposed. Our continuing success with funding was helped by my preference for a small lab, which

allowed me to be hands-on day to day and, most centrally, to focus on problems I judged to be important rather than on many incremental publications needed to justify large lab funding. Long experience has made it clear to me that the most important element for doing creative science is the continuous interaction of a small group of devoted researchers. I think three is the optimum.

The development of hydrogen exchange science and its applications have accounted for much of our activity over the years. That may seem limiting, but actually the broad applicability of HX to all things molecular has allowed us to roam and to explore widely. We developed the methods, the chemistry, and the interpretation of HX in terms of macromolecular structure and dynamics and used it to study nucleic acid systems ranging over synthetic polynucleotides, tRNA, ribosomes, and chromatin; protein systems large and small ranging over synthetic polypeptides, RNAse A and H, myoglobin, hemoglobin allostery, sickle cell hemoglobin, haptoglobin, maltose binding protein, collagen, amyloid, frog and bacterial rhodopsin, antibody epitopes, GroEL, Hsp104, and even active muscle; and lipoproteins A-1, C-3, E-3, and E-4.

HYDROGEN EXCHANGE MEASUREMENT, CHEMISTRY, AND PROTEIN DYNAMICS

Starting from the seminal work of Linderstrøm-Lang and his colleagues, our development of HX methodology has progressed from the tritium-gel filtration method (18), through fast dialysis separations (21) and optical absorption differences (13), into site-resolved NMR (75), then a kinetically resolved functional labeling method (65), and then a fragment separation method using high-performance liquid chromatography (HPLC) (16). It became clear that we needed to add a second dimension of peptide fragment separation that used MS. Efforts with Steve Carr at SmithKline in Philadelphia and then Ed Burlingame at UCSF failed because electrospray ionization had not yet come along. When it did (28), we managed, in collaboration with David Smith at Purdue and Nebraska, to add a final MS fragment resolution and analysis stage, now known as HDX-MS, or HX MS in my preferred terms. After several more collaborations, we were able to purchase our own MS equipment, which allowed us to develop needed improvements (39, 40, 52, 74). HX MS has now grown to become a leading biophysical technology for many hundreds of biomolecular studies (12) and has even led to its own International Society for HDX-MS.

In order to interpret measured HX rates in terms of molecular structure and dynamics, one has to know the basic HX rates in the absence of structure and their dependence on environmental parameters. Bob Molday, Yawen Bai, and coworkers calibrated in detail the exchange behavior of the main chain protein amide hydrogen (3, 10, 26, 53, 55). Amide HX is catalyzed only by hydroxide and by hydronium ion, according to the principles laid out in Manfred Eigen's proton transfer theory (11). HX rate also depends on temperature, isotope effects, size of the molecule, some polypeptide end effects, and neighboring side chain effects, all piled onto the basic peptide rate. We calibrated all of these, including the effects of all 20 amino acid side chains to both the left and the right of the main chain amide. We showed how to efficiently aggregate all of these effects to produce an accurate prediction of structurally unprotected HX rates (3, 53). A spreadsheet that calculates these rates is available for download from our website (**http://HX2.med.upenn.edu**). These values underpin all efforts to interpret protein HX rates, as indicated by several thousand citations. Buzz Baldwin has written a scholarly account of these developments based on the talk he gave at my 80th birthday celebration (6).

The rich variety of protein motions translates into HX behavior that is piled onto the basic unhindered rate. After a long period of uncertainty and controversy, Linderstrøm-Lang's early intuition about HX mechanism and the equations he wrote have turned out to be spot on (see 35, 50). Structurally protected hydrogens exchange slowly because they are protected by H-bonding.

They must be exposed to exchange by a transient H-bond-breaking structural opening reaction (k_{op}) (9, 70). Given the calibrated HX rate for the unprotected state, $k_{ex} = k_{ch}$[cat], where [cat] is [OH^-] above pH ~ 3 or [H^+] at lower pH, the measured HX rate then gives the equilibrium constant of the opening-closing reaction ($k_{ex} = k_{op}/k_{cl} \times k_{ch}$[cat] = $K_{op}\ k_{ch}$[cat]) and therefore the stabilization free energy opposing opening ($\Delta G^0_{op} = -RT \ln K_{op} = +RT \ln Pf$). $Pf = k_{ch}[cat]/k_{ex}$ is the HX slowing or protection factor. This is the EX2 bimolecular case, where $k_{cl} >> k_{ch}$[cat]. If k_{ch}[cat] ≫ k_{cl}, exchange occurs on every opening. This is the EX1 or monomolecular exchange case, where the exchange rate gives the structural opening rate ($k_{ex} = k_{op}$). (Subscripts op, cl, and ch indicate opening, closing, and chemical, respectively; [cat] is catalyst concentration.)

We have abundantly verified these relationships (see below). Thus, site-resolved HX measurement now offers the extraordinary capability for specifying in detail structure, dynamics, thermodynamics, and change therein throughout any protein molecule.

ALLOSTERIC STRUCTURE CHANGE AND ENERGETICS

In 1970, we set out to test the ability of HX methods to detect in hemoglobin (Hb), the classical allosteric protein, its individual structure changes, the energetics of each change, and the transmission of allosteric energy though the protein (19). I take this opportunity to summarize the results we obtained and outline an energy-centric perspective that has been absent before.

Our Hb effort was inspired by the structural work of Max Perutz (58, 59), the energy-related insights of Wyman (76) and Monod (54), and the supposed connection of HX with structure and energetics postulated by Linderstrøm-Lang (see 35, 50). Regulatory structure change in Hb and other proteins is intimately connected with energy transduction and transfer. In Hb, some of the binding free energy of initially bound oxygens is diverted into structure change, leading to reduced initial oxygen binding affinity. The energy is carried through the protein by structure changes and applied to release constraints that inhibit binding by not-yet-liganded hemes, resulting in enhanced binding affinity for later oxygens. This strategy produces the functional sigmoid O_2-binding curve. Understanding how all of that works requires the ability not only to identify the working parts of the machinery but also to measure site-resolved energy changes, and trace the pathways of energy transfer through the protein. We found that HX can provide this capability.

As always, it is first essential to know the detailed structure. The momentous contributions of Max Perutz and his coworkers supply that information (58, 60, 61). The homologous α and β subunits in the Hb $\alpha_2\beta_2$ tetramer comprise seven and eight helical segments, respectively, labeled A to H, as shown for the β-subunit in **Figure 3*a***. Helix E and F form a V-shaped cradle for the heme group. Perutz's work imaged Hb structure and the structure changes that accompany allostery, but imaging is blind to the energetics that power and stabilize interactions. Even today, explanations of protein function usually depend on rationalizations inferred from structural images rather than on firm energetic information.

