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Hydrogen-deuterium exchange coupled to mass spectrometry: A multifaceted tool to decipher the molecular mechanism of transporters

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A B S T R A C T

Transporters regulate trafficking through the biological membrane of living cells and organelles. Therefore, these proteins play an important role in key cellular processes. Obtaining a molecular-level description of the mechanism of transporters is highly desirable to understand and modulate such processes. Different challenges currently complicate this effort, mostly due to transporters' intrinsic properties. They are dynamic and often averse to *in vitro* characterization. The crossing of the membrane via a transporter depends on both global and local structural changes that will enable substrate binding from one side of the membrane and release on the other. Dedicated approaches are required to monitor these dynamic changes, ideally within the complex membrane environment. Hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) has recently emerged as a powerful biophysical tool to understand transporters' mechanism. This mini-review aims to offer to the reader an overview of the field of HDX-MS applied to transporters. It first summarizes the current workflow for HDX-MS measurements on transporters. It then provides illustrative examples on the molecular insights that are accessible thanks to the technique; following conformational transitions between different states, observing structural changes upon ligand binding and finally understanding the role of lipid-protein interactions.

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1. Introduction

The transport of hydrophilic molecules through the biological membrane is an essential feature of any living cell. Specialized proteins aptly named transporters carry out this task by allowing a

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passage through the lipid bilayer to specific substrates. Because of their fundamental role, the study of transporters has always been an intense area of research. They play a role not only in sustaining a cell's basic functions but also in defining drugs pharmacokinetics properties and therapeutic efficiency [1–3]. A well-known example is the role played by efflux pumps in the resistance phenotype of cancer cells [4] or pathogenic bacteria [5]. Furthermore, transporter dysfunction often has dramatic consequences in humans. More than 80 recessive disorders are associated with transporter loss of function [1,6].

According to the alternating-access model of transport, these membrane proteins switch between conformations exposing a central binding site to opposite sides of the membrane [7]. This model has been validated through several structural snapshots of transporters in various stages of their conformational cycle [8,9]. Despite these advances, several questions remain unanswered regarding the fundamentals of transmembrane transport. Contemporary study of the molecular mechanism of transporters consists in describing at the molecular level what causes the structural rearrangements, both locally and globally, that enable the conformational transition, and to deduce the sequence of events leading to such changes. The trend is shifting toward integrative structural biology approaches, to unpick various aspects of a protein's function and put them back together.

Recently, hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS) has emerged as a powerful tool to study the molecular mechanism of transporters [10,11]. This method reports on the rate of exchange of deuterons with labile hydrogens of the protein amide backbone [12]. The H/D exchange is directly related to amide H-bond stability and solvent accessibility. HDX-MS gives insight into protein local and global structural dynamics [13,14]. The steep increase in the use of HDX-MS as a tool to study membrane proteins in general and transporters in particular is linked to the gigantic leap forward in structural resolution afforded by cryo-electron microscopy (cryo-EM) [15] and computational methods, namely the Alphafold2 prediction algorithm [16]. Now that static snapshots can be obtained and or predicted, the structural framework in which the technique reveals its capabilities is available.

In this mini-review, we summarize and provide examples of the recent insight that was obtained about the way transporters work using HDX-MS. Specifically, we will focus on three aspects (Fig. 1a). First, what are the structural consequences of ligand binding? Second, what are the conformational dynamics supporting the alternating-access model of transport? Finally, what is the role of the lipid environment in modulating local and global dynamics? A clear understanding of the experimental workflow and the nature of the data obtained by this technique is necessary to appreciate and interpret the results of HDX-MS experiments (Fig. 1b).

2. The HDX-MS workflow

A typical HDX-MS experiment comprises four steps. 1. Isotopic labeling of the protein with deuterated buffer. 2. Quenching of the exchange reaction and digestion 3. Liquid chromatography couped to mass spectrometry (LC-MS) and 4. data analysis. Each step of this workflow needs to be adapted and optimized for membrane proteins.

