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Mini Review

Using NMR-detected hydrogen-deuterium exchange to quantify protein stability in cosolutes, under crowded conditions in vitro and in cells

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ABSTRACT

We review the use of nuclear magnetic resonance (NMR) spectroscopy to assess the exchange of amide protons for deuterons (HDX) in efforts to understand how high concentration of cosolutes, especially macromolecules, affect the equilibrium thermodynamics of protein stability. HDX NMR is the only method that can routinely provide such data at the level of individual amino acids. We begin by discussing the properties of the protein systems required to yield equilibrium thermodynamic data and then review publications using osmolytes, sugars, denaturants, synthetic polymers, proteins, cytoplasm and in cells.

1. Introduction

The cytoplasm is crowded with a variety of proteins, nucleic acids, and small molecules, which can occupy 30–40% of a cell's volume, with a total concentration exceeding 300 g/L [1]. In this complicated environment, proteins experience interactions with macromolecules that are absent in dilute buffer where proteins are most often studied [2]. Crowding effects on protein- and protein complex-stability are explained by a combination of hard repulsions [3,4] and so-called "soft" chemical interactions [3,5–10]. Simple crowding theory treats macromolecules as inert spheres that exclude volume due to steric repulsion. These hard repulsions are entropic and favor more compact protein conformations under crowded conditions. Therefore, simple theory predicts that crowding will stabilize folded proteins and protein complexes. Chemical interactions can be both enthalpic and entropic (by affecting the number of bound solvent or cosolute molecules), and modulate hard repulsions *via* repulsive or attractive interactions [10,11]. Repulsive chemical interactions (i.e., non-complementary charges) reinforce the hard- repulsions and are therefore stabilizing; attractive chemical interactions (e.g., opposite charges, hydrogen bonds) weaken the hard repulsions and are destabilizing because they favor expanded conformations [10].

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It is important to study proteins in their native environments—living cells, to gain the most physiologically-relevant information. However, before the advent of methods for in-cell studies, protein- and protein complex-stability were, and often still are, studied in concentrated solutions of cosolutes. We begin by discussing cosolutes with molecular weights up to several hundred Daltons (Da). These molecules include natural osmolytes [12] and the monomers that comprise synthetic polymers. We then focus on cosolutes with molecular weights from a few kDa to MDa, including synthetic polymers, individual proteins, and cell lysates. Among these larger cosolutes, synthetic polymers are often used to mimic the cellular interior. Although less physiologically relevant, synthetic polymers are useful for protecting proteins, biological drugs, and vaccines [13–15]. Synthetic polymers commonly used to protect proteins and in studies of macromolecular crowding include polyethylene glycols (PEGs), polyvinylpyrrolidones (also called povidones, PVPs), the sucrose polymer Ficoll™, and the glucose polymer dextran.

2. Measuring stability

Many single-domain globular proteins undergo the following reversible, two-state reaction

$$N \rightleftharpoons U \tag{1}$$

where N represents the biologically active native state, and U represents the unfolded (i.e., biologically inactive) state [16]. The conformational stability of a globular protein is defined as the modified standard-state Gibbs free energy of unfolding ($\Delta G^{\circ\prime}_U$).

$$\Delta G^{\circ\prime}_U = G^{\circ\prime}_U - G^{\circ\prime}_N \tag{2}$$

where $G^{\circ\prime}_U$ is the unfolded state free energy and $G^{\circ\prime}_N$ the native state free energy. Protein stability is quantified by determining the populations, or relative populations, of native and unfolded states at equilibrium with a variety of tools [17], and calculating the Gibbs standard-state free energy of unfolding,

$$\Delta G^{\circ\prime}_U = -RTln\frac{[U]}{[N]} = -RTlnK_U \tag{3}$$

where R is the gas constant and T is temperature in Kelvin, and K_U is the equilibrium constant for unfolding. Typical globular proteins have $\Delta G^{\circ\prime}_U$ values of ~7 kcal/mol at room temperature, which means that only one out of 10^5 protein molecules are not in the native state. Christian Anfinsen developed the "thermodynamic hypothesis" to explain how a protein folds into its native conformation [18]. His efforts on ribonuclease A showed that the native state is the thermodynamically most stable structure in physiologically relevant conditions.

