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Hydrogen/Deuterium Exchange Mass Spectrometry Applied to IL-23 Interaction Characteristics: Potential Impact for Therapeutics

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Abstract

Interleukin-23 (IL-23) is an important therapeutic target for the treatment of inflammatory diseases. Adnectins are targeted protein therapeutics that are derived from domain III of human fibronectin, and have similar protein scaffold to antibodies. A specific adnectin (Adnectin 2) was identified to bind to IL-23 and compete with IL-23/IL-23R interaction, being a potential protein therapeutic. Hydrogen/deuterium exchange mass spectrometry (HDX MS) and computational methods were applied to probe the binding interactions between IL-23 and Adnectin2 and to determine the correlation between the two orthogonal methods. This review article summarizes the current structural knowledge about Il-23 and it focuses on the applicability of HDX MS to investigate the higher order structure of proteins, which plays an important role for the discovery of new and improved biotherapeutics.

Keywords

Interleukins; structure; protein-protein interactions; hydrogen/deuterium exchange; mass spectrometry; protein binding; biotherapeutics

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Interleukins are secreted proteins that bind to their specific receptors and play important roles in the communication among leukocytes. Subsets of immune and inflammatory cells interact via interleukins. There are many interleukins with different functions [1]. For example, IL-1 family has 11 members which have different functions and can bind to IL-1 type I or type II receptors [2]. Interleukin 23 (IL-23) is an important part of the inflammatory response against infection and it is a member of the IL-12 cytokine family [3]. Its role is to promote up-regulation of the matrix metalloprotease MMP9, to increase angiogenesis and to reduce CD8+ T-cell infiltration. In conjunction with IL-6 and TGF-β1, IL-23 stimulates naive CD4+ T cells to differentiate into a novel subset of cells called T_h17 cells, which produce IL-17, a pro-inflammatory cytokine that enhances T cells and stimulates the production of other pro-inflammatory molecules such as IL-1, IL-6, TNF-alpha, NOS-2, and chemokines resulting in inflammation [4, 5]. IL-23 is a heterodimeric cytokine consisting of two subunits: p40, which is also a component of the IL-12 cytokine, and p19, which is considered the IL-23 alpha subunit [6]. A functional receptor for IL-23 (the IL-23 receptor) is composed of IL-12R β1 and IL-23R [5]. IL-23 has been implicated in several autoimmune inflammatory disorders such as gastritis, psoriasis and arthritis [4, 7, 8] and is an important therapeutic target for the treatment of different inflammatory diseases [3, 9].

Over last several years there have been significant developments in finding new therapeutics to target IL-23. Ustekinumab represents a novel class of immuno-modulating agents whose pharmacologic effects are restricted to specific immunologic pathways. This monoclonal antibody (mAb) has been approved for the treatment of inflammatory diseases such as plaque psoriasis and psoriatic arthritis [10–14]. Although this antibody was designed to bind to p40 subunit of IL-12, it was discovered that it also modulates IL-23 by binding to the p40 subunit of IL-23. Clinical observations established that IL-12/23 p40 subunit is integral to the pathogenesis of psoriasis, psoriatic arthritis and Crohn’s disease [15]. Ustekinumab was shown to bind to IL-12, IL-23 and IL12/23p40 with identical interactions [16].

While antibodies are effective therapeutics, as an alternative to them, several small protein-based drugs and alternative antibody formats are currently being investigated. Such protein scaffolds, with highly specific binding properties derived from natural human proteins have now entered clinical trials [17–20]. Less complex molecules that elicit the same extracellular blocking effects are desirable, including antibody mimetics [21]. Adnectins are one type of antibody mimetic that have shown tight and specific target binding with low toxicity, high thermal stability, good solubility, and relative ease of manufacturing [22]. Adnectins are derived from the 10th fibronectin type III domain (^{10}Fn3) [23, 24] containing complementarity-determining regions (CDRs) (BC, DE and FG loops) that are structurally analogous to the antibody heavy chain CDRs H1, H2 and H3 [21, 23, 25, 26]. In the early drug discovery, Adnectins can be designed to bind with high affinity (low nM range) and specificity to relevant targets such as IL-23 [21, 27, 28]. Adnectins provide a binding surface area that is similar in size to that of Fvs of an antibody. More importantly, the smaller size of adnectins allow to bind to surfaces on their targets that would be sterically inaccessible to Fvs, therefore making the adnectins a viable drugs alternative to antibodies which may expose epitopes that would be otherwise inaccessible to Fvs. To better understand the

