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Type 1 and Type 2 scenarios in hydrogen exchange mass spectrometry studies on protein–ligand complexes

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Hydrogen/deuterium exchange (HDX) mass spectrometry (MS) is a widely used technique for probing protein structure and dynamics. Exposure to D_2O induces the deuteration of backbone N–H groups *via* a process that involves transient excursions to partially unfolded protein conformers. The resulting mass shifts can be probed by MS, usually in combination with proteolytic digestion and/or electron-based fragmentation. Studies on protein–ligand complexes represent a particularly important HDX/MS application. The prevailing view is that ligand binding should reduce deuteration rates, and it is often expected that this reduction will be most pronounced in the vicinity of the interaction site. Many protein–ligand systems do indeed behave in a fashion that is consistent with this paradigm. In this review we point out that the opposite effect may be encountered as well. Also, mixed scenarios are possible where ligand binding induces elevated HDX rates in some protein regions, whereas rates in other segments are reduced. We present a framework that links ligand-induced changes in HDX kinetics to alterations in the occupancy of excited protein conformers. Spontaneous ligand binding will always lower the free energy of the ground state. In contrast, the corresponding free energy shifts of excited states are largely unpredictable, giving rise to a range of possible HDX responses. "Type 1" scenarios, characterized by a reduction of HDX rates are just as feasible as "Type 2" behavior where deuteration is accelerated. Even "Type 0" phenomena may be encountered, where HDX rates are unaffected by the presence of ligand. Type 0/1/2 scenarios can coexist in the same protein (these terms are not to be confused with the EX1/EX2 expressions which refer to a different aspect of protein HDX). Allosteric effects and ligand-induced protein–protein contacts can affect the outcome of protein–ligand binding studies as well. In summary, comparative HDX measurements conducted in the presence and in the absence ligand provide a detailed fingerprint of biomolecular interactions. However, protein–ligand interactions can elicit a wide range of responses, and the interpretation of binding site mapping experiments may not always be straightforward.

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Introduction

Numerous biological events are mediated by noncovalent protein–ligand interactions.[1] Ligand binding can induce dramatic changes in the structure and activity of protein receptors.[2–4] Many cellular regulation and communication processes, as well as drug action mechanisms rely on the fact that proteins can be switched on or off *via* changes in ligand concentration.[5] For example, signal transmission across the nerve–muscle synapse is mediated by the nicotinic acetylcholine (ACh) receptor. Binding of this protein to ACh induces pore opening, allowing ions to traverse the membrane.[6] Subsequent hydrolysis of ACh removes the ligand, causing the receptor to switch back to its closed state. Ligands that are important for switching events in other protein systems include metal ions, hormones, and various inhibitors. Protein–protein interactions play a key role as well.[7]

Analytical tools that are capable of detecting protein–ligand interactions and that report on the underlying structural changes are essential for many biochemical, pharmacological, and clinical applications.[8] Titration experiments with optical,[9] nuclear magnetic resonance (NMR),[10] or mass spectrometric detection[8,11] play a major role in this context. Isothermal titration calorimetry (ITC) provides dissociation constants and thermodynamic parameters.[12] Surface plasmon resonance assays yield information on binding and dissociation kinetics.[13]

Hydrogen/deuterium exchange (HDX) mass spectrometry (MS)[14–21] represents another important tool that is widely used for probing protein–ligand interactions. The number of HDX/MS practitioners has surged in recent years, partly as the result of integrated commercial systems that have now become available.[22] Applications that are of particular interest include epitope mapping,[23–25] conformational studies on biopharmaceuticals,[26,27] and investigations on protein–drug interactions.[1,28,29] HDX/MS-based binding studies follow a simple strategy that involves comparative measurements of protein deuteration kinetics in the presence and in the absence of ligand.

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Unfortunately, the interpretation of HDX/MS data is not always straightforward, and novices may have some misconceptions. For example, it is too simplistic to assume that changes in protein HDX rates are primarily due to steric shielding by the ligand.[28] Instead, HDX changes are often caused by alterations in the structure and dynamics of the protein after ligand binding.[30] It is also incorrect to assume that binding sites can always be mapped by looking for protein regions that exhibit the most pronounced ligand-induced HDX reduction. In reality, allosteric effects[28,29] and newly formed protein–protein contacts[31] can result in protection patterns that are quite complex, extending to regions remote from the interaction site. In some cases ligand binding can even cause an increase in deuteration rates.[32]

The current article attempts to develop a comprehensive view of the various scenarios that may be encountered in HDX-based binding assays. We will first review some basic aspects of protein–ligand interactions, as well as a few HDX fundamentals. Subsequently, we propose a general framework that accounts for the fact that ligand binding can cause a decrease ("Type 1") or an increase ("Type 2") in deuteration rates, along with various hybrid scenarios. These concepts will be illustrated by highlighting recent data from the literature.