The most common tools for detecting the equilibrium between U and N include fluorescence- and absorbance- spectroscopy, circular dichroism (CD) spectropolarimetry, and differential scanning calorimetry (DSC) [19–21]. Perturbants used to populate the unfolded state include guanidinium chloride, urea, and heat. Values of $\Delta G^{\circ\prime}_U$ can then be obtained from K_U using Equation (3). The observation that different tools give similar thermodynamic values provides confidence that the protein shows two-state behavior [22].

Protein stability can also be measured under non-perturbing conditions by using nuclear magnetic resonance (NMR) - or mass spectrometry (MS)- detected hydrogen-deuterium exchange (HDX) [23–26]. HDX MS is fast, sensitive, and provides information about protein dynamics, but may not routinely provide information at the residue level. However, NMR-detected HDX can be used to assess stability at the level of individual backbone amide protons [27] for proteins up to about 200 residues [28].

NMR-detected HDX is usually performed using an ^{15}N-enriched test protein and the ^{15}N–^{1}H heteronuclear single quantum coherence (HSQC) experiment or one of its close relatives [27]. In this experiment, every amide nitrogen-proton pair gives rise to a cross peak at the coordinates of its ^{15}N, ^{1}H chemical shifts. To a first approximation, cross peak volume is proportional to the concentration of the amide linkage. When the protein is resuspended in D_2O, the amide proton exchanges irreversibly with deuterons. Deuterium is not detected at the same frequency as a proton. Therefore, when a proton is exchanged for a deuteron, its cross-peak volume decreases with time, and finally disappears. These properties make NMR-detected HDX amenable to studying protein stability. If HDX occurs on a time scale of minutes or longer, the exponential decay in the volume of each amide proton cross peak can be used to obtain a rate constant for exchange (k_{obs}), which, as described next, can be connected to protein stability.

Each amide proton has an open and a closed state. HDX only occurs from the open state [25,26]:

$$\mathrm{N-H(closed)} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \mathrm{N-H(open)} \xrightarrow{k_{int}} \mathrm{N-D(open)} \underset{k_{op}}{\overset{k_{cl}}{\rightleftharpoons}} \mathrm{N-D(closed)} \tag{4}$$

where k_{op} and k_{cl} are the opening- and closing- rate constants, and k_{int} is the intrinsic rate constant from the open state. k_{int} values are obtained from data on unstructured peptides [29].

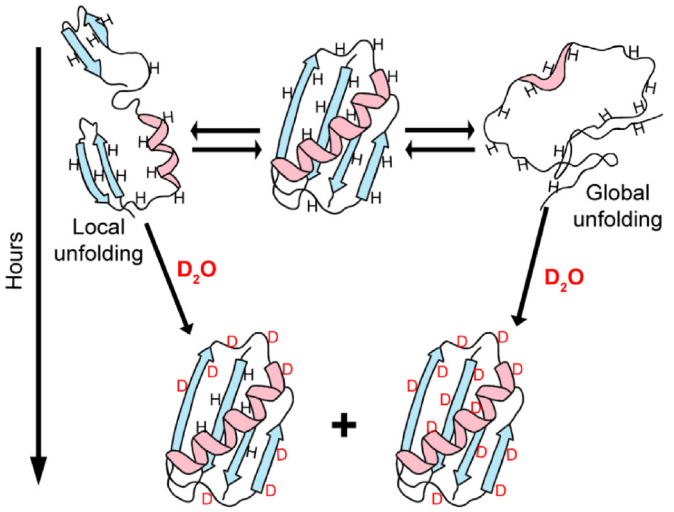

Fig. 1. Hydrogen-deuterium exchange (HDX) and local/global unfolding.