HX is able to read out structural energetics because the same interactions that stabilize against allosteric transitions also stabilize against local unfolding reactions that can determine HX behavior. We explored this essential matter by deriving the relationships that connect the ΔG, ΔH, ΔS, and ΔCp parameters of individual bonding interactions to the same parameters that contribute to the stability of the protein (68). The stabilization free energy provided to the host protein [$\Delta G_{prot} = -RT \ln (K_{bond} + 1)$] is close to, although a bit greater than, the bond free energy ($\Delta G_{bond} = -RT \ln K_{bond}$).

We developed the functional labeling and fragment separation methods and used them to identify the segments that change and measure their HX, as illustrated in **Figure 3**. Most of Hb's exchangeable hydrogens, approximately 3/4, are insensitive to allosteric change and tend to exchange slowly, perhaps because they represent the stable protein framework. Hydrogens that do

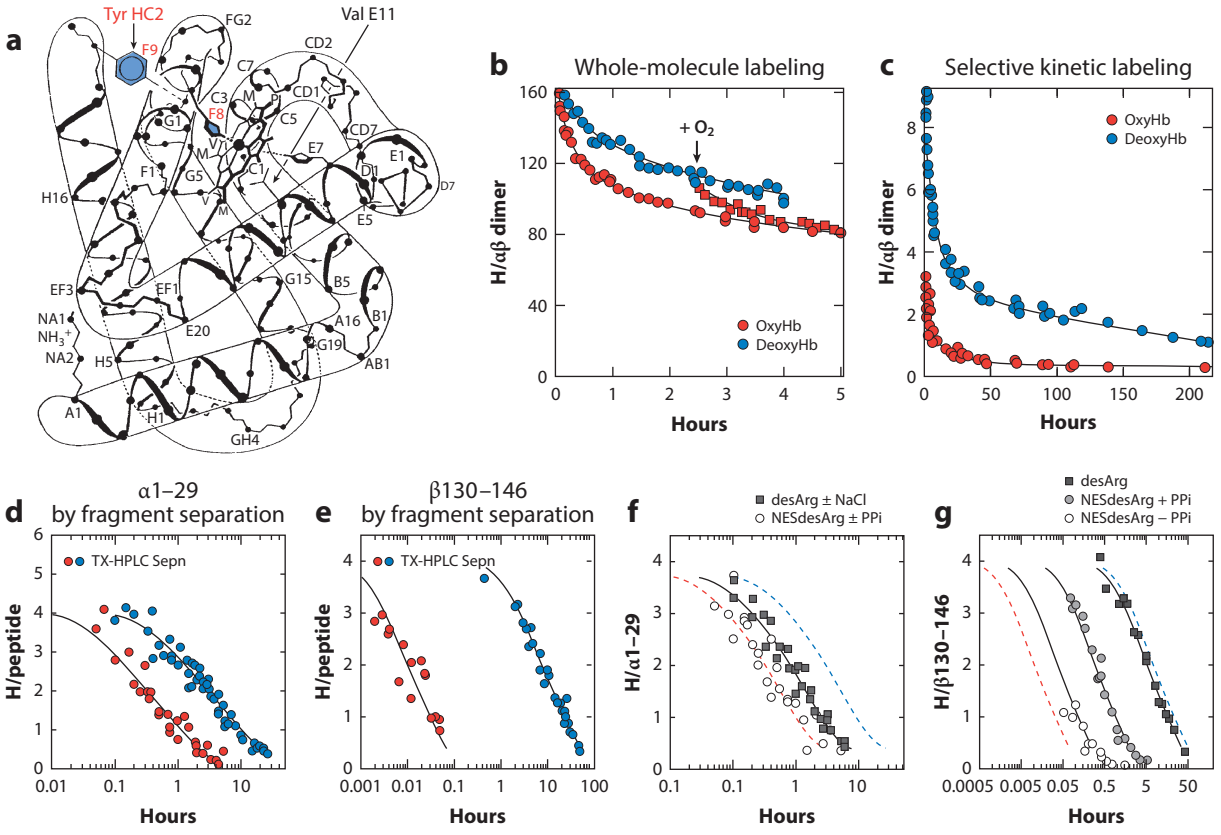

Figure 3

Allosterically sensitive segments of hemoglobin (Hb) seen by selective H-T exchange labeling and fragment separation. (*a*) The Hb β chain with the important intrasubunit energy transmission pathway from the heme binding site to the β-chain C terminus at the upper left. Panel adapted with permission from Reference 61. (*b–g*) The course of methods development that revealed structure changes at increasing resolution. (*b*) H-T exchange at a whole-molecule level. Allosterically sensitive amide sites exchange more rapidly in oxyHb (*red*) when important salt links are broken, but in a whole-molecule experiment they are seen only above a large background of allosterically insensitive sites. (*c*) The functional labeling kinetic selection method. In functional labeling a short exchange-in selectively tags allosterically sensitive sites that exchange-in fast in oxyHb and exchange-out much more slowly in deoxyHb. The large obscuring background of sites that exchange at the same rate in oxy- and deoxyHb is practically removed. (*d*, *e*) After selective labeling, proteolytic fragmentation with the peptide fragments separated by high-performance liquid chromatography then isolates allosterically sensitive segments and records their hydrogen exchange. The chain terminal fragments α1–29 and β130–146 are shown as they exchange in oxyHb (*red*) and deoxyHb (*blue*). All sets are kinetically single exponential, diagnostic for cooperative segmental unfolding. (*f*, *g*) The effects of some detailed modifications while Hb is still in the T-state. Dashed curves trace the position of the unmodified red and blue curves.

change appear in small coherent sets. The amides in each set, although located sequentially along α-helical segments with varied environments, exchange at close to the same rate in deoxyHb and each whole set moves in unison to a faster rate when stabilizing cross-links are broken in oxyHb. These results reveal cooperative segmental unfolding reactions. **Figure 3*d*** and **Figure 3*e*** show results for two especially interesting allosterically sensitive sets, at the α-chain N terminus (helix αA) and the β-chain C terminus (helix βH).