Protein–ligand interactions: enthalpy and entropy effects

The interaction between a protein P and its ligand L is governed by the equilibrium

$$\mathrm{PL} \rightleftharpoons \mathrm{P} + \mathrm{L} \tag{1}$$

with a dissociation constant

$$K_\mathrm{d} = [\mathrm{P}][\mathrm{L}]/[\mathrm{PL}] \tag{2}$$

that corresponds to the standard dissociation free energy

$$\Delta_\mathrm{d}G^0 = -RT \ln K_\mathrm{d} \tag{3}$$

For PL to be stable the dissociation step has to be endergonic, *i.e.*, $\Delta_\mathrm{d}G^0$ has to be positive. Typical protein–ligand dissociation free energies range from *ca.* +15 kJ mol^{-1} to +70 kJ mol^{-1}, corresponding to millimolar to sub-picomolar K_d values.

Some protein receptors comprise multiple subunits. Also, ligand binding does not always follow a 1 : 1 stoichiometry, leading to equilibria of the type $\mathrm{P}_m\mathrm{L}_n \leftrightarrow \mathrm{P}_m + n\mathrm{L}$ where m is the number of protomers in the complex, and n is the number of ligand molecules involved. For simplicity, our discussion will mainly focus on simple PL systems with $m = n = 1$, but most of the concepts outlined below can be extended to other cases as well.

It can be quite difficult to dissect the various enthalpic ($\Delta_\mathrm{d}H^0$) and entropic ($\Delta_\mathrm{d}S^0$) factors that govern the dissociation free energy ($\Delta_\mathrm{d}G^0$) of a PL complex according to[27,28]

$$\Delta_\mathrm{d}G^0 = \Delta_\mathrm{d}H^0 - T\Delta_\mathrm{d}S^0 \tag{4}$$

Numerous contributions have to be considered that originate from the protein, the ligand, and the surrounding solvent.[12,33,34] We will briefly touch on the concepts of enthalpic and entropic stabilization, skirting around the contentious issue of enthalpy–entropy compensation.[35–40]

Enthalpic stabilization

The thermodynamic stability ($\Delta_d G^0 > 0$) of most PL complexes arises from the dominance of enthalpic factors. To appreciate the role of $\Delta_d H^0$, one has to understand that entropy effects usually push towards dissociation of PL, largely because the translational, rotational, and conformational freedom of the ligand is enhanced upon disruption of the complex ($\Delta_d S^0 > 0$). This entropy gain lowers the stability of PL because ($-T\Delta_d S^0$) in eqn (4) will be negative. Complexes of this kind can only be stable if $\Delta_d H^0 \gg 0$, thereby ensuring that the overall dissociation free energy in eqn (4) remains positive. $\Delta_d H^0 \gg 0$ implies that a substantial amount of heat is required to rupture the hydrogen bonds, salt bridges, van der Waals contacts, and other interactions that stabilize the protein–ligand complex. ITC is capable of measuring this heat energy directly,[9] by reporting on the association enthalpy which is equal to $-\Delta_d H^0$.

Entropic stabilization

As pointed out, the entropy gain associated with dissociation of the ligand is a major factor that tends to compromise the stability of many complexes. Efforts aimed at designing high affinity interactions therefore frequently employ conformationally restricted ligands for which the magnitude of $\Delta_d S^0$ is reduced. An example of this strategy is the use of cyclized peptide ligands.[41] Also, some proteins compensate for the entropy gain associated with ligand release by providing enhanced conformational freedom to their polypeptide chain in the bound state.[34,38,42]

Surprisingly, the dissociation of some complexes is exothermic ($\Delta_d H^0 < 0$), implying that these systems must be stabilized by a negative $\Delta_d S^0$.[9] Such a scenario seems peculiar, considering that we just noted the preponderance of PL systems with $\Delta_d S^0 > 0$. To understand why dissociation of a complex can be entropically unfavorable, one has to consider that both the free ligand and the free protein are surrounded by partially immobilized water molecules. In the case of hydrophobic moieties this phenomenon is particularly pronounced, as envisioned in the iceberg model which assumes that nonpolar groups are surrounded by a clathrate-like water shell.[43] As long as a hydrophobic ligand is bound to a nonpolar site on the protein, formation of this clathrate-like water is greatly reduced because the hydrophobic surfaces are shielded from the solvent. Dissociation of PL leads to formation of an ordered solvent layer at the newly exposed binding site on the protein, as well as around the ligand. This represents an entropically unfavorable contribution ($\Delta_d S^0 < 0$). Scenarios with $\Delta_d S^0 < 0$ and $\Delta_d H^0 < 0$ are thus often considered to be a hallmark of hydrophobically bound complexes.[39] However, similar effects can also be encountered for protein–metal interactions, where entropic stabilization of PL may arise from tightly bound waters in the hydration shell of free metal ions.[40]