Provided the assumptions described below are valid, k_{obs} can be linked to stability. The first assumption is that the protein is stable, i.e., $k_{cl} \gg k_{op}$. If this assumption is valid, then exchange occurs at one of two limits, EX1 or EX2 [26]. Under the EX1 limit, k_{cl} is rate-determining, and $k_{obs} \approx k_{op}$. The EX1 limit is usually associated with less stable proteins or proteins at basic pH, and the resulting HDX data cannot be used to quantify stability [30]. At the EX2 limit,[a] exchange from the open state, k_{int}, is rate-determining. In this case, the ratio of k_{obs} to k_{int} defines the equilibrium opening constant of an amide proton, K_{op} (Equation (5)), which can be converted to equilibrium opening free energy, $\Delta G^{\circ\prime}_{op}$ [23] *via* Equation (6)

$$k_{obs} = \frac{k_{op}}{k_{cl}} k_{int} = K_{op} k_{int} \tag{5}$$

$$\Delta G^{\circ\prime}_{op} = -RTlnK_{op} = -RTln\frac{k_{obs}}{k_{int}} \tag{6}$$

The largest value of $\Delta G^{\circ\prime}_{op}$ approximates the free energy of unfolding, $\Delta G^{\circ\prime}_{U}$, as shown on the right-hand side of Fig. 1, providing information on global stability [31–33]. Local fluctuations, of the native state, as shown on the left-hand side of Fig. 1, result in $\Delta G^{\circ\prime}_{op}$ values less than that for global stability [34]. If a residue is in a disordered region [35], there is no protection, and $\Delta G^{\circ\prime}_{op}$ is zero. Key to this review, cosolutes are essentially invisible to NMR-detected HDX and other ^{15}N-directed NMR experiments if they are not enriched in ^{15}N.

3. Calorimetry measures global unfolding

DSC measures temperature dependence of heat capacity of a protein solution and thus provides a direct measure of $\Delta H^{\circ\prime}_{U}$ [36]. Assuming a two-state transition, the shape of the curve can be used to assess the degree of unfolding to provide a measure of $\Delta G^{\circ\prime}_{U}$ as a function of temperature.

The constant pressure heat capacity of the unfolded state is greater than that of the native state, such that $\Delta C_{p,U}$ is greater than zero [36]. The sign of $\Delta C_{p,U}$ is considered evidence that hydrophobic surface is exposed upon unfolding [37]. Knowing $\Delta C_{p,U}$ for unfolding is positive and assuming a two-state unfolding mechanism, we can write the Gibbs-Helmholtz equation for protein stability [38,39].

$$\Delta G^{\circ}_{U}(T) = \Delta H^{\circ}_{U,cal}\left(1 - \frac{T}{T_m}\right) + \Delta C_{p,U}\left[(T - T_m) - Tln\left(\frac{T}{T_m}\right)\right] \tag{7}$$

where $\Delta H^{\circ\prime}_{U,cal}$ is the enthalpy at the transition and T_m is the temperature where $\Delta G^{\circ\prime}_{U} = 0$ and $\Delta S^{\circ\prime}_{m} = \Delta H^{\circ\prime}_{U,cal}/T_m$.

Calorimetric enthalpy, $\Delta H^{\circ\prime}_{U,cal}$, is the area under a transition peak. By assuming a two-state transition, the van't Hoff enthalpy ($\Delta H^{\circ\prime}_{U,vH}$) can be derived from the shape DSC thermogram, as it can from other spectroscopic measurements. The

[a] An easy way to tell if your protein is in the EX2 regime is to change the sample pH by one unit. Since exchange is base catalyzed, k_{obs} should change 10-fold.

observation that $\Delta H^{\circ\prime}_{cal}/\Delta H^{\circ\prime}_{vH}$ equals 1 —meaning that the directly measured DSC enthalpy equals the van't Hoff enthalpy, which requires assuming a two-state transition— is strong evidence that the protein unfolds *via* a two-state reaction.

4. Stability in dilute solution

NMR-detected HDX (Fig. 1) has been used to study protein stability for decades [27]. For global unfolding residues, the opening free energy, $\Delta G^{\circ\prime}_{op}$, approximates $\Delta G^{\circ\prime}_{U}$, the free energy of unfolding from calorimetric data [31]. The $\Delta G^{\circ\prime}_{op}$ values for local unfolding residues are, by definition, less than $\Delta G^{\circ\prime}_{U}$, because they undergo smaller fluctuations from the native state [34].