binding interactions between IL-23 and adnectin, a detailed structural characterization has been carried out with an anti-IL-23 adnectin (Adnectin 2) [29].

Structural studies: known crystal structure

There are many ways to investigate protein structure. Amongst them, X-ray crystallography remains the gold standard to this day. The molecular structures on many cytokine-receptor systems are well established. The p35 subunit of IL-12 was crystallized and it consists of a four-helix-bundle sharing homology with IL-6 and GCSF [30]. It is linked to the p40 subunit via a disulfide bond. On the contrary, the p40 subunit adopts a completely different structure featuring three β-sheet sandwich domains homologues to the IL-6 receptor α-chain [31]. The crystal structure of free IL-23 and bound to a Fab of a neutralizing antibody was solved by Beyer *et al.* [32]. IL-23 forms a heterodimeric complex between p40 and p19. The p40 subunit is comprised of three domains D1, D2 and D3 that consist of β-strands. They resemble the S-type immunoglobulin fold [30]. The p19 subunit consists of a four α-helix bundle with the four α-helices named A, B, C, and D. IL-23 has 15 cysteine residues, 10 located within the p19 subunit and 5 in the p40 subunit. All the cysteine residues are involved in disulfide bonds, with exception of three cysteine residues. In addition, p40 subunit contains four potential N-glycosylation sites. However, N113 remains unmodified as shown by the crystal structure [32]. The N200 site was shown to be glycosylated in the IL-12, although this site was removed in the current IL-23 crystal structure and N200 was mutated to a glutamine. Beyer *et al.* also described the crystal structure of IL-23 upon binding to the 7G10 Fab [32]. This antibody adopts the canonical immunoglobulin fold and it binds very tight to IL-23 (Kd of 1.1 nM). The crystal structure shows that 7G10 Fab is a p19 specific neutralizing antibody that targets only the p19 domain and could eventually compete with IL-23 receptor.

Recently, a crystal structure of the IL-23 and Adnectin 2 complex was described [29]. Adnectin 2 binds to IL-23 with a high affinity (Kd of 2nM). It binds at the junction between the p40 and p19 subunits, making interactions with both subunits including domain 2 and 3 of the p40 subunit. Interestingly, although the diversified loops are located towards the center of the interface, interactions extend along the β-strands away from the diversified loops. The interaction between Adnectin 2 and IL-23 is large, burying 1320 $Å^2$ on the Adnectin surface and 1370 $Å^2$ on the IL-23 surface. The principal interactions occur through the FG loop (610 $Å^2$ of buried surface) and the BC loop (380 $Å^2$) [29].

Although a crystal structure of a protein complex can provide valuable information on the protein interface, the detailed protein dynamics information is not present, as a crystal structure captures just one, most energetic favorable conformation of the molecule in the solid state. Thus, our focus was to investigate if we can use different methods to probe the conformational dynamics of IL-23 free in solution and upon binding to Adnectin 2.