A fresh look at HDX fundamentals

Hydrogen has two stable isotopes, *i.e.*, protium (^{1}H) and deuterium (^{2}H). Strictly speaking, the term "hydrogen/deuterium exchange" thus represents a misnomer, and "protium–deuterium exchange" should be used instead.[44] However, the latter is uncommon in the literature because most practitioners equate "hydrogen" with ^{1}H.

HDX measurements can be conducted with MS or with NMR spectroscopic detection. The labeling chemistry is the same for both techniques, although the detection methods are obviously dissimilar. NMR takes advantage of the different ^{1}H/^{2}H nuclear spins, yielding deuteration levels at individual N–H sites. MS studies are based on the mass difference between ^{1}H and ^{2}H, and measurements are typically conducted by monitoring the deuteration kinetics at the level of proteolytic peptides. With electron-based fragmentation[17,45,46] and other complementary strategies[47,48] the spatial resolution of HDX/MS can approach the single-residue level. For technical and practical aspects of HDX/MS[14–17,28,29,47] and HDX/NMR[49–59] readers are referred to other reviews. HDX measurements can also be conducted in the reverse direction, *i.e.*, by labeling a fully deuterated protein with H_2O.[60] The same basic principles apply to both cases, but the following considerations assume that exchange takes place in the more commonly used "exchange in" (H → D) direction.

Exposure of a protein to D_2O induces the replacement of backbone N–H with deuterium. Most backbone amide groups in natively folded proteins are engaged in hydrogen bonds that act to stabilize secondary structure (mainly α-helices and β-sheets). HDX at these N–H sites is thought to be mediated by opening/closing fluctuations that transiently rupture hydrogen bonds, concomitant with exposure of N–H groups to the solvent. The exchange mechanism at each site can be expressed as[61,62]

$$\mathrm{N-H_{closed}} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \mathrm{N-H_{open}} \xrightarrow[D_2O]{k_{ch}} \text{exchanged} \quad (5)$$

where the opening and closing rate constants are designated as k_{op} and k_{cl}, respectively, and where k_{ch} is the chemical rate constant. The latter is governed by the pH (or pD) of the solution, the temperature, and the chemical nature of the side chains adjacent to the N–H site.[63] Under typical physiological conditions k_{ch} is on the order of $\sim 1\ s^{-1}$.

Most HDX studies are carried out under conditions where the opening/closing events of eqn (5) are very short-lived, such that $k_{cl} \gg k_{ch}$.[62] In this review we will limit ourselves to a discussion of this so-called EX2 regime.[64] The deuteration rate constant k_{HDX} of a backbone N–H within the framework of eqn (1) can be expressed as

$$k_{HDX} = p_{op} k_{ch} \quad (6)$$

where p_{op} is the fraction of time that the site spends in the open state.[62] Boltzmann statistics[65] imply that p_{op} is given by

$$p_{op} = \frac{e^{-\frac{\Delta_{op}G^0}{RT}}}{Z} \quad (7)$$

with the opening free energy $\Delta_{op}G^0$ and the partition function $Z = 1 + \exp(-\Delta_{op}G^0/RT)$.[66] For a stable protein $k_{cl} \gg k_{op}$, such that $\Delta_{op}G^0 \gg 0$ and $Z \approx 1$. The occupancy of the closed state is $1/Z \approx 1$. One way to deal with eqn (7) is by introducing the opening equilibrium constant $K_{op} = \exp(-\Delta_{op}G^0/RT)$, yielding the well-known expression $k_{HDX} = K_{op}k_{ch}$.[17]

For the present discussion it is advantageous to use an alternative approach that retains the exponential notation of eqn (7). When expressing free energy of the excited state relative to the ground state in units of RT (*i.e.*, $\Delta_{op}G^0 = \Delta j \times RT$) the excited state occupancy becomes $p_{op} = \exp(-\Delta j)$, and eqn (6) turns into

$$k_{HDX} = e^{-\Delta j}k_{ch} \quad (8)$$

Let's consider an experiment where a particular amide exhibits a reduced rate constant k_{HDX}, *e.g.*, as the result of ligand binding to the protein. The only unambiguous conclusion that can be drawn from such an observation is that the occupancy of the open state is reduced because the free energy gap Δj between the closed and the open state has increased. Contrary to common belief, a lowering of k_{HDX} does not necessarily imply that the protein becomes "more rigid" in the sense that it loses conformational entropy, or that it has less extensive root-mean-square fluctuations.