Temperature-dependent HDX experiments have been performed on several proteins [40–43]. At high temperatures, exchange rates cannot be measured because the protein is unstable (making the assumptions $k_{cl} \gg k_{op}$ and $k_{cl} \gg k_{int}$ invalid). In the temperature range consistent with the EX2 mechanism, $\Delta G^{\circ\prime}_{op}$ for globally unfolding residues approximates $\Delta G^{\circ\prime}_{U}$. Under these conditions, $\Delta G^{\circ\prime}_{op}$ for locally unfolding residues is, by definition, less than $\Delta G^{\circ\prime}_{U}$. At high temperatures, $\Delta G^{\circ\prime}_{op}$ for locally unfolding residues converges on $\Delta G^{\circ\prime}_{U}$, suggesting that global unfolding becomes the dominant exchange mechanism. Orban et al. showed that the $\Delta G^{\circ\prime}_{op}$ values obtained from HDX results and calorimetric $\Delta G^{\circ\prime}_{U}$ provide highly complementary information on the $\Delta G^{\circ\prime}_{U}$-versus temperature profiles of the B1 and B2 IgG-binding domains [42]. Furthermore, the temperature-dependent HDX data can be used to obtain the van't Hoff enthalpies of opening ($\Delta H^{\circ\prime}_{op}$) [43] by plotting $-RlnK_{op}$ against $1/T$. Which, given the standard-state free energy, provides access to the entropy of opening *via* the Gibbs equation (Equation (8)).

$$\Delta G^{\circ\prime}_{op} = \Delta H^{\circ\prime}_{op} - T\Delta S^{\circ\prime}_{op} \tag{8}$$

5. Osmolytes and monomers of synthetic polymers

Many tools, including NMR and spectrophotometric methods (e.g., CD, UV-Vis), have been used to study protein stability under crowded conditions. Zwitterionic osmolytes [e.g., trimethylamine *N*-oxide (TMAO), glycine betaine (*N*,*N*,*N*-trimethylglycine)] stabilize globular proteins and their complexes [44]. However, lowering the pH imparts a net positive charge on TMAO, which can change it from a stabilizing cosolute to a destabilizing cosolute [45], showing the importance of chemical interactions. Sucrose, glucose, and ethylene glycol, the monomers of Ficoll™, dextran, and PEG, respectively, also tend to stabilize proteins and protein-protein interactions [44,46], as do polyols (e.g., sorbitol, xylitol, mannitol) [46].

Osmolytes usually stabilize proteins enthalpically. Glycerol, glucose, and trehalose enthalpically stabilize a β-hairpin structure [46,47]. Similar data show that ethylene glycol stabilizes SH3 [48,49]. However, stabilization by sugars can be entropic [48,50–52]. The predominance of an enthalpic effect suggests a role for chemical interactions, but for sugar-based polymers at high concentrations, an entropic effect arising from hard repulsions is expected.

The destabilizing osmolyte, urea, is the most used small cosolute for NMR detected HDX studies [40,53–55]. Its concentration, and other conditions (e.g., pH, temperature), must be chosen carefully to prevent HDX from moving to the EX1 regime [53,54]. Urea destabilizes proteins because it interacts favorably with the backbone, and more backbone is exposed in the unfolded state [56]. The unfolded-state free energy decreases more than the native state free energy, resulting in a decrease in stability [57–60]. Therefore, urea promotes exposure to the protein backbone, causing the HDX rates to increase.

In contrast to urea, TMAO stabilizes proteins [54]. TMAO interacts unfavorably with the protein backbone, increasing the free energy of the unfolded state relative to the native state, shifting the N⇌U equilibrium toward N [60,61]. TMAO suppresses structural fluctuations that expose protein backbone, decreasing the rate of HDX. Another osmolyte, glycine betaine, is also a protein stabilizer. It stabilizes proteins in buffer, and is even more effective in cell lysates [62].

Sucrose [43], the monomer of Ficoll™, and trehalose [55] stabilize proteins by increasing the free energy of the unfolded state relative to the native state [50]. *N*-ethylpyrrolidone, the monomer of PVP, is destabilizing [63]. Studies of the temperature dependence of NMR detected HDX show that sucrose stabilizes chymotrypsin inhibitor 2 (CI2) enthalpically [43]. The details of their polymers are discussed next [64].