The labeling step is the H/D exchange reaction. This is done by diluting the transporter at least fivefold into a deuterated buffer for a specific amount of time. A minimum of 4 time points is recommended to cover different timescales of exchange (second, minutes, hours) and capture significant differences between conditions. This wide range of timescales reflects the variety of observed H/D exchange rates of the backbone amides H-bond. Typically, the backbone of an unfolded loop that is solvent accessible will exchange immediately (<seconds), while an H-bond involved in secondary structural elements such as an alpha-helix will exchange slowly or not at all (>hours). This rule of thumb should be used with caution as experience has shown many times that regions that were predicted as protected from exchange based on structural models were exchanging significantly, and vice-versa [17]. H/D exchange is *in fine* a measure of an H-bond “willingness” to exchange. A special care should be taken to ensure that no aggregation happens during the labeling step, as such a phenomenon would greatly complicate both the following experimental steps (quench, digestion, and LC-MS) as well as data interpretation. The time during which the protein is stable at the labeling temperature has to be determined before the experiment, using analytical size exclusion chromatography. Unstable transporters will aggregate over time, leading to a peak just after the void volume—distinct from that of the monomeric folded protein. Alternatively, spectroscopic approaches such as Circular Dichroism (CD) or Attenuated Reflection Fourier Transform infrared spectroscopy (ATR-FTIR) can also be used to monitor global changes in secondary structure.

The labeling reaction is quenched by lowering the temperature to 0°C and the pH to 2.5, conditions at which the rate of exchange of amide hydrogens is the slowest [18]. The quench buffer can be complemented with additives to facilitate enzymatic digestion step. Typically, denaturants such as Guanidium hydrochloride (Gu-HCl) and urea, and reducing agents such as tris (2-carboxyethyl) phosphine (TCEP) can help, although this is transporter-dependent [19]. The addition of detergent such as dodecylmaltoside has been shown to improve digestion efficiency [20]. The digestion itself is done by pepsin or another enzyme active at acidic pH. The use of an enzymatic column on-line with the chromatography system is the current standard approach [10,11]. The peptic peptides are then separated by liquid chromatography, and analyzed for identification and quantification of deuterium uptake by mass spectrometry. To minimize back-exchange and subsequent loss of signal, the chromatography step is typically done in ~6 min and at 0 °C using an ultraperformance liquid chromatography (UPLC) system.

HDX-MS experiments on transporters typically compare the protein under different conditions (apo vs ligand, mutant vs wild-type) to study the effect of a variable on the structural dynamics. The difference in deuterium uptake (ΔHDX) contains the structural information. The data analysis comprises two steps. First, quantification of deuterium uptake per peptide per time point. This step can be done by a variety of software either freely available or licensed [21–23]. The second step is a statistical analysis to evaluate the significance of the ΔHDX values. Series of approaches have been developed, ranging from the “rule-of-thumb” that any difference above 0.5 Da is likely significant (this has now fallen into disuse) to more sophisticated and accurate approaches that integrate both peptide level and protein-level significance [24,25]. Ultimately, the ΔHDX values are plotted on a 3D model of the transporter (determined experimentally or predicted), to visualize how a specific variable affects structural dynamics. And that's when the fun starts.

3. Structural changes upon ligand binding

The structural consequence of ligand binding can be captured in high-resolution structures but such static snapshots often represent only a substate of a varied structural ensemble and miss how the binding affects the protein at the dynamic level. Local unfolding, transient states and allosteric networks within the transporter together determine changes in the free energy conformational landscape that will eventually result in substrate translocation. Transporters can be promiscuous regarding ligand binding, and HDX-MS has proven quite powerful at pointing out differences in