Ligand binding and HDX kinetics: a two-state model

HDX-based ligand binding investigations are usually conducted with the expectation that a protein–ligand complex will exhibit reduced deuteration rates relative to the free protein. This view is consistent with a large number of MS[14–17,28,29] and NMR studies.[49–58] To rationalize this behavior one can consider a minimalist two-state model (Fig. 1), which envisions that a protein undergoes conformational fluctuations between its native state (N) and the fully unfolded conformation (U). No other conformers are allowed. N can form a complex NL, whereas U is incapable of ligand binding due to the absence of a structured interaction site.[17,32] Within this model, the opening/closing transitions of eqn (5) are equivalent to global unfolding/refolding. Fig. 1A illustrates a scenario where in the absence of ligand $\Delta_{op}G^0 = 4RT$, such that $p_{op} = e^{-4}$. Lowering the free energy of the ground state by $\Delta_dG^0 = 2RT$ *via* ligand binding increases the free energy gap between ground state and excited state, thereby changing p_{op} from e^{-4} to e^{-6}. This reduces k_{HDX} from $(e^{-4}k_{ch})$ to $(e^{-6}k_{ch})$, as dictated by eqn (8) (Fig. 1B). It is seen that ligand-induced alterations of excited state occupancies are key to understanding changes in HDX kinetics.

The two-state model of Fig. 1 represents a simple didactic tool,[67] but its applicability is limited to very specific

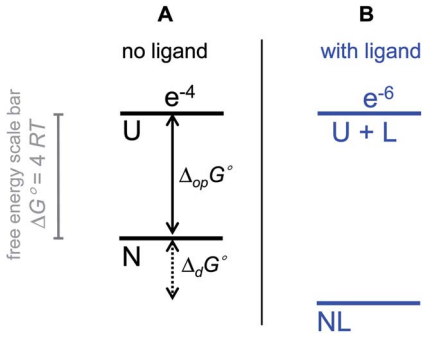

Fig. 1 Free energy level diagram of a two-state protein that can bind a ligand L in the ground state only. (A) No ligand present. The "open" unfolded state U is separated from the "closed" ground state N by a free energy gap of $4RT$, resulting in an excited state occupancy of e^{-4}. (B) Ligand binding lowers the energy of the ground state by $\Delta_dG^0 = 2RT$, thereby widening the gap to the excited state U to $6RT$. The excited state occupancy drops to e^{-6}, and k_{HDX} decreases as dictated by eqn (8). Modified with permission from ref. 32. Copyright 2014, American Chemical Society.

experimental scenarios. For example, SUPREX measurements examine protein–ligand systems in denaturant-containing solutions close to the unfolding midpoint, where the assumption of two-state behavior is often adequate.[16] For binding studies conducted in non-denaturing solution, however, the use of two-state models is too restrictive. The following section outlines an extended framework that addresses this limitation.

Type 1 and Type 2 binding scenarios

Under native solvent conditions the opening/closing transitions of most proteins (eqn (5)) are dominated by a multitude of sub-global events such as foldon fluctuations, fraying, and local dynamics down to the individual amide level.[20,62,68,69] Most of these thermally activated events can proceed without dissociation of the ligand from the protein. Excursions to a ligand-free unfolded state U (as in Fig. 1) are the exception. Realistic descriptions of protein conformational dynamics must therefore go beyond the two-state model described above. A more suitable model has to comprise a large number of ligand-bound excited states, each of which corresponds to the opening of a certain group of backbone N–H sites.

It is undisputed that many proteins are "more tightly folded" after accommodating a ligand, resulting in a greater resilience towards structural fluctuations of the type described in eqn (5), implying a strengthening of hydrogen bonds.[14,15,17,20,28,29,49–58,70] This behavior may be attributed to the fact that intermolecular binding can promote the reinforcement of intramolecular bonds,[71,72] often driven by local concentration effects.[67] N–H sites that fall into this category show reduced HDX rates in the presence of ligand, and they are said to follow Type 1 behavior. Alternatively, ligand binding can cause more rapid deuteration, representing a so-called Type 2 scenario. A third case, where ligand binding does not affect the HDX kinetics is referred to as Type 0.[32] It is emphasized again that all three scenarios refer to conditions where deuteration proceeds in the commonly

encountered EX2 regime.[62] For the sake of clarity, we repeat that the Type 1 and Type 2 effects discussed here are not to be confused with EX1 and EX2 exchange mechanisms (a discussion of the latter can be found in ref. 64). We will now outline a framework that links the different ligand binding types to alterations in the Boltzmann occupancy of partially unfolded conformers.