In the Linderstrøm-Lang model (35, 50), the HX rate of each set of exchangeable hydrogens proportions to the equilibrium unfolding of that segment (EX2 HX) and therefore provides a

direct read-out of equilibrium segmental stability and change in stability. Upon the deoxy-to-oxy transition, the α-chain N-terminal helical segment exchanges ninefold faster (**Figure 3*d***) indicating a stability loss of 1.2 kcal/segment (2.4 kcal/tetramer). The β-chain C-terminal segment is 190-fold faster (**Figure 3*e***) indicating a loss in stability of 2.8 kcal/segment (5.6 kcal/tetramer). We compared these energy changes measured by HX with the values found by the Gary Ackers and Stan Gill labs, who used whole-molecule methods to measure the difference in subunit dissociation and in O_2 dissociation (22, 73) between deoxy and oxy states. The total ΔG measured by HX, 8.1 kcal/tetramer (at 0°C), compares to the overall stability change of 8.3 kcal/tetramer (5°C) measured by whole-molecule methods. Thus, the total difference in allosteric free energy between the oxy and deoxy states (called R- and T-states) is realized in the terminal intersubunit cross-linked segments!

The important involvement of chain termini in both Hb allostery and kinetic protein folding (see below) encouraged Krishna Mallela to pursue a broad analysis of the entire Protein Data Bank. Krishna's results point to some special and widespread role for protein chain termini interactions (42).

We also found a set of exchanging hydrogens that signals an allosteric energy change not at the chain termini but on the F-FG segment between the heme and the C-terminal helix. Upon heme liganding, a seven-residue length of the F-FG segment in the β-chain is destabilized by 2 kcal, signaled by a 30-fold increase in its local unfolding (15). The F-FG segment in the α-chain behaves similarly but was not studied in detail. Surprisingly, the measured 2-kcal change in β-chain F-FG segmental stability does not make a separate contribution to the overall T/R equilibrium. The reason appears to be that the F-FG segment functions as a connector in the intrasubunit energy transmission pathway. It registers the same strain energy that is subtracted from O_2 binding in the T-state and delivers it to the intersubunit bonds.

Detailed probing by imposing mutations and chemical modifications at suspected allosterically sensitive sites while still in the T-state subdivides these energies, as illustrated in **Figure 3*f*,*g***. These more localized values, tabulated in Reference 14, also agree with conventionally measured whole-molecule changes, although it is noteworthy that individual salt link energies cluster around 0.6 kcal/site, below the value often used in computational studies.

These results add the energy dimension to the structural studies of Perutz and others and suggest a coherent picture for how Hb mobilizes energy transfer and energetic structure change to manipulate its O_2 binding affinity. Perutz found that the heme group in deoxyHb is domed and that O_2 binding makes it more planar. HX showed that heme flattening is made difficult by the resistance of the adjacent F helix liganded to the heme iron by His92(F8)β (**Figure 3*a***). We measured the strain imposed on the F-FG segment in terms of its change in stability upon heme liganding (15). The 30-fold increase in F-FG HX rate corresponds to 2 kcal in segmental destabilization. At these same conditions (0°C, pH 7.4) Yonetani et al. measured a reduction in T-state O_2-binding affinity by 30-fold (83), i.e., by the same 2 kcal destabilization measured for the strain induced in the resisting F-FG segment. Evidently the strain energy is subtracted from and equivalently reduces the binding energy of initially bound oxygens.

The same energy is transmitted through the protein to the intersubunit salt links and deployed to power the T-to-R quaternary allosteric transition. Transfer of the strain energy through the protein is initially accomplished by an intrasubunit chain of directly adjacent residues (**Figure 3*a***). Perutz noted that the pull on HisF8 shifts the F-FG bridge, which frees TyrHC2(145)β from its pocket, impacting the neighboring HisHC3(146)β (**Figure 3*a***, *upper left*). This locally connected intrasubunit energy transfer chain carries the O_2-induced strain to the cross-subunit salt links at the chain terminus. Analogous changes impact the cross-linking salt links that connect the two α-subunits at their N- and C-terminal residues. When the cross-subunit salt link system is

sufficiently destabilized, the R-state becomes the lower free energy form and a whole-molecule T-to-R transition then occurs.

The global allosteric transition represents a second kind of energy transfer strategy. The transition accomplishes long-distance (intersubunit) energy transfer, communicating with other links that normally act to stabilize the T-state. We found an analogous situation in the Hsp104 AAA+ system described below (80), suggesting that the characteristic allosteric quaternary structure change may serve as a common strategy for long-range energy transfer. One can imagine that the evolutionary development of a long residue-to-residue transmission chain would be much more difficult.

This picture also provides a possible explanation for some related issues. Yonetani et al. (83) demonstrated an extraordinary range of Hb O_2 affinities, approximately 50-fold in K_T, as a function of various allosteric effectors while Hb is still in the T-state. This appears to reflect a direct connection to the HisF8 constraint without a T-to-R transition. The same view may also bear on the various models that have been proposed to account for aspects of Hb allostery (for a review, see 31) by recourse to unspecified tertiary structure changes labeled t and r. It seems likely that taut and relaxed forms of the HisF8 constraint and its associated local chain (**Figure 3**) can account for the postulated tertiary structures.

In summary, these HX studies illuminate Hb's energy economy. Hb subtracts some of the energy of initial O_2 binding, transduces it into mechanochemical form, and transmits the energy through the protein by way of a short chain of local residue contacts. The chain connects the ligand site to intersubunit interactions that govern the quaternary structure change, which then connects to distant liganding constraints. The inter- and intrasubunit mechanisms (T to R and t to r) commonly occur together in one cooperative whole-molecule transition, but they can be separated, identified, and characterized, structurally and energetically, by site-resolved HX measurements.

Our Hb project accomplished several more general advances—the functional labeling method; the fragment separation methodology, which led us to the modern HX MS method; the demonstration of locally cooperative unfolding reactions; their use for locating structure changes and measuring the energetics of each; the suggestion of a special role of chain terminal interactions; and the discovery of two different classes of energy transfer pathways, an intrasubunit push-pull chain of directly adjacent residues and a long-range intersubunit global transition system. Other results were found that seem quite interesting including cross-subunit tertiary structure effects while still in the T-state (17).

PROTEIN FOLDING

The folding problem has long occupied a central place in protein biophysics and it continues to impact many current issues. Yet there is still no consensus about how proteins fold and why they fold in that way. The problem is that during folding the system is heterogeneous (U, N, I_i) and the intermediates (I_i) that carry proteins from their unfolded (U) to their native (N) state are only minor constituents, they have brief lifetimes, and they cannot be isolated for study.