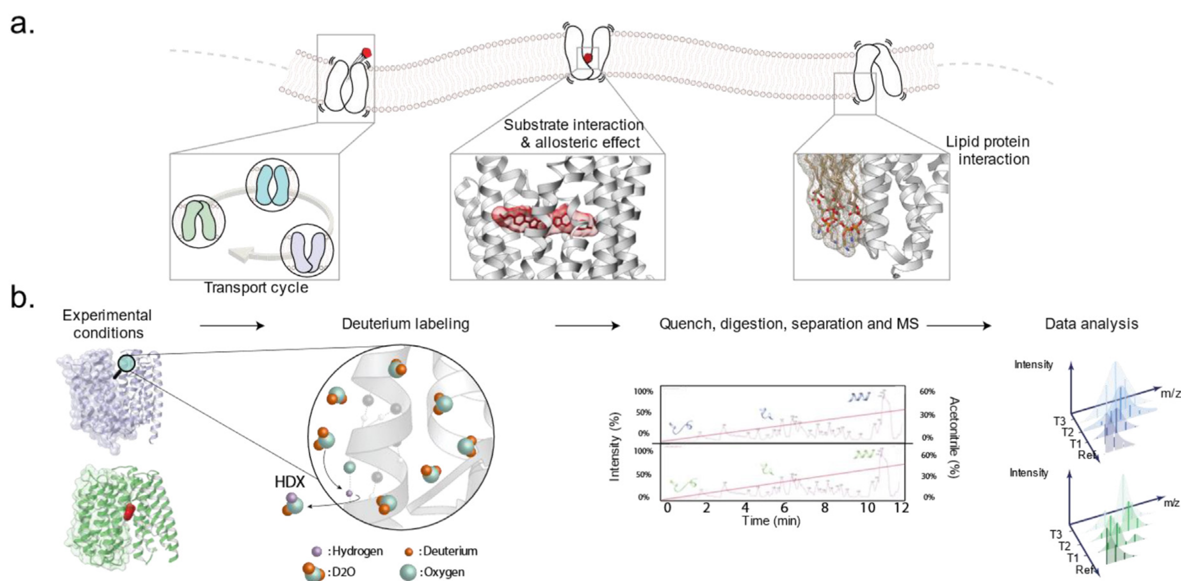

Fig. 1. HDX-MS to study the mechanism of transporters. a. The conformational transition between distinct states of the transport cycle (left), the molecular consequences of substrate binding (middle) and the role of lipid-protein interactions (right) are three key aspects of transport function that can be studied by HDX-MS experiments. b. Typical workflow of a differential HDX-MS experiment on a transporter. The transporter in apo (purple) and bound (green) conditions is labeled in a deuterated buffer. The labeling reaction is quenched, the proteins are enzymatically digested and peptides are separated and analyzed by LC-MS. Data analysis of the deuterium uptake reveals differences between conditions that contain structural dynamics information.

dynamics as a function of the bound substrate, counter-ion, or nucleotide.

Such efforts have been ongoing for more than 15 years now, starting with the mitochondrial ADP/ATP transporter bAnc1p [26], then ABC transporters BmrA [27] and P-gP [28,29], the neurotransmitters transporters LeuT [30,31], DAT [32] and SERT [33], and EAAT1 [34], and bacterial secondary transporters XylE [35], NhaA [36] and NorM_PS [37], as well as the Na^+/Ca^{2+} exchanger NCX [38]. A nice illustrative example about how HDX-MS can be used to fingerprint differences in inhibitors mode of action is provided by the work of Möller and colleagues [33]. The team studied the HDX-MS profile of the neurotransmitter SERT in the presence and absence of different molecules, namely the antidepressant S-citalopram, the neurotransmitter serotonin (5-HT) and psychotropic drugs cocaine and ibogain. They observed that specific regions of the protein displayed distinct patterns of H/D exchange upon binding of different compounds. Significant changes in ΔHDX values were observed mostly on the intracellular end of helix TM1, and at the extracellular loop EL4. Both S-citalopram (S) and cocaine displayed reduced exchange on TM1 compared to the Na^+ bound state. In contrast, ibogaine caused a significant destabilization of that region coupled to a decrease in exchange (protection) of EL4. Framed in the context of the high-resolution structures obtained with S-citalopram [39] and ibogain [40], the deprotection of TM1 extracellular side seems to be the hallmark of a substrate that acts like a non-competitive inhibitor of serotonin. Indeed, ibogain binds into the same central binding site as serotonin but its binding does not prevent the transporter isomerization. In contrast, S-citalopram locks the transporter in the OF conformation and directly prevents serotonin binding. Such observations can be used to predict the effect of other substrates for which no high-resolution structural data is available. For example, it is reasonable to suppose that the structural effect of cocaine is similar to S-citalopram in terms of binding location and ensuing structural effects, given the very high similarity between both HDX profiles. This prediction is corroborated by cysteine accessibility data that shows that cocaine favors the similar conformational outward-facing state [41].