Fig. 2A illustrates the excited states that are accessible to a native protein in the absence of ligand. Each of these excited levels (only three out of the countless different states are shown) represents a conformation where a certain group of N–H sites is open, while the ground state is all-closed. Every conformer is characterized by a unique value of Δj which designates the free energy gap relative to the ground state. Hence, the occupancy of each level is given by eqn (7), where the partition function $Z = 1 + \Sigma \exp(-\Delta j)$ extends over all possible states. The approximation $Z \approx 1$ still holds because the experiment is conducted under native solvent conditions where the ground state represents the dominant species.[23,24] The occupancy of each excited state is $p \approx \exp(-\Delta j)$, such that eqn (8) remains valid. Ligand binding lowers the free energy of the ground state NL by $\Delta_d G^0$ (assumed to be $4RT$ for the three cases outlined in Fig. 2B–D). The HDX response to ligand binding depends on the extent to which this lowering of the ground state free energy is accompanied by free energy shifts of the excited states.

For the Type 1 scenario of Fig. 2B it is assumed that ligand binding triggers a strengthening of contacts within the protein, such that transitions to excited conformers are associated with wider free energy gaps (larger Δj values) than in the absence of ligand. The resulting lower occupancy of partially unfolded conformers decreases HDX rates throughout the protein (eqn (8)). Simply speaking, deuteration is reduced under these conditions because the ligand-induced downward shift in free energy is larger for the ground state than for the excited states.

Spontaneous ligand binding will always lower the free energy of the NL ground state relative to that of the ligand-free species N. However, the accompanying shifts in excited state levels do not necessarily have to take place as suggested in Fig. 2B. Many other scenarios are possible. For example, in Fig. 2C we assume that the free energy of all protein conformers (ground state and excited states) is lowered by the same amount. In this case all Δj values remain unaltered, such that ligand binding will cause no change in the HDX kinetics. Such a Type 0 scenario will be encountered if the ligand does not affect the energy landscape of the protein, *e.g.*, by binding to a remote solvent-exposed side chain.[73]

Fig. 2D illustrates Type 2 behavior, where the presence of ligand increases HDX rates. Such a situation can arise if the protein possesses a large intrinsic binding affinity, but where the ligand can only be accommodated after an unfavorable structural change has taken place. The ligand-induced distortion causes conformers with open N–H sites to be more readily accessible, thereby lowering Δj values such that deuteration occurs more rapidly (eqn (8)).

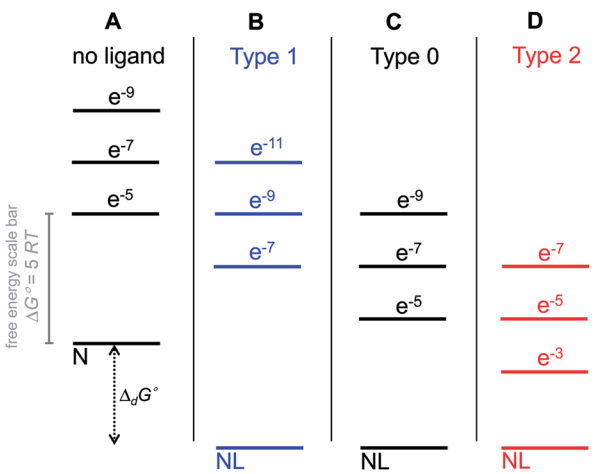

Fig. 2 Schematic free energy level diagram of a protein that can adopt many partially unfolded ligand-bound states. Only three of these are shown. The occupancy of each state is $e^{-\Delta j}$. (A) No ligand present. Excited states are characterized by $\Delta j = 5$, 7, and $9RT$. For panels B–D it is assumed that ligand binding lowers the free energy of the ground state by $\Delta_d G^0 = 4RT$. (B) Type 1 scenario: ligand binding reduces HDX rates because excited state occupancies are decreased. (C) Type 0 scenario: excited state populations and HDX kinetics remain unchanged after binding. (D) Type 2 scenario: excited state populations are increased, such that deuteration proceeds more rapidly after binding. Modified with permission from ref. 32. Copyright 2014, American Chemical Society.

Ligand-induced folding: a special case of Type 1 behavior

The three scenarios outlined in Fig. 2 apply to HDX events that take place within the framework of eqn (5),[61,62] where N–H sites are hydrogen bonded both in the presence and in the absence of ligand. Changes in the deuteration rates of these sites arise from ligand-induced alterations in the occupancy of open states.