Wolynes, Onuchic, and others have developed a statistical-mechanical energy landscape model retaining the early idea (8) that protein folding proceeds through innumerable tracks that progress by incorporating one amino acid at a time in random order (7, 46, 57). The model is often represented in terms of a funnel-shaped energy landscape, as in **Figure 4*a***, which simply graphs the primary thermodynamic requirement that spontaneous folding must proceed energetically downhill, losing conformational entropy as it goes. The funnel alone, like any thermodynamic statement, contains no mechanistic information.

Cyrus Levinthal considered and rejected the possibility that proteins might fold one amino acid at a time (47, 48) except in the case of very small proteins since the difficulty scales

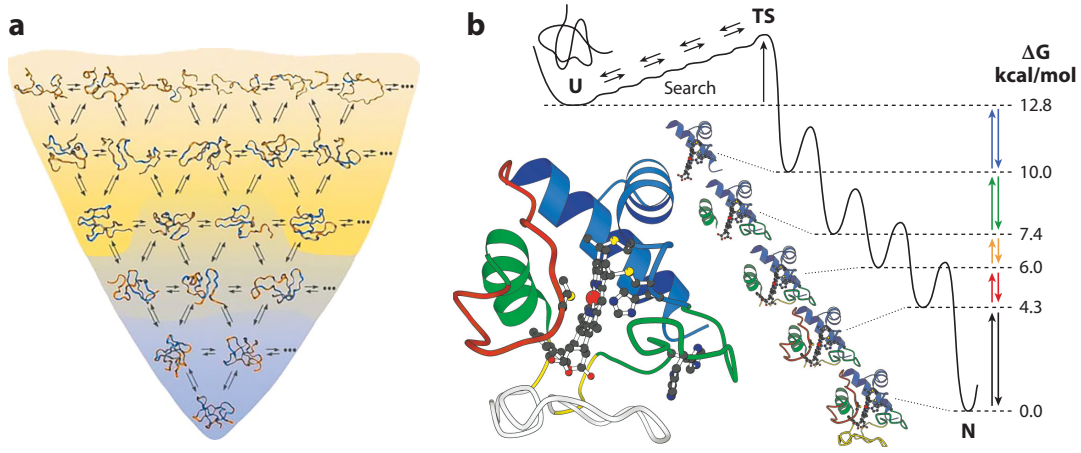

Figure 4

Protein folding models. (*a*) The funneled multipathway model (adapted from Reference 56). Unfolded proteins navigate to the native state through many amino acid–level pathways by diffusing down a funnel-shaped landscape, driven by decreasing energy, plotted as the vertical *Z* axis of the funnel, and opposed by decreasing conformational entropy plotted in the *XY* plane. (*b*) The foldon model exemplified by Cytochrome c (Cyt c) (104 amino acids). Experiment shows that Cyt c folds through the stepwise energetically downhill formation of native-like cooperative foldon units, referred to here as blue, green, yellow, red, and infrared, pictured in the molecular model and named in their energetically descending order of formation.

exponentially with protein length. However, such small proteins account for only approximately 5% of the proteome. For more sizeable proteins, finding correct amino acid partners by chance diffusional encounter alone among the astronomical number of competing choices would take nearly forever whereas real proteins often fold in seconds. In answer to the Levinthal problem, a novel feature of the energy landscape proposal is the assertion that the residue-level search for correct partners is able to succeed due to a bias against wrong partners, explained as a hypothetical minimal frustration principle imposed by evolution (57). The physical-chemical basis for such a bias has never been found, with one partial exception advanced by George Rose (see below) (66, 67).

To understand folding, we and others have instead relied on experiment. The most thorough analyses using HX in both equilibrium and kinetic modes have been done for the model protein Cyt c. Extensive results consistently demonstrate that Cyt c is composed of cooperative units called foldons, as diagrammed in **Figure 4*b***, which pictures a different, more defined energy landscape. During kinetic folding, Cyt c folds in a sequence of native-like foldon steps. Thus the same interactions and cooperative units that establish the native state provide built-in instructions for its kinetic pathway formation. Other proteins have now also been shown to fold in that way, albeit often with confounding side issues, especially the ubiquitous appearance of transient folding errors due to the low energy of the interactions that guide foldon formation.

We first found the foldon units of native Cyt c as sets of amino acids that exchange together in cooperative reversible unfolding and refolding reactions (5). Conditions were found where HX is so slow in N that the various amides only exchange and become H to D-labeled (in D_2O) when they are deprotected in repeated transient visits to higher free energy partially or fully unfolded states (2, 4, 23). The H to D label accumulates during many such visits over time and can then be read out in the dominant native state by 2D NMR to reveal the structure of the normally invisible transient states. For example, **Figure 4*b*** indicates that HX detects the transient infinitesimally populated Green-unfolded/Blue-folded state with 10^{-7} steady state population. In this case, after some H to D exchange time one sees that only sites in the blue foldon remain NMR-visible (protonated), whereas amides in the lower lying foldons have all been deuterated.

The free energy level of each partially unfolded state above the native state can be determined from its measured D-labeling rate (fraction of time unfolded) and our calibrated unhindered rates according to the Linderstrøm-Lang equations. The transient high energy states found represent a ladder of discrete partially folded native-like forms, well separated in ΔG, as in **Figure 4*b***. These and similar results for other proteins portray the steady-state Boltzmann distribution of protein forms as they cycle through the high-free-energy space above N. Under native conditions, the space is dominated by a few energy basins occupied by combinations of native-like foldons, as in **Figure 4*b***. The randomly distributed HX behavior that would be produced by forms like those in **Figure 4*a*** is not seen.

Independently, kinetic HX pulse labeling experiments (33, 64) showed that Cyt c folds by stepping down the energy ladder through these same foldon-based intermediates. In these experiments the unfolded protein is diluted into folding conditions in D_2O for increasing refolding times, then subjected to a brief high-pH H-to-D exchange labeling pulse, and then allowed to fold to N. Protein regions not yet protected by folding at the time of the pulse become labeled with D. Regions already folded and protected retain their H. To define the already-folded and not-yet-folded regions, the residue-resolved H/D occupation as a function of pre-pulse folding time was read out by 2D NMR of the refolded protein (5) or at the level of identifiable segments by the fragment separation method directly after the labeling pulse (33). The results display a stepwise succession of foldon folding steps, first B (blue), then B + G (green), then B + G + Y (yellow), and finally all + red. Residues in the large bottom infrared loop exchange rapidly and were only captured in later work (32). Comparison of the folding sequence with Cyt c structure suggests that, at each folding step, prior native-like structure templates the addition of an incoming foldon with complementary structure, called sequential stabilization, much like the later-named folding upon binding phenomenon.