Another illustrative example of how HDX-MS can help deciphering molecular subtleties with important functional consequences is illustrated by the work of Jia et al., on the proton-coupled transporter XylE [28]. High-resolution structures of XylE bound to its substrate xylose or to the inhibitor glucose show that both molecules bind at the same location, and favors the same outward-facing conformation [42]. The two structures are almost superimposable but functional assays reveal that only xylose can be transported by the protein. Extensive differential HDX-MS experiments revealed that the binding of the inhibitor leads to a difference in structural dynamics compared to the xylose-bound structure that is detectable by HDX-MS. Practically, to detect such difference Jia and colleagues first looked at the effect of xylose and glucose on the WT transporter. In both cases, sugar binding led to a stabilization of the OF conformation. They then hypothesized that a specific coupling between the proton and the substrate xylose was driving the transport cycle. To emulate such coupling, they introduced a mutation at a conserved acidic residue (D27) that had been previously identified as part of the proton translocation pathway [43]. They then compared the wild-type protein with the D27 N mutant—dubbed protonation mimic—in the presence of either xylose or glucose. They observed an overall increase in H/D exchange on the entire transporter, a pattern that had not been observed in any other condition (Fig. 2a). This indicates an increase in solvent accessibility and in conformational heterogeneity. By contrast, binding of glucose to the mutant blocks the transporter in an OF conformation (Fig. 2b). The authors hypothesize that this increase in dynamics is the signature of a "transition-competent conformational state" that results from a specific allosteric coupling between D27 and the substrate binding site. The authors couple these experimental findings with all-atom MD simulations of XylE in the IF conformation embedded in a bilayer, with and without a proton on D27, and in the presence and absence of xylose or glucose. Only the combination of a proton on residue D27 and xylose in the binding pocket makes the transporter more dynamic, with local unfolding observed in specific helices and increased solvent accessibility both in the binding pocket and at the proton