A somewhat different situation is encountered for proteins that are unfolded (or that comprise unfolded regions) in the absence of ligand. Intrinsically disordered proteins (IDPs) represent an important class of receptors that fall into this category. Many IDPs form well organized backbone hydrogen bonds only in the presence of their binding partners.[74–76] Eqn (5) does not apply to unprotected N–H sites. Instead, the lack of hydrogen bonds results in very rapid HDX, with rates approaching those of peptide model compounds ($k_{HDX} \approx k_{ch}$).[63] Proteins that undergo ligand-induced folding switch from this unprotected regime to a situation where exchange occurs much more slowly.[77] Specifically, eqn (8) predicts that k_{HDX} will be reduced by a factor of $e^{-\Delta j} \ll 1$ upon addition of ligand.

Hybrid scenarios

For the three cases considered in Fig. 2B–D all amide sites in a given protein show the same kind of response upon ligand binding, *i.e.*, either Type 2, 1 or 0. Such global changes in deuteration rates are indeed observed in some cases.[28,32]

However, many other proteins display hybrid scenarios where certain N–H sites exhibit slower HDX kinetics in the presence of ligand, while others undergo faster deuteration, or remain unaffected.[78–82] In other words, Type 2, Type 1, and Type 0 effects can coexist in the same protein.

Fig. 3 outlines how such a mixed HDX response can be attributed to changes in excited state occupancies of individual protein segments. For illustrative purposes we assume that in the absence of ligand all N–H sites undergo deuteration with $k_{HDX} = e^{-5}k_{ch}$ (eqn (8)). Some hydrogen bonds get stabilized upon ligand binding, resulting in slower deuteration with $k_{HDX} = e^{-9}k_{ch}$ (Type 1, blue). Sites in another region of the protein get destabilized, such that the corresponding HDX rates increase to $k_{HDX} = e^{-3}k_{ch}$ (Type 2, red). The remainder of the protein is unaffected by the presence of ligand, and the corresponding deuteration rates remain unchanged at $e^{-5}k_{ch}$ (Type 0, black).

We once again emphasize that Fig. 3 only represents a schematic cartoon. For real proteins the number of free energy levels both in the presence and in the absence of ligand will be much larger than in this example. Fig. 3 nonetheless illustrates how the model proposed here can account for the entire spectrum of conceivable ligand-induced changes in HDX kinetics. Mixed scenarios are most informative when it comes to pinpointing structural and dynamic changes of a protein in response to ligand binding. Such differential free energy shifts provide an opportunity for binding site mapping. However, readers are reminded that allosteric effects may take place, where ligand interactions influence protein dynamics in regions remote from the binding site.[28,29,79]

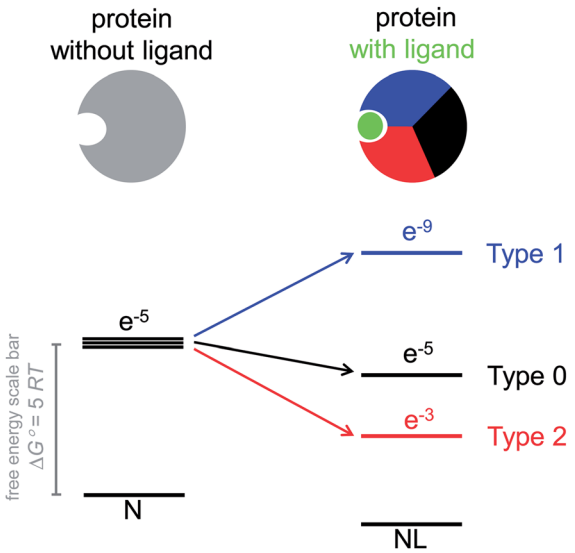

Fig. 3 Schematic illustration of a protein that displays a mixed HDX response to ligand binding. The left hand side displays the situation without ligand, assuming that $k_{HDX} = e^{-5}k_{ch}$ for all backbone amides. The right hand side shows the excited state free energy levels in the presence of ligand. Deuteration slows down for amides in the "blue" protein segment (Type 1) because that particular region gets stabilized upon ligand binding. Amides in the "red" segment exhibit faster HDX (Type 2) due to ligand-induced destabilization, whereas the remaining N–H sites do not change their deuteration behavior (Type 0, black). Similar to the preceding figures, the occupancy $p = e^{-\Delta j}$ is indicated for each excited conformer. The ligand is displayed as green circle in the protein cartoon at the top of the figure.