6. Synthetic polymers

PEG, with its high water solubility, is commonly used to understand macromolecular crowding. It has well-studied physical properties and is commercially available at high purity in more than ten molecular weights ranging from 0.1 to 2000 kDa [65–67]. Dextrans are bacterially-synthesized glucose polymers available commercially in at least six sizes from 6 to 150 kDa [68,69]. Ficoll™, which is made from sucrose, is a crosslinked polymer and available in two sizes, 70- and 400- kDa [70,71]. PVP is well characterized and available in at least five sizes from 10 kDa to 1300 kDa [64,69,72].

An essential property of synthetic polymer solutions changes with concentration [73]. At low concentrations (the so-called dilute region), synthetic polymers act as individual molecules, and simple hard-particle exclusion may be used to explain their effect on protein stability [74]. Above a certain concentration, called c^*, which is defined by the polymer's molecular weight, the polymer no longer acts like a collection of individual molecules, but instead becomes entangled with its neighbors, causing the macroscopic solution viscosity to increase steeply [69,75]. Above c^*, confinement is used to explain crowding effects on protein stability, in that the test protein molecule exists in a cavity created by polymers big enough to

contain the protein and its water of hydration. Confinement is most often considered stabilizing because there is no room for the globular protein to unfold. In confinement, changes in stability depend on the size, shape, and chemical nature of the cavity [76–78].

Contrary to simple expectations, even early work on crowding showed that PEGs can decrease globular protein stability [79]. Destabilization can grow with PEG size, and destabilization is dominated by PEG's hydrophobic nature and the increasing hydrophobicity of the test protein. However, the more hydrophilic sugar polymers, Ficoll™ and dextran stabilize α-lactalbumin and lysozyme [80], and the stabilization is often enthalpic [81,82]. The Ebbinghaus group also showed that concentrated dextran solutions enthalpically stabilize ubiquitin [83], but other groups interpret the dextran-induced stabilization as purely entropic [84–86]. A systematic investigation of a β-hairpin peptide shows that the stabilization by PEGs, dextrans, and glucose switches from entropic to enthalpic with increasing cosolute concentration [46].

Turning to residue-level HDX, our lab reported the systematic study of PVPs on the equilibrium stability of the small globular protein, CI2 [64]. HDX in PVP solutions occurs under the EX2 mechanism, and PVP does not change k_{int}. In solutions of PVP from 10 kDa to 55 kDa, almost all residues experience a stabilizing effect. The exceptions are residues for which stabilities are the same in the presence and absence of PVP. Below c^*, protein stabilities in PVP solutions are consistent with the predictions of stability changes from hard-particle exclusion. Stability increases with increases in PVP concentrations, but higher-molecular weights of PVPs show a weaker stabilization. Above c^*, the data are independent of PVP molecular weights, consistent with ideas about confinement. In addition to hard interactions, the data for some residues show that PVP stabilizes CI2 by weakly binding the folded state, which is a soft interaction.

Ficoll™, like its monomer sucrose, also stabilizes CI2 [43]. Ficoll™ increases $\Delta G^{\circ\prime}_{op}$ and $\Delta H^{\circ\prime}_{op}$ in general and increases with increasing Ficoll™ concentration. Both Ficoll™ and sucrose stabilize CI2 enthalpically. In summary, observations of stabilization in concentrated solutions of PVP and Ficoll™ show that the stabilization is driven by enthalpy, not entropy. That is, we must consider not just hard repulsions but also polymer-protein and polymer-solvent interactions [87].