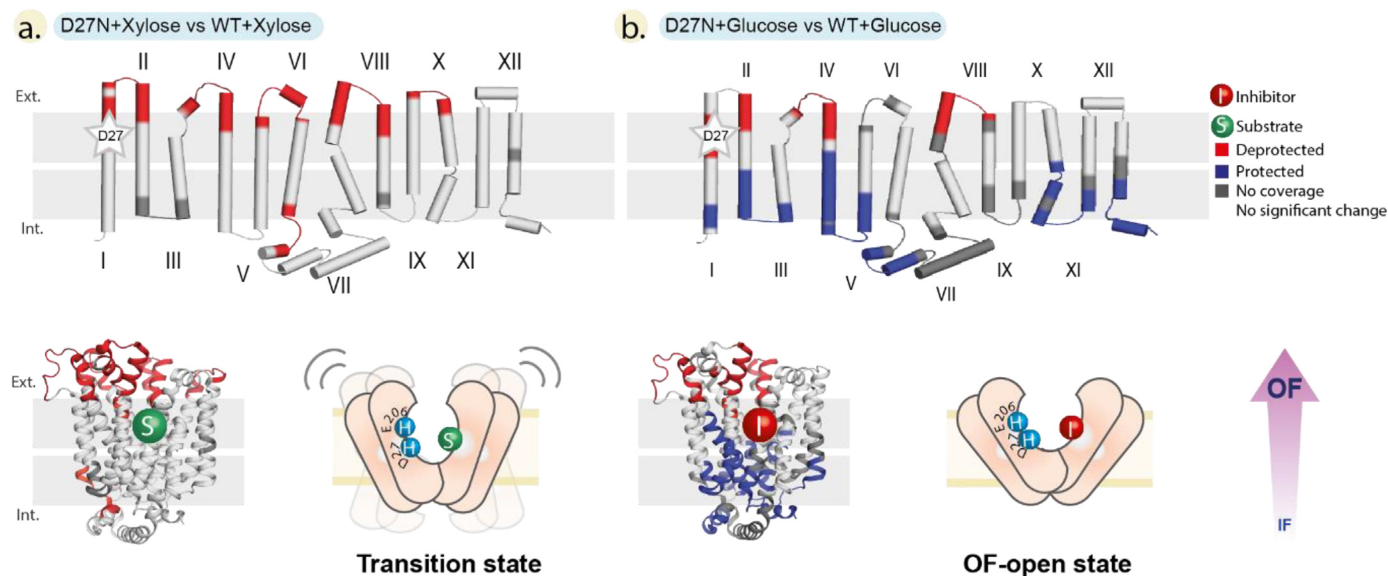

Fig. 2. Differential uptake maps (ΔHDX) of XylE WT and protonation mimic D27 N reveal a specific coupling between xylose and D27. A. ΔHDX maps of the mutant vs WT in the presence of xylose. b. ΔHDX maps of the mutant vs WT in the presence of glucose. The ΔHDX values are plotted on topological representation and 3D structure (PDB: 4GBY) of XylE. Blue and red regions indicate a negative (protected) and a positive (deprotected) deuterium uptake difference, respectively. Mutated residues are indicated by a star.

binding site. This work provides a fitting example of the complementarity between MD simulations and structural mass spectrometry.

4. Conformational switch underlying the alternating access

The catalytic cycle of transporters is based on a conformational transition between states open to opposite sides of the membrane, the outward-facing (OF) and inward-facing (IF) states. This conformational switch results from important structural rearrangements that affect the pattern of solvent accessibility. Such changes in accessibility can be measured by HDX-MS and used to identify the molecular switches leading to the changes in conformational equilibrium. The systematic validation of such approach comes from a study by this author and colleagues [44]. By using conformationally restricted mutants, they showed on the three homologous transporters LacY, GlpT, and XylE that the changes in solvent accessibility leads to a specific ΔHDX pattern. The mutants were blocked in the OF conformation using a glycine to tryptophan mutation at the extracellular end of a transmembrane alpha-helix. Deuterium uptake of the mutants was compared to that of the wild-type proteins. The ΔHDX maps (mutants minus WT) showed a significant increase in HDX on the extracellular side coupled to a decrease on the intracellular side, for all three transporters. The team then used this signature pattern as a guide to study the role of a conserved charge-relay network on the cytoplasmic side of the three transporters. This network, known as the A-motif, is a conserved feature of transporters from the widespread Major Facilitator Superfamily (MFS) [9]. Disruption of the charge-relay networks favors the IF conformation, whatever the initial conformational ensemble. This finding confirms the results of other studies aimed at following dynamics of MFS transporters with fluorescence [45] or EPR spectroscopy [46,47], as well as predictions based on the high-resolution structures of various MFS transporters [9].

Prior to this systematic investigation, changes in conformational equilibrium of transporters had already been detected by HDX-MS. Different studies have used HDX-MS to probe the changes in conformational equilibrium in the presence of different ligands such as endogenous metabolites, co-transported ions or exogenous drugs on the neurotransmitter homolog LeuT [30,31], the sodium/proton antiporter NhaA [36] and the multidrug efflux pump P-gP [22]. One of the first studies that captured the conformational switch of a transporter with HDX-MS was that of BmrA. in 2012 [27]. In this work, Mehmood and colleagues measured the exchange of BmrA purified in detergent micelles. They compare the protein mutated at location E504 in the presence of ATP, a condition known to lock the transporter in the closed conformation, with the protein in its apo form, when the two intracellular NBDS should be away from each other. They observe an important increase in deuteration of the NBDs and the intracellular domains that link the NBDs to the transmembrane domain in the apo form compared to the mutant. This finding confirms the structural predictions based on models, 10 years before a high-resolution structure became available [48]. This work also provided proof-of-principle that HDX-MS is able to detect finer mechanistic details that are not captured by high-resolution structures. For example, based on the structural data available, the deuteration of the intracellular domains (ICDs 1 and 2) should be similar in both bound and apo forms. However, there is a clear asymmetry between both domains. One domain (ICD1) shows a decrease in deuterium uptake in the closed form compared to the apo form, while the other one is more heterogenous. Such asymmetry in the local dynamics was later observed with and EPR spectroscopy on other ABCs transporters [49,50].