Examples of different HDX responses to ligand binding

The scenarios discussed above can be illustrated using some recent data from the literature. Fig. 4 provides a few examples, using a color scheme to indicate if HDX is reduced (blue – Type 1) or enhanced (red – Type 2) in the presence of ligand. The first ligand–protein system to be studied by HDX/MS in the early 1990s was the binding of heme to apo-myoglobin.[83] We revisited this protein to examine how it fits into the framework discussed above.[32] The deuteration difference map reveals that heme binding induces a global reduction in HDX rates, thus signifying a clear case of Type 1 behavior (Fig. 4A). Closer examination reveals that stability enhancements are most pronounced for helix F which undergoes ligand-induced folding.[32,84]

Hemoglobin (Hb) is a tetramer that comprises two α and two β subunits, each of which can bind an O_2 molecule. Hb oxygenation dramatically accelerates the HDX kinetics in most regions of the protein (Fig. 4B). This Type 2 behavior is consistent with the well-known T (deoxy) → R (oxy) transition which comprises a ~15° rotation of the two αβ pairs relative to each other, along with a weakening of inter-subunit

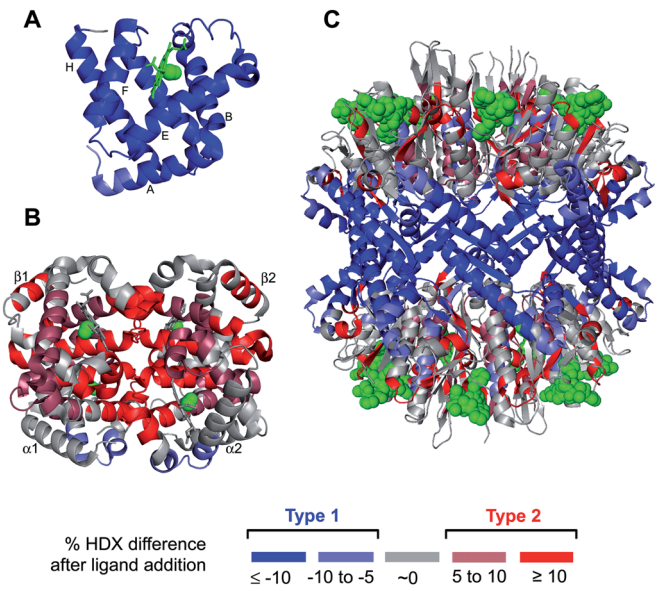

Fig. 4 HDX response of three protein systems to ligand binding. Deuteration differences are color-coded. Blue represents Type 1 behavior, where deuteration is reduced in the presence of ligand. Red signifies deuteration enhancements (Type 2). (A) Heme binding to apo-myoglobin. (B) Oxygen binding to hemoglobin.[32] (C) ADEP binding to the ClpP.[79] All ligands are depicted in green. Panels A and B were reproduced with permission from ref. 32. Copyright 2014, American Chemical Society.

contacts.[85–87] The Type 2 behavior of Hb can be attributed to free energy partitioning upon oxygenation. The large intrinsic binding free energy for each of the four O_2 ligands arises from interactions with the Fe(II) centers, and from hydrogen bonding with the distal His.[88] A significant fraction of this free energy is "reinvested" to drive the T → R transition,[89] thereby destabilizing the oxy-Hb ground state and promoting HDX. On the basis of these Hb data it is tempting to speculate that Type 2 behavior might be limited to cooperative multi-subunit systems. However, ligand-induced HDX enhancements can take place for monomeric proteins as well.[32]

The bacterial protease ClpP consists of fourteen subunits that assemble into two stacked rings.[90] The central degradation chamber can be accessed *via* axial pores which are obstructed as long as the protein does not interact with any binding partners. Acyldepsipeptides (ADEPs) are antibacterial compounds that bind in hydrophobic clefts surrounding the pores. Binding causes the pores to open up, thereby triggering uncontrolled hydrolysis of intracellular proteins.[91] Interestingly, ADEP binding destabilizes hydrogen bonds in the vicinity of the ligand binding sites, while stabilizing the equatorial region of the complex. This behavior is apparent from the color pattern in Fig. 4C, which signifies a mixed Type 1/Type 2 response.[79] At the same time, these ClpP data provide a cautionary example that highlights the occurrence of allosteric effects, where ligand binding sites do not coincide with the regions of strongest HDX protection.[28,29,79]