HDX MS: tool to study protein conformational dynamics

While X-ray crystallography and NMR structural analyses of protein complexes are desirable especially for providing information about binding interactions at atomic level resolution, it is not always possible to obtain such data. As many have pointed out before

[33–41], there are several advantages of studying protein/protein interactions in solution by HDX MS. A comprehensive review on HDX MS has been described in the literature [40]. Briefly, HDX MS is a versatile tool for probing protein structure, dynamics, and binding interfaces [42, 43]. This technique relies on protein backbone amide protons that are in constant exchange with solvent protons, or deuteriums if the protein is in deuterated solvent. The number of exchangeable protons and their rates of exchange depend on several factors including pH, temperature, chemical environment, and the three-dimensional protein architecture [33, 40, 43, 44], thus reflecting the structure and dynamics of the protein. The labeling is a function of H-bonding and solvent accessibility (Figure 1A). Typically, protein backbone amide protons exchange rapidly with deuterons if they are involved in weak or suboptimal hydrogen bonds, reside at/near the surface, or are readily accessible to the solvent; the exchange rates are slower if they are involved in strong intra-molecular hydrogen bonds and/or are less accessible to solvent [33]. In addition, by locating where exchange occurs, the specific parts of the protein that are most dynamic can be determined. Furthermore, regions of proteins that are heavily solvent exposed and unstructured will be labeled much more quickly than those protected from solvent or highly structured. HDX MS has been successfully applied to determine protein-protein binding sites (epitopes), based on the reduced solvent exposure in regions that constitute the binding interface [36, 45]. However, if there are changes to protein conformation as a result of binding, the exchange may be altered. These conformational changes can be detected and localized to particular regions of a protein at peptide or amino acid resolution [46–48]. Care must be exercised in these kinds of experiments as the location of changes might be remote to the site of the perturbation (i.e. allosteric effects) [49].

A typical workflow is shown in Figure 1B. The protein of interest is usually exposed to D_2O buffer for various periods of time and the reaction is quenched by adjusting the pH to 2.5 and the temperature to 0 °C. The quenched protein is digested with pepsin or another aspartic protease [50–52], online or offline, and deuterium incorporation into each pepsin fragment is analyzed. Chromatographic separation before MS analysis is accomplished with ultrahigh pressure liquid chromatography (UPLC) that provides high-resolution separations under typical HDX MS experimental conditions [53]. The obtained relative deuterium uptake levels can be plotted versus the exchange time.

HDX MS: IL-23 free and bound to Adnectin 2

IL-23 shows a dynamic behavior in solution

To form the basis for comparison of the exchange into the Adnectin 2 bound form, the incorporation of deuterium into IL-23 (molecular weight (MW) 59 kDa) in solution was measured. Undeuterated IL-23 was digested with pepsin using the same experimental conditions as those used for deuterium labeling. Peptides constituting 93.8% sequence coverage of the p19 fragment (MW 19 kDa) and 82.8% sequence coverage of the p40 fragment (MW 40 kDa) were followed by HDX MS experiments. The HDX results for IL-23 are summarized in Figure 2A. The p40 subunit consists of three domains D1, D2 and D3 that adopt an IgG like fold, whereas p19 subunit consists of a four-helix bundle A, B, C and D. Both subunits are dynamic in solution. For example, D1 and D2 of the p40 subunit

are quite dynamic and these two domains become rapidly deuterated. On the contrary, some parts of D3 are more solvent protected and this domain becomes deuterated at later time points. On the other hand, the helices that constitute the p19 subunit have different exchange patterns. Parts of the A and C helices are more solvent exposed as they incorporate deuterium at early time points, while parts of the B and D helices remain solvent protected and only exchange up to 40% of their entire exchangeable hydrogens even after three hours of labeling. These data indicate that the helices are not rigid structures and they have different levels of flexibility. The HDX MS data provide insight into the solution dynamics of IL-23.

HDX MS Analysis of Adnectin 2

Adnectin 2 was designed to bind to IL-23. HDX MS data for Adnectin 2 alone were also obtained. Upon pepsin digestion, a total of 13 peptides covering 90.3% of the amino acid sequences were identified and exchange into all of these peptides was followed (Figure 2B). Adnectin's higher-order structure is mainly composed of anti-parallel β-sheets, with connecting loops (BC, DE and FG) that are equivalent to the CDRs of an antibody heavy chain [23, 26, 27]. After 10 sec of deuteration, some β-strands of Adnectin 2 were protected from exchange with the exception of the BC and FG loops that exchanged up to 40% and 50% respectively of their available backbone amide hydrogens. After 3 hours of labeling, Adnectin 2 was more than 50% labeled including all three loops FG, BC and DE, indicating a dynamic solution conformation in spite of a heavily β-stranded structure. This dynamic behavior of Adnectin 2 is related to its function and the ability to bind to its targets.