In these experiments, each kinetic folding step is seen to be single exponential, indicating stepwise folding of the cooperatively structured foldon units. Each intermediate state, once formed, persists as the later foldons join, indicating a well-defined sequential pathway. However, as might be expected due to the sequential stabilization phenomenon, kinetic steps tend to be closely packed, potentially confusing their sequentiality. Foldon steps are more clearly separated in the equilibrium ΔG dimension (**Figure 4*b***, *far right*) measured as described above.

Independently, mutational experiments in which the equilibrium stability of one or another of these states was changed, called stability labeling, demonstrate the same sequentiality (43, 45, 51, 77). Each lower energy state contains one more foldon. Other experiments under HX EX1 *but still native* conditions measured the sequence of unfolding reactions. The native protein walks back up the ladder unfolding one more foldon at each step (32).

Experimental results for other proteins have now detected one or more distinct N-like intermediates in both analogous and very different types of experiments. Proteins investigated include RNAse H (34); maltose binding protein in free solution and inside the GroEL cavity (82); apoCyt b562 (27, 72); HEW lysozyme (1); OspA from *Borrelia burgdorferi* (78); apomyoglobin (37); triose phosphate isomerase by SH labeling (69); the TIM barrel protein DapA inside GroEL (30); and calmodulin by optical tweezers (71). The folding pathways observed tend to be linear but need not be depending on how the foldon units interact in the native protein (43, 44). For a video discussion, the reader is referred to Reference 20.

A particularly interesting case is presented by the folding of repeat proteins with linearly arranged repeat subunits. The subunits tend to fold cooperatively, guided and stabilized by interaction with neighboring subunits (62), just like the cooperative folding of individual foldons and the inter-foldon sequential stabilization process seen for the folding of Cyt c and other

proteins. The clear suggestion is that the foldon-based construction of contemporary proteins and their foldon-based folding pathway have evolved co-dependently from these beginnings (24).

A variety of factors have misled many, I would say most, folding investigations. One widespread problem is the intervention of kinetic barriers. Misfolding steps are ubiquitous (25) and have led to significant interpretative confusion—proline misisomerization, heme misligation, alternative disulfide bond formation, domain swapping, non-native interactions, alternative docking modes, aggregation, and statistical chance when alternative steps are close in energy. Slow error repair produces non-two-state folding and determines the measured folding rate, negating any effort to predict folding rates from basic principles. Experiments that find alternative parallel pathways, usually two, are often misrepresented as supporting the many-pathway model. Fast reaction experiments that look at very early prefolding or simulations that observe residues not yet involved in structure naturally find chaotic residue-level dynamics. It is misleading to interpret this prefolding behavior in terms of multiple folding pathways. Experiments that have attempted to study folding by studying unfolding (denaturant, temperature, pressure, optical tweezers) will tend to fail. Unfolding must go through the same stages as for folding only under native conditions where microscopic reversibility holds.

I do not understand the claim that the nonspecific depiction of basic thermodynamic principles graphed in the funneled landscape icon, which applies equally even to the folding of RNA or any other polymer, can provide specific mechanistic details about protein folding. In contrast, extensive experimental evidence now clearly displays the foldon-based nature of protein folding, as illustrated in **Figure 4*b***, and an apparent absence of the many random intermediate forms and multiple pathways suggested in **Figure 4*a***.

A different thermodynamic requirement considered by George Rose (66, 67) dovetails with the foldon hypothesis. Rose reasons that H-bonding, either to water or internally, must be maintained for almost every polar group before, during, and after folding because each unpaired polar group would cost several kcals. In the one case I know of, a nearly complete H-bonding inventory (70) examined by NMR and HX, shows that this requirement is met in the native staph nuclease protein (70). How can this extremely difficult requirement be satisfied also during kinetic folding? The foldon model provides a general solution. The problem of properly H-bonding every polar group at every folding step in every protein, while meeting all other stability and structural requirements along the way, can be universally solved by replicating the same substructures that have been evolved to satisfy these requirements for the native protein.

These considerations dictate that folding pathway intermediates must be native-like. The Levinthal kinetic considerations dictate that folding intermediates must be composed of relatively small modules. Biophysical considerations dictate their pathway ordering. Extensive evidence using specialized experimental capabilities has now demonstrated distinct folding intermediates and pathways for many proteins with just these properties. This is the foldon story.

PROTEIN MACHINES

The vast superfamily of AAA+ proteins function to convert the chemical potential of cytosolic ATP into physicochemical form and use it to perform an extraordinary variety of energy-requiring physical operations on proteins, nucleic acids, and membrane systems throughout all of biology (41, 63). The newly available high-resolution capability of cryo-electron microscopy has drawn most attention to the close details of AAA+ protein–substrate interaction. The ability of HX MS to describe changes in structure and energetics enables a broader study of AAA+ function. We applied the full HX MS capability to structure-function studies of the hexameric Hsp104 (6 × 908 residues) AAA+ protein machine (79–81). By measuring the whole sweep of HX behavior