The power of HDX-MS to follow conformational switching of transporters is also elegantly illustrated in the work on the glutamate/aspartate transporter EAAT1 by Canul-Tec and colleagues [34]. EAAT1 was the first human neurotransmitter from SLC1 family to have its high-resolution structure solved. The team captured the transporter bound to the allosteric inhibitor UCPH101. The inhibitor is located in a crevice between two structural domains, known as the scaffold domain and the transport domain. This site is 15 Å away from the Na^+ ion and L-aspartate binding site, indicating a novel allosteric mechanism of inhibition. The authors predicted that the mechanism of inhibition was caused by a “blocking” of the transporter in the OF conformation, preventing the conformational switching necessary for transport. To confirm their hypothesis, they performed HDX-MS measurements with and without UCPH101 and

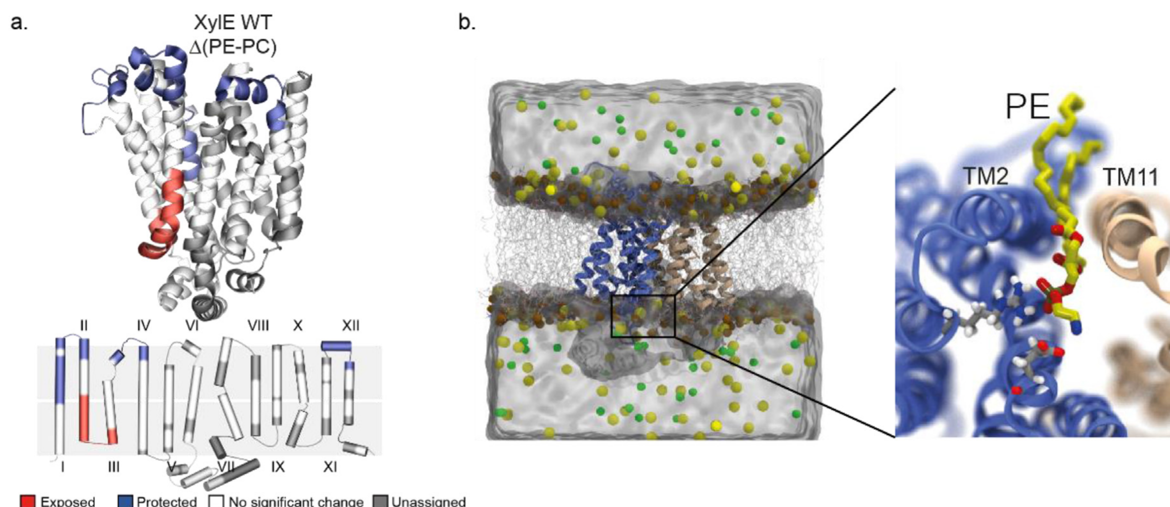

Fig. 3. Differential HDX-MS measurements of the transporter XylE in nanodiscs and all-atom MD simulations in bilayer reveal a direct lipid-protein interaction modulating the conformation equilibrium. a. ΔHDX map of XylE in PE nanodiscs vs PC nanodiscs. b. Simulation box. Representative snapshot of XylE (PDB: 4JA4) embedded in an explicit lipid bilayer and water box with 100 mM NaCl. The polar headgroup of phospholipid PE interacts directly with charged residues of a conserved cytoplasmic charge relay network.

observed a decrease in uptake upon binding on several regions. They then plotted the regions showing a decrease on the structure of the EAAT1 in the OF conformation and on a model of the transporter in the IF conformation. The patten of protection is consistent with a switch from the IF to OF conformation, where the peptides transition from a solvent-exposed region to a solvent-protected region. This experiment validated the allosteric inhibition model, and provided another illustrative case of how HDX-MS experiments combined with other biophysical tools can discriminate between mechanistic hypotheses.

5. Lipid-protein interactions in transport mechanism

It is no secret that the experimental study of transporters is complicated by the fact that they are embedded a lipid membrane. Extraction from that environment is difficult and often detrimental to the transporter structure and function. Hence, different methods that allow to preserve a lipid bilayer while allowing biophysical studies have been developed [51]. So far, HDX-MS studies of transporters in membrane mimetic systems have been performed using the nanodisc technology [52]. This approach uses a derivative of the ApoA1 human protein (termed Membrane Scaffold Protein MSP) that spontaneously rearranges as a belt around a discoidal lipid bilayer, leading to nanoscale protein-containing discs of sizes ranging from 10 nm to 16 nm depending on the type of MSP scaffold used [53].