HDX MS of IL-23 upon Adnectin 2 binding

Adnectin 2 was incubated with IL-23 prior to HDX MS experiments. The HDX MS results indicated strong protection from exchange in the p19 subunit of IL-23, especially in the N-terminal region (overlapping peptides between amino acids 18–37 in α-helix A) with a deuterium uptake difference of more than three Dalton upon binding (Figure 3A). Interestingly, the dynamics of the molecule also play a role in the binding. For example, the peptide 18–23 shows no protection at the 10 second time point, while at 1 minute labeling time point, the difference in uptake levels is visible and at least one amide H becomes protected. Another peptide 24–37 gets rapidly deuterated and upon binding, this peptide becomes protected even at the earliest time point (10 second labeling), suggesting an immediate occlusion from the solvent [54–56] upon Adnectin 2 binding. The mass spectra in Figure 3B exhibit how the isotopic distribution patterns are shifted towards lower *m/z* upon Adnectin 2 binding. Moderate protection from deuteration was also observed in the α-helix C in peptide 89–105, where one amide hydrogen atom becomes protected upon Adnectin 2 binding at the earlier time points, and the effect of the binding on deterium uptake diminished after 60 minute labeling. The HDX behaviors for C-terminal region of the p19 subunit (peptide 145–153) in the middle of the α-helix D illustrate that this peptide is occluded from deuteration at earlier time point, however, the hydrogen bonds start to break allowing deuterium to get into this peptide at later time point. Upon Adnectin 2 binding, this peptide shows protection only at later time points. Figure 3C summarizes the location and the magnitude of the deuterium uptake in p19 upon Adnectin 2 binding.

In the case of p40, only several peptides become stabilized upon Adnectin 2 binding. The peptides 196–218 and 263–286 from the D2 and D3 domains show moderate protection upon binding, suggesting that these regions were either not directly involved in binding or their conformation was only modestly altered in the presence of Adnectin 2 (data not shown). The peptide 285–294 from the D3 domain of IL-23 p40 is dynamic and occluded from solvent at earlier time points. Overall, the protection upon Adnectin 2 binding on p40 is not as drastic as in the case of p19.

Computational data: IL-23/Adnectin 2 complex

A computational approach is an additional strategy in probing protein/protein interactions. It relies on various databases and software tools. Prediction of protein structure consists of calculation of the 3D structures of a protein starting with its amino acid sequence [57]. It is well established that the native structures of proteins correspond to minimum-energy states. Currently, there are several software tools available that can be used to apply different calculation methods to proteins allowing the prediction of a protein structure and study of the molecular interactions between proteins. Empirically, the interactions between proteins can be studied from several experimental techniques, such as alanine scanning, peptide panning, yeast two-hybrid systems [58], affinity purification/mass spectrometry [59], and protein microarrays [60]. A number of computational methods for prediction of protein/protein interactions have been developed in the recent years [61, 62]. As already reviewed by others [63], these computational methods can be clustered in Bayesian network modeling, 3D template-based protein complex modeling, identification of homologous interacting pairs and identification of structural patterns [64].