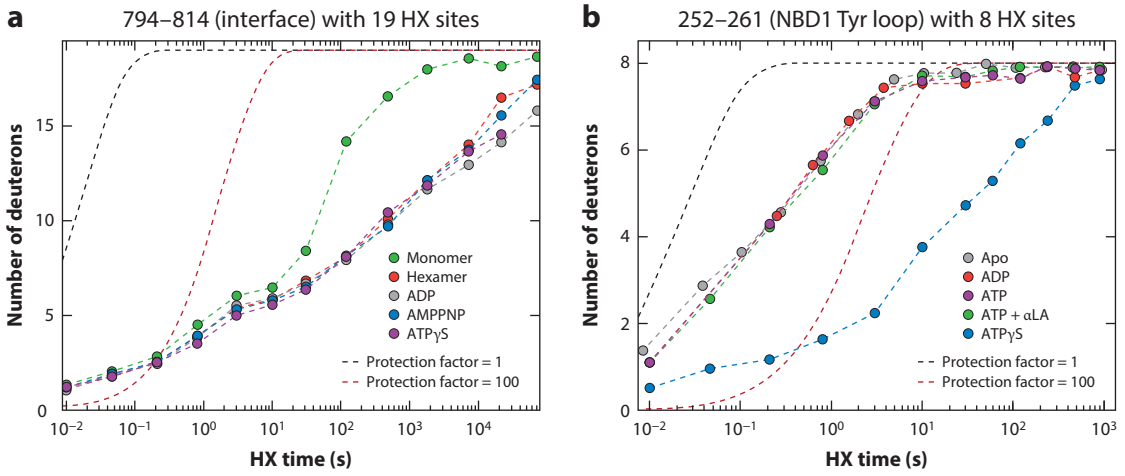

Figure 5

Example hydrogen exchange (HX) mass spectrometry (MS) peptide data. HX MS curves are shown for two out of 350 fragments obtained for the Hsp104 homohexamer (908 residues/protomer) with different nucleotide ligands bound. The Hsp104 hexamer adopts a spiraled, loosely structured open state in the apo and ADP-bound forms. It adopts a flatter, more tightly structured spiral in the closed state upon binding the ATP analog ATPγS. In the presence of ATP it actively cycles between open and closed states. (*a*) A segment placed at a subunit interface that responds to hexamer dissociation but does not change when the hexamer experiences the global open-to-closed transition. (*b*) A segment that includes the axial pore loop in the NBD1 domain, which functions to seize protein substrates and pull them into and through the central pore. These experiments used methods we have described and illustrate the reproducibility, resolution, and detail now obtainable (38–40, 52, 74, 79, 80). For extensive examples, see supplementary information in References 79 and 80.

(10 ms to 20 h) monitored in several hundred peptide fragments, HX MS was able to report on the entire 0.6 MDa Hsp104 protein, with various nucleotides bound, in open and closed states, while at rest and during active processing. **Figure 5** shows some examples. These experiments have led to a list of new insights.

We found that active Hsp104 and some other AAA+ proteins cycle through the closed state and the previously known but largely ignored open state. In fact, the large majority of cycle time is spent in the open state because the rate-limiting step in the functional cycle turns out to be ADP dissociation from the open state ($k_{off} = 1/s$). This allows cellular control of Hsp104 activity to be exercised by regulating the ADP off rate and thus the time needed for each cycle. The ADP off rate is regulated by Hsp70 interaction with the malleable M domain, which shields the nucleotide binding site. The long-lived open state accounts for the major but previously unexplained dwell phase in optical tweezer experiments. To minimize the cycle time lost to slow ADP dissociation, AAA+ proteins can complete several ATPase-dependent substrate translocation steps in the brief closed state before switching back to the open state for collective ADP ejection and ATP replenishment. The same property appears to explain the ability to adjust the force of the translocation power stroke on demand by accumulating the relaxation energy released by the hydrolysis of several ATPs.

In addition, the role of ATP has been ambiguous. We found a somewhat unexpected behavior. Following ADP ejection, the favorable rebinding of ATP to the low-free-energy Hsp104 open state allows ATP to act as an allosteric effector to drive the protein to its higher-free-energy closed state, just as Hb diverts some O_2 binding energy to drive its T to R transition. Also as in Hb, the major quaternary structure change acts to transmit the energy subtracted from ATP binding to distant sites. However, opposite to Hb, HX is slowed in the higher energy Hsp104 closed state

indicating the stabilization of some protein segments (e.g., **Figure 5*b***), in this case to prepare for substrate processing.

The closed state is held poised metastably at the higher free energy level by an unfavorable cross-protomer clash of the ATP γ-PO_4 in the open state. The function of ATP hydrolysis is to release the γ-PO_4 and permit rather than drive energetically downhill protein relaxation, which couples to and powers substrate translocation. The hydrolysis of sufficient ATPs allows the transition back to the open state where spent ADP is ejected and fresh ATP is rebound.

This sequence of events, newly described by our HX MS studies, integrates the ATP and structure change cycles to carry Hsp104 around a repeating functional cycle. Remarkably, the cycle is unidirectional because each step around the cycle is energetically downhill (ADP off, ATP on plus open to closed, ATP hydrolysis, PO_4 release, substrate translocation, closed to open). Thus, substrate translocation driven by the cycle is naturally unidirectional without the need for additional rectification machinery such as a Brownian ratchet (36). Comparison of this functional strategy with other proteins suggests that a similar cycle is widely conserved among protein machines, enabling the core AAA+ motif to serve as a general adaptor for transducing the chemical potential of cytosolic ATP into a wide range of physical operations.

A FOUNDATION FOR THE FUTURE

I have taken this opportunity to single out a few studies taken from our past HX adventures, stemming from my career-long fascination with HX technology and interpretation. Our earlier studies of hemoglobin allostery established the ability of HX to localize and measure functional energy transmission and change throughout a protein. An overview of the current protein folding story underscores the exceptional ability of HX to examine the higher-energy excited state manifold of protein molecules including the normally invisible intermediate forms that occupy that space and account for the protein folding process. Recent work on protein machines demonstrates the ability of modern HX MS methodology to map out the important structural and energetic features of complex protein functions. These results exemplify the wealth of fundamental biophysical information that HX studies are now able to provide and illustrate where protein biophysics can now go.

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LITERATURE CITED

1. Bai Y. 2000. Kinetic evidence of an on-pathway intermediate in the folding of lysozyme. *Protein Sci.* 9:194–96
2. Bai Y, Englander JJ, Mayne L, Milne JS, Englander SW. 1995. Thermodynamic parameters from hydrogen exchange measurements. *Methods Enzymol.* 259:344–56