HDX studies of protein stability in synthetic polymers are incomplete but promise to reveal new information. For example, little is known about the effect of PEGs, which are widely used to protect protein-based drugs, on local unfolding. Gaining such knowledge is important because partial protein unfolding can presage aggregation, which harms these increasingly important medicines. We used HDX NMR to assess the effects of PEGs and ethylene glycol on GB1 stability and structure [88]. The data show that PEGs and ethylene glycol stabilize the protein differently. Chemical shift and CD data show that ethylene glycol interacts with GB1 more strongly than PEGs but neither affects the structure of the folded state. In terms of stability, ethylene glycol and 12000 g/mol PEG stabilize GB1 more than PEGs of intermediate size. Temperature dependent HDX data show that ethylene glycol and smaller PEGs stabilize GB1 enthalpically while the largest PEG acts entropically. The key finding is that PEGs turn local unfolding into global unfolding, and this conclusion is supported by meta-analysis of published NMR-detected protein HDX data acquired in the presence of a number of small and large cosolutes [88]. In other words, there are fundamental differences between how small and large synthetic polymers affect stability.

7. Protein crowders and in-cell efforts

Cells are not crowded with synthetic polymers. If one is interested in biology, it is better to use globular proteins as crowders. Unlike PEG, Ficoll™, PVP or dextran, globular proteins are compact [89], and can be treated more like spheres. Another key difference between protein crowders and most common synthetic polymers is that proteins are charged. Destabilization is often observed in high concentrations of globular proteins [82,90], but there are exceptions [91]. At least part of the destabilization comes from the attraction of the protein crowder to the backbone of the test protein [2].

The ultimate goal is to study protein stability in living cells, but that is a challenging task. Cells must remain alive during the experiment. Another problem is test protein leakage from dead cells [92,93], which can cause artifacts, but control experiments can be performed to identify this problem [94]. Protein stability in cells is assessed using several approaches, including fluorescence [95,96], ^{19}F NMR [48] and heteronuclear NMR [82,97]. Cellular environments tend to destabilize test proteins compared to buffer alone due to the attractive interactions [82,98,99], and charge plays an important role in this destabilization [98,100]. However, increased stability has also been reported [97,101,102].

HDX NMR has also been applied to study stability in solutions of protein crowders and in cells [62,103,104]. Reconstituted cytosol and lysate destabilize CI2 [62,104]. However, the stabilizing osmolyte glycine betaine can relieve this destabilization [62]. Our lab also studied the stability of the B1 domain of protein G (GB1) in living *Escherichia coli* cells and in solutions of either bovine serum albumin (BSA) or lysozyme [103]. The pH in *E. coli* cells in the stationary phase is close to pH 5.8. At pH 5.8 and 37 °C, the cytoplasm of *E. coli* destabilizes GB1 compared to buffer. The destabilization arises from the attractive interactions. The accumulation of positive charge on GB1 with the decreased intracellular pH strengthens interactions with the majority of negatively charged *E. coli* proteins [105,106]. BSA and lysozyme also destabilize GB1 compared with buffer alone. The destabilization by lysozyme can be explained by the attractive interactions between positively charged lysozyme and anionic GB1. The destabilization by BSA and lysozyme is in agreement with the HDX results for CI2 [90]. The explanation for destabilization by BSA is that nonspecific attractive backbone interactions overcome charge-charge interactions and hard repulsions [81,90,104,107].

The temperature dependence of ubiquitin stability in the solutions of BSA and lysozyme was used to quantify the enthalpic and entropic components [81]. The results suggest that stabilizing hard repulsions can be completely offset by attractive test protein-crowder protein interactions. These chemical interactions can arise from charge-charge interactions, the hydrophobic

effect, and hydrogen bonding. In summary, although there are few studies of protein stability in solutions of protein crowders and in cells, the observation of both increased and decreased stability points to a key role in chemical interactions under physiologically relevant conditions.

8. What's next

Efforts should consider how the shape and charge of protein crowders affect stability, which is a challenging task because there is no easy way to keep the surface properties of a crowder protein constant while changing its molecular weight. Perhaps we should start with charged synthetic polymers even though synthetic polymers are not globular. Such efforts will yield a more nuanced view of macromolecular crowding that will increase both our understanding of nature and improve our ability to protect protein-based drugs and vaccines.

CRediT authorship contribution statement

I-Te Chu: Formal analysis, Writing – original draft, Writing – review & editing. **Gary J. Pielak:** Funding acquisition, Project administration, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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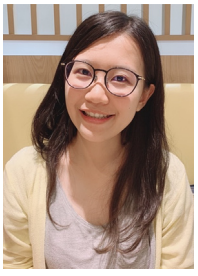

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