Based on the interfaces determined in solution between IL-23 and Adnectin 2 by HDX MS, we conducted computational assessment to calculate the interactions energies between the interface residues in IL-23/Adnectin 2 complex and determine if there is a correlation between the two methods. The initial step was to analyze the various properties of amino acid residues that are located at the interface between IL-23 and Adnectin 2, and to elucidate the interactions occurring at that interface. To better understand the overall complex, the interaction energies between IL-23 (p19 and p40) and Adnectin 2 were calculated for the protein complex by subtracting the sum of the free energy of binding (ΔG) for the two states (bound and unbound) from the energy values for the individual states. This value takes into account multiple parameters such as: change in surface accessibility, change in Van der Waals (VDW) interactions, change in hydrogen bonds and electrostatic interactions, and change in solvation. The major components of interaction energies are VDW energies (which correlate to contact surface areas and hydrophobic interactions) and electrostatic interaction energies. A good correlation was found between the energy calculations and the HDX MS data. Regions that showed protection in IL-23 upon binding to Adnectin 2 as identified by HDX MS had reduced total interaction energies. Figure 4 summarizes the data obtained by the two orthogonal methods. The HDX MS data that showed decreased exchange upon Adnectin 2 binding is represented for both p19 and p40 subunits. In order to establish what are the important amino acids involved in Adnectin 2 binding, the cutoff was set at ΔG >5kcal/ mol. For the p19 subunit 7 amino acids were found important for the binding. Those residues are H29, M35, W26, L37, R148, L31 and D36. Their ΔG values

range from 14.6 kcal/mol to 5.2 kcal/ mol. For the p40 subunit, 8 amino acids were found to be important for the Adnectin 2 binding. Those residues are K263, R266, R285, K264, S294, E100, P101 and S204. Their ΔG values range from 19.7 kcal/mol to 5.2 kcal/ mol. The amino acids that were found to be important for Adnectin 2 binding based on interaction energy calculation are represented in Figure 4.

What does it all mean?

The unambiguous characterization of the binding interfaces of a protein to its ligand(s) can play a significant role in the discovery and development of new and improved biotherapeutic agents. Such information can best be obtained with a crystal or NMR structure wherein the position of the atoms from each member of the complex becomes apparent. However, it is not always possible to obtain a crystal structure of a protein complex and if one can be obtained, defining the interface could be confounded by crystal packing effects. Furthermore, protein complexes may not be amenable to NMR for various reasons, especially for complexes where the total size of the complex exceeds that possible for conventional NMR analysis. From this perspective, several alternative approaches combining experimental procedures and computational methods have been developed. Some of these techniques, e.g., screening peptide libraries for binding, may not be ideal because they might only detect short, linear stretches of amino acids that are recognized by a certain ligand/ antibody which may or may not adopt the relevant conformation.

In the present work, amide hydrogen exchange of IL-23 free and in complex with Adnectin 2 was first interrogated by HDX MS. The final output of HDX MS data is a description of where in the protein(s) deuterium incorporation became altered upon complex formation [65]. The HDX MS data indicates that IL-23 p19 subunit is less dynamic in solution, compared with the p40 subunit which is less structured. HDX MS data also shows that there are some peptides with reduced deuterium uptake when Adnectin 2 is bound to IL-23. The epitope of IL-23 appears to be discontinuous with different structurally important regions of the molecule allowing less exchange in the presence of Adnectin 2.

The crystal structure of Adnectin 2 bound to IL-23 has been published and the contact areas are known [29]. Based on the structure of the complex, one should be able to predict with some certainty what the HDX MS data might look like, assuming that the crystal conformation is also found in solution. Not surprisingly, the HDX-MS data reveal the regions of IL-23 with reduced deuteration upon binding to be similar to regions as indicated by the crystal structure (Figure 5). As demonstrated here, it is relatively straightforward to rationalize the HDX MS data when the structure is known. When the structure is not known, one would need to be careful when interpreting HDX data for the binding interface, considering other factors such as allostery [66]. Subsequent experiments such as site-directed mutagenesis might be necessary to confirm which regions were critical for direct binding. Alternatively, computational methods could be utilized to validate HDX MS data. In this study, the interaction energies were calculated for the regions that showed reduced deuterium uptake in order to validate the binding interface. Overall, the data obtained from these two methods are in good agreement. Figure 5 summarizes the results from all three methods. Although there is a good overall overlap in identifying binding interfaces among