3. Bai Y, Milne JS, Mayne L, Englander SW. 1993. Primary structure effects on peptide group hydrogen exchange. *Proteins Struct. Funct. Genet.* 17:75–86
4. Bai Y, Milne JS, Mayne L, Englander SW. 1994. Protein stability parameters measured by hydrogen exchange. *Proteins Struct. Funct. Genet.* 20:4–14
5. Bai Y, Sosnick TR, Mayne L, Englander SW. 1995. Protein folding intermediates: native-state hydrogen exchange. *Science* 269:192–97
6. Baldwin RL. 2011. Early days of protein hydrogen exchange: 1954–1972. *Proteins Struct. Funct. Bioinform.* 79:2021–26
7. Bryngelson JD, Onuchic JN, Socci ND, Wolynes PG. 1995. Funnels, pathways, and the energy landscape of protein folding: a synthesis. *Proteins* 21:167–95
8. Bryngelson JD, Wolynes PG. 1987. Spin glasses and the statistical mechanics of protein folding. *PNAS* 84:7524–28
9. Calhoun DB, Vanderkooi JM, Englander SW. 1983. Penetration of small molecules into proteins studied by quenching of phosphorescence and fluorescence. *Biochemistry* 22:1533–39
10. Connelly GP, Bai Y, Jeng MF, Englander SW. 1993. Isotope effects in peptide group hydrogen exchange. *Proteins Struct. Funct. Genet.* 17:87–92
11. Eigen M. 1964. Proton transfer, acid-base catalysis, and enzymatic hydrolysis. Part I: elementary processes. *Angew. Chem. Int. Ed.* 3:1–19
12. Engen JR, Botzanowski T, Peterle D, Georgescauld F, Wales TE. 2021. Developments in hydrogen/deuterium exchange mass spectrometry. *Anal. Chem.* 93:567–82
13. Englander JJ, Calhoun DB, Englander SW. 1979. Measurement and calibration of peptide group hydrogen-deuterium exchange by ultraviolet spectrophotometry. *Anal. Biochem.* 92:517–24
14. Englander JJ, Louie G, McKinnie RE, Englander SW. 1998. Energetic components of the allosteric machinery in hemoglobin measured by hydrogen exchange. *J. Mol. Biol.* 284:1695–706
15. Englander JJ, Rogero JR, Englander SW. 1983. Identification of an allosterically sensitive unfolding unit in hemoglobin. *J. Mol. Biol.* 169:325–44
16. Englander JJ, Rogero JR, Englander SW. 1985. Protein hydrogen exchange studied by the fragment separation method. *Anal. Biochem.* 147:234–44
17. Englander JJ, Rumbley JN, Englander SW. 1998. Signal transmission between subunits in the hemoglobin T-state. *J. Mol. Biol.* 284:1707–16
18. Englander SW. 1963. A hydrogen method using tritium and sephadex. Application to ribonuclease. *Biochemistry* 2:798–807
19. Englander SW. 1971. Oxygen changes hemoglobin's breathing. In *Probes of Structure and Function of Macromolecules and Membranes*, ed. B Chance, C Lee, JK Blasie, pp. 389–92. New York: Academic
20. Englander SW. 2007. *How do proteins fold and why?* The Biomedical and Life Sciences Collection, Henry Steward Talks, London. **https://hstalks.com/bs/636/**
21. Englander SW, Crowe D. 1965. Rapid microdialysis and hydrogen exchange. *Anal. Biochem.* 12:579–84
22. Englander SW, Englander JJ, McKinnie RE, Ackers GK, Turner GJ, et al. 1992. Hydrogen exchange measurement of the free energy of structural and allosteric change in hemoglobin. *Science* 256:1684–87
23. Englander SW, Kallenbach NR. 1983. Hydrogen exchange and structural dynamics of proteins and nucleic acids. *Q. Rev. Biophys.* 16:521–55
24. Englander SW, Mayne L. 2017. The case for defined protein folding pathways. *PNAS* 114:8253–58
25. Englander SW, Mayne L, Krishna MMG. 2007. Protein folding and misfolding: mechanism and principles. *Q. Rev. Biophys.* 40:287–326
26. Englander SW, Poulsen A. 1969. Hydrogen-tritium exchange of the random chain polypeptide. *Biopolymers* 7:329–39
27. Feng H, Zhou Z, Bai Y. 2005. A protein folding pathway with multiple folding intermediates at atomic resolution. *PNAS* 102:5026–31
28. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science* 246:64–71
29. Gellert MF, Englander SW. 1963. The molecular weight of rabbit myosin A by light scattering. *Biochemistry* 2:39–42

30. Georgescauld F, Popova K, Gupta AJ, Bracher A, Engen JR, et al. 2014. GroEL/ES chaperonin modulates the mechanism and accelerates the rate of TIM-barrel domain folding. *Cell* 157:922–34
31. Henry ER, Mozzarelli A, Viappiani C, Abbruzzetti S, Bettati S, et al. 2015. Experiments on hemoglobin in single crystals and silica gels distinguish among allosteric models. *Biophys. J.* 109:1264–72
32. Hoang L, Bédard S, Krishna MMG, Lin Y, Englander SW. 2002. Cytochrome *c* folding pathway: kinetic native-state hydrogen exchange. *PNAS* 99:12173–78
33. Hu W, Kan ZY, Mayne L, Englander SW. 2016. Cytochrome c folds through foldon-dependent native-like intermediates in an ordered pathway. *PNAS* 113:3809–14
34. Hu W, Walters BT, Kan ZY, Mayne L, Rosen LE, et al. 2013. Stepwise protein folding at near amino acid resolution by hydrogen exchange and mass spectrometry. *PNAS* 110:7684–89
35. Hvidt A, Nielsen SO. 1966. Hydrogen exchange in proteins. *Adv. Protein Chem.* 21:287–386
36. Hwang W, Karplus M. 2019. Structural basis for power stroke vs. Brownian ratchet mechanisms of motor proteins. *PNAS* 116:19777–85
37. Jennings PA, Wright PE. 1993. Formation of a molten globule intermediate early in the kinetic folding pathway of apomyoglobin. *Science* 262:892–96
38. Kan ZY, Mayne L, Sevugan Chetty P, Englander SW. 2011. ExMS: data analysis for HX MS experiments. *J. Am. Soc. Mass. Spectrom.* 22:1906–15
39. Kan ZY, Walters BT, Mayne L, Englander SW. 2013. Protein hydrogen exchange at residue resolution by proteolytic fragmentation mass spectrometry analysis. *PNAS* 110:16438–43
40. Kan ZY, Ye X, Skinner JJ, Mayne L, Englander SW. 2019. ExMS2: An integrated solution for hydrogen-deuterium exchange mass spectrometry data analysis. *Anal. Chem.* 91:7474–81
41. Khan YA, White KI, Brunger AT. 2021. The AAA+ superfamily: a review of the structural and mechanistic principles of these molecular machines. *Crit. Rev. Biochem. Mol. Biol.* 57:156–87
42. Krishna MMG, Englander SW. 2005. The N-terminal to C-terminal motif in protein folding and function. *PNAS* 102:1053–58
43. Krishna MMG, Englander SW. 2007. A unified mechanism for protein folding: predetermined pathways with optional errors. *Protein Sci.* 16:449–64
44. Krishna MMG, Lin Y, Englander SW. 2004. Protein misfolding: optional barriers, misfolded intermediates, and pathway heterogeneity. *J. Mol. Biol.* 343:1095–109
45. Krishna MMG, Maity H, Rumbley JN, Lin Y, Englander SW. 2006. Order of steps in the cytochrome *c* folding pathway: evidence for a sequential stabilization mechanism. *J. Mol. Biol.* 359:1411–20
46. Leopold PE, Montal M, Onuchic JN. 1992. Protein folding funnels: a kinetic approach to the sequence-structure relationship. *PNAS* 89:8721–25
47. Levinthal C. 1968. Are there pathways for protein folding. *J. Chim. Phys.* 65:44–45
48. Levinthal C. 1969. How to fold graciously. *Univ. Illinois Bull.* 67(41):22–24
49. Linderstrøm-Lang K. 1958. Deuterium exchange and protein structure. In *Symposium on Protein Structure*, ed. A Neuberger. London: Methuen
50. Linderstrøm-Lang KU, Schellman JA. 1959. Protein structure and enzyme activity. In *The Enzymes*, ed. PD Boyer, H Lardy, K Myrback, pp. 443–510. New York: Academic
51. Maity H, Maity M, Krishna MM, Mayne L, Englander SW. 2005. Protein folding: the stepwise assembly of foldon units. *PNAS* 102:4741–46
52. Mayne L, Kan ZY, Chetty PS, Ricciuti A, Walters BT, Englander SW. 2011. Many overlapping peptides for protein hydrogen exchange experiments by the fragment separation-mass spectrometry method. *J. Am. Soc. Mass. Spectrom.* 22:1898–905
53. Molday RS, Englander SW, Kallen RG. 1972. Primary structure effects on peptide group hydrogen exchange. *Biochemistry* 11:150–58
54. Monod J, Wyman J, Changeaux JP. 1965. On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* 12:88–118
55. Nguyen D, Mayne L, Phillips MC, Englander SW. 2018. Reference parameters for protein hydrogen exchange rates. *J. Am. Soc. Mass. Spectrom.* 29:1936–39
56. Oliveberg M, Wolynes PG. 2005. The experimental survey of protein-folding energy landscapes. *Q. Rev. Biophys.* 38:245–88