The use of HDX-MS on transporters reconstituted in a lipidic environment has revealed important aspects about the role of lipid-protein interactions. Observations gathered with HDX-MS do not require the introduction of probes, that can affect the dynamics, structure and function. While the first HDX-MS experiment of a membrane protein in nanodiscs dates back from 2010 [54], the first mention of an HDX-MS study of a reconstituted transporter is from 2017 [31]. Adhikary and colleagues compared the deuterium uptake patterns of reconstituted LeuT, in conditions known to favor the OF (Na^+-bound) and IF (Y268A mutant) conformations. They confirmed that the conformational preference for IF and OF was similar in the more native lipid environment. Confident that this system can yield relevant insights, the authors have a deeper look into local structural dynamics. They identify structural elements that differ between the OF and IF conditions. Similar to what was observed on the human neurotransmitter SERT (see previous section), TM1a and EL4 are important conformational markers that display specific dynamics depending on the state they are in, with TM1a exchanging more in IF and EL4 more in OF. Another research group compared the dynamics of LeuT in detergent micelles and nanodiscs [30]. They observed that TM1a displays the hallmark of local unfolding in both environments (known as EX1 kinetics, characterized by a bimodal isotope pattern reflecting simultaneous exchange of all the amides protons)), and propose that local unfolding is necessary for the conformational switch. Li and colleagues carried out a similar study on the multidrug efflux pump P-glycoprotein and also observed a similar HDX behavior in micelles and nanodiscs. These studies suggest that conformational dynamics observed in micelles are representative of what can happen in a more complex bilayer environment. It is worth noting that local differences in rates of H/D of exchange and kinetics are observed between both systems and might manifest into important functional differences [28]. Whether such differences are reflecting changes in structural dynamics of the protein or are caused by differences in the experimental systems (in terms of solvent accessibility, local pH, etc.) is hard to establish. These works underline the need for orthogonal approaches to understand the role of lipid-protein interactions in the mechanism of transporters.

This author and colleagues took advantage of the nanodisc approach to specifically interrogate the role of lipid-protein interactions in the mechanisms of transporters. To this end, they reconstituted the transporters XylE and LacY into nanodiscs of different lipid compositions, one that mimics *E. coli* membranes and contains 70% of phosphatidylethanolamine (PE), and another one with 70% OF the non-bacterial lipid phosphatidylcholine (PC) [44]. They performed ΔHDX-MS measurements and observed that the *E. coli*-like nanodisc favored the IF conformation of both proteins compared to the control composition (Fig. 3a). With the help of MD simulations, they identified a predicted site for a direct interaction between the lipid PE and the transporters that would stabilize the IF conformation (Fig. 3b). The experimental validation of the predicted binding site was done by introducing a mutation at the site of interaction. Upon mutation, the lipid-dependent change in the conformational equilibrium was lost. This work shows that a direct lipid-protein interaction can be involved in the stabilization of a specific conformational state. The combination of HDX-MS and MD is especially powerful to probe the role of lipids in the mechanism of transporters with a molecular level of resolution. These

studies lay the ground for further work on the role of the membrane in the molecular mechanism of transport.

6. Conclusions

Biophysical studies of transporters are picking up speed as the realization sinks in that their fundamental physiological roles are poorly characterized, in regard to their acknowledged importance in health and disease. Compared to other membranes proteins such as ion channels and GPCRs, they are lagging behind in terms of biochemical and structural description. Several initiatives, such as the transporter consortium [1,55] and the ReSolute initiative [56], have emerged with the clear goal to catch up with their membrane counterparts, and are making great efforts to provide biochemical and biophysical tools to reach that goal.

In that context, HDX-MS has proven extremely valuable is to detect differences in dynamics that are lost in translation during the process of structural resolution. Disorder is an important functional feature and HDX-MS is a valuable tool to detect such aspect. A better understanding of how transporters work at a molecular and dynamic level is desirable for two main reasons: First, to provide a better understanding of fundamental physiological principles, such as homeostasis, nutrient uptake and signalling. Second, to provide a rational basis for drug design and drug pharmacokinetics. Such knowledge can be used to answer important questions such as: how can we stop an efflux pump from exporting antibiotics? How can we design a drug molecule that will be transported efficiently? Can we find an allosteric inhibitor specific to this neurotransmitter? The answer to these questions needs to be rooted in a fundamental description of molecular level interactions and dynamics of the transporter within the complex environment of the cell, starting with the membrane.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2022.08.014.

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