HDX/MS experiments on the ε subunit of ATP synthase from *Bacillus PS3* highlight additional issues that may be encountered in ligand binding experiments. The protein comprises a two-helix domain and a β sandwich. Isolated ε undergoes a major structural transition upon ATP binding. In the presence of ligand the two helices switch from an extended to a compact conformation. The earlier literature implies that the isolated protein acts as a monomer, although the ATP-bound state crystallizes in a dimeric form.[92] In the presence of ATP the helical region displays strongly reduced deuteration, consistent with its involvement in ligand binding. Unexpectedly, a second protected region is seen in the β sandwich which does not interact with ATP (Fig. 5A). As noted, allosteric effects represent one possible reason for the occurrence of such Type 1 behavior in two widely separated regions.[28,29,79] However, in the current case a different phenomenon is encountered. Analytical ultracentrifugation revealed that ε undergoes ATP-induced dimerization. When considered in the context of the dimeric X-ray structure, it becomes clear that HDX protection in the β sandwich results from ATP-induced protein–protein contacts (Fig. 5B).[31] This finding highlights the fact that ligand-induced oligomerization phenomena may play a major role for the HDX behavior of proteins. For this reason it can be essential to conduct ligand binding assays not only with HDX/MS detection, but in conjunction with complementary analytical approaches such as analytical ultracentrifugation, gel filtration, or native mass spectrometry.[93–95]

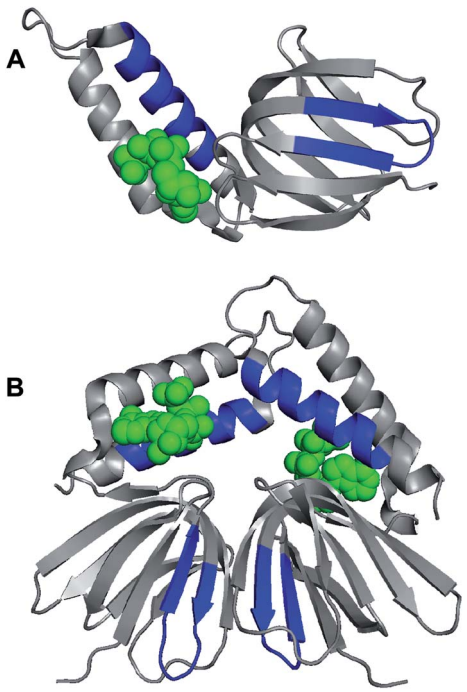

Fig. 5 ATP binding to the isolated ε subunit of *Bacillus PS3* ATP synthase monitored by HDX/MS. Regions exhibiting the most pronounced reduction in deuteration after addition of ATP are highlighted in blue. The ATP ligand is shown in green. (A) A depiction of the protection pattern in the context of a single polypeptide chain. (B) Actual (dimeric) form of the protein in the presence of ATP, highlighting the fact that protection arises from both ATP binding and from protein–protein contacts.[31]

Conclusions

This review demonstrates that protein–ligand interactions can give rise to a wide range of HDX scenarios. Binding can cause a decrease (Type 1) or an increase (Type 2) of deuteration rates. Examples of Type 0 behavior (*i.e.*, a lack of HDX changes) are quite rare, although HDX/MS is occasionally used for verifying the absence of ligand-induced perturbations.[27] All of these scenarios are thermodynamically feasible because the ligand binding behavior of a protein is dominated by the ground state. Binding is a spontaneous process as long as the free energy of NL is lower than that of N (Fig. 2). This criterion for spontaneity makes no prediction regarding the excited state behavior, because the cumulative Boltzmann occupancy of "open" conformers is low.[62] Nonetheless, these sparsely populated excited states govern the HDX properties of the protein according to eqn (5) and (8). The largely unpredictable ligand-induced changes of Δj values translate into a range of possible deuteration scenarios (Fig. 2 and 3).

Extensive discussions in the literature have examined the question whether ligand binding processes are best described by lock-and-key or induced-fit mechanisms, or whether conformational selection models are more appropriate.[3,96] The Type 2/1/0 framework outlined here is compatible with any of these mechanisms. The various types of HDX response arise from differences in structure and dynamics of the free protein

vs. the bound state. The mechanism of the actual binding process, therefore, appears to be of secondary importance for the type of HDX response.

The prevalence of Type 1 binding tends to foster the view that reduced deuteration rates are a general hallmark of protein–ligand interactions.[14,15,17,20,28,29,49–58,70] It has now become clear that biologically important interactions are easily overlooked when using screening approaches that exclusively focus on such Type 1 scenarios. Type 2 (and even Type 0) behavior may be more common than currently thought.[32] Allosteric effects and ligand-induced oligomerization can contribute to the HDX response as well. The combination of HDX/MS with complementary structural techniques may therefore be required to garner a full understanding of protein–ligand interactions. Overall, it is hoped that this review will help practitioners decipher the complexities of ligand-induced changes in deuteration patterns.

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