57. Onuchic JN, Wolynes PG. 2004. Theory of protein folding. *Curr. Opin. Struct. Biol.* 14:70–75
58. Perutz MF. 1963. X-ray analysis of hemoglobin. *Science* 140:863–69
59. Perutz MF. 1970. Stereochemistry of cooperative effects in haemoglobin. *Nature* 228:726–39
60. Perutz MF. 1989. Mechanisms of cooperativity and allosteric regulation in proteins. *Q. Rev. Biochem.* 22:130–236
61. Perutz MF, Wilkinson AJ, Paoli M, Dodson GG. 1998. The stereochemical mechanism of the cooperative effects in hemoglobin revisited. *Annu. Rev. Biophys. Biomol. Struct.* 27:1–34
62. Petersen M, Barrick D. 2021. Analysis of tandem repeat protein folding using nearest-neighbor models. *Annu. Rev. Biophys.* 50:245–65
63. Puchades C, Sandate CR, Lander GC. 2020. The molecular principles governing the activity and functional diversity of AAA+ proteins. *Nat. Rev. Mol. Cell Biol.* 21:43–58
64. Roder H, Elove GA, Englander SW. 1988. Structural characterization of folding intermediates in cytochrome c by H-exchange labelling and proton NMR. *Nature* 335:700–4
65. Rogero JR, Englander JJ, Englander SW. 1986. Individual breathing reactions measured by functional labeling and hydrogen exchange methods. *Methods Enzymol.* 131:508–17
66. Rose GD. 2021. Protein folding—seeing is deceiving. *Protein Sci.* 30:1606–16
67. Rose GD. 2021. Reframing the protein folding problem: entropy as organizer. *Biochemistry* 60:3753–61
68. Sharp KA, Honig B. 1990. Electrostatic interactions in macromolecules: theory and applications. *Annu. Rev. Biophys. Biophys. Chem.* 19:301–32
69. Silverman JA, Harbury PB. 2002. The equilibrium unfolding pathway of a $(\beta/\alpha)_8$ barrel. *J. Mol. Biol.* 324:1031–40
70. Skinner JJ, Lim WK, Bedard S, Black BE, Englander SW. 2012. Protein dynamics viewed by hydrogen exchange. *Protein Sci.* 21:996–1005
71. Stigler J, Ziegler F, Gieseke A, Gebhardt JC, Rief M. 2011. The complex folding network of single calmodulin molecules. *Science* 334:512–16
72. Takei J, Pei W, Vu D, Bai Y. 2002. Populating partially unfolded forms by hydrogen exchange-directed protein engineering. *Biochemistry* 41:12308–12
73. Turner GJ, Galacteros F, Doyle ML, Hedlund B, Pettigrew DW, et al. 1992. Mutagenic dissection of hemoglobin cooperativity: effects of amino acid alteration on subunit assembly of oxy and deoxy tetramers. *Proteins* 14:333–50
74. Walters BT, Ricciuti A, Mayne L, Englander SW. 2012. Minimizing back exchange in the hydrogen exchange-mass spectrometry experiment. *J. Am. Soc. Mass. Spectrom.* 23:2132–39
75. Wand AJ, Englander SW. 1985. Two-dimensional proton NMR studies of cytochrome c: assignment of the N-terminal helix. *Biochemistry* 25:5290–94
76. Wyman J Jr. 1964. Linked functions and reciprocal effects in hemoglobin: a second look. *Adv. Protein Chem.* 19:223–86
77. Xu Y, Mayne LC, Englander SW. 1998. Evidence for an unfolding and refolding pathway in cytochrome c. *Nat. Struct. Biol* 5:774–78
78. Yan S, Kennedy SD, Koide S. 2002. Thermodynamic and kinetic exploration of the energy landscape of *B. burgdorferi* OspA by native-state hydrogen exchange. *J. Mol. Biol.* 323:363–75
79. Ye X, Lin J, Mayne L, Shorter J, Englander SW. 2019. Hydrogen exchange reveals Hsp104 architecture, structural dynamics, and energetics in physiological solution. *PNAS* 116:7333–42
80. Ye X, Lin J, Mayne L, Shorter J, Englander SW. 2020. Structural and kinetic basis for the regulation and potentiation of Hsp104 function. *PNAS* 117:9384–92
81. Ye X, Mayne L, Englander SW. 2021. A conserved strategy for structure change and energy transduction in Hsp104 and other AAA+ protein motors. *J. Biol. Chem.* 297:101066
82. Ye X, Mayne L, Kan ZY, Englander SW. 2018. Folding of maltose binding protein outside of and in GroEL. *PNAS* 115:519–24
83. Yonetani T, Park SI, Tsuneshige A, Imai K, Kanaori K. 2002. Global allostery model of hemoglobin: modulation of O_2 affinity, cooperativity, and Bohr effect by heterotropic allosteric effectors. *J. Biol. Chem.* 277:34508–20

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