the three methods, there are regions showing differences in binding as probed by different methods. For example, the data from interaction energies and the crystal structure indicate that residues Glu100 and Pro101 from p40 are important for Adnectin 2 binding, while HDX MS data showed no differences in deuterium uptake upon binding. Based on the crystal structure, Pro87 that belongs to the Adnectin 2's FG loop is required for retaining the FG loop conformation and it directly contacts residues Glu100 and Pro101 from the p40 subunit. As Pro residues don't have amide hydrogens available to exchange with deuterium, it is not possible to detect any differences in deuterium uptake in this region of p40 subunit. Another region showing differences among the three methods is located in the p19 subunit between amino acids 89–105. The HDX MS data indicate a subtle protection from exchange at the early time point, and these differences disappear at late time points. This peptide consists of a loop that is very dynamic as shown by HDX MS data. As a result of its dynamic nature, this peptide can partially bind to Adnectin 2. Those subtle movements are not captured in the crystal structure or the computational method.

To the best of our knowledge, the dataset presented in this manuscript represents the first report on the solution conformational dynamics of IL-23, opening insights into how the dynamics influence the function of this medically important target protein. Furthermore, binding of IL-23 to Adnectin 2 reveals discontinuous conformational epitopes with contribution from both p19 and p40 subunits. As a complementary method, computational analysis supports the characterization of binding interfaces in connection to experimentally obtained HDX MS data. This combination approach can lead to a better understanding of the structure-function relationship in protein/protein interactions, offering new strategies for discovery of improved biotherapeutics.

Expert commentary & five-year view

Significant advancements have been made in the field of HDX MS over the last 10 years, including instrumentation and software development. This technique is now widely adopted by scientific community with many groups using the technique on a routine basis to study proteins for many applications [67]. HDX MS experiments offer insight into how proteins move in solution by revealing information about protein dynamics. A wide range of labeling times can be monitored, from milliseconds to days, thereby providing access to many types of protein motions, including fast and slow. There are certain limitations associated with this technique. For example, the deuterium label itself is labile and can revert back to hydrogen if exposed to a solution or gas rich in hydrogen. In addition, HDX cannot be used to determine the structure of a protein. Because exchange is a function of the combined effects of solvent exposure and hydrogen bonding, exchange into an α-helix may look the same as exchange into an unstructured region of a protein that is inaccessible to the solvent. Typically, exchange at all potential exchangeable sites (both side chains and backbones) cannot be determined in the same experiment because the rates of exchange of backbone amide hydrogens and hydrogens in more rapidly exchanging side chain positions are usually very different. Some positions are too fast to be measured at all. Protein complexes driven mainly by electrostatic interactions of the side chains of very structured elements may have no changes to backbone amide hydrogen rates and therefore, such interactions are not detectable by HDX MS experiments.

With all this being said, there are more advantages than limitations in using HDX MS. This technique is well-suited for the characterization of interactions between protein targets and small or large molecules that bind to them, as illustrated in the IL-23/Adnectin 2 study. This highlights the importance of HDX MS as a technique to investigate structure–function relationship of biologically and therapeutically important proteins.

As the field continues to grow, there are challenges to be addressed in the future. Obtaining the protein of interest in its highest purity possible is an ongoing issue that is protein dependent. Dealing with data interpretation for heavily post-translationally modified proteins (such as heterogeneity due to glycosylation) remains challenging. Coupling HDX MS with MS fragmentation techniques (i.e. electron-transfer dissociation, electron-capture dissociation) to obtain amino acid resolution needs further development. Refinement in automation (including easy-to-use and reliable software) for higher-throughput is an ongoing endeavor. It is expected that HDX MS technique will evolve with advances in instrumentation, software and methodologies and continue to play important roles in understanding protein structure and dynamics.

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Key issues

- HDX MS is a powerful tool to investigate protein conformation and dynamics
- Unambiguous identification of binding interfaces is critical for rational design of protein therapeutics
- Epitope mapping can now routinely be performed with HDX MS
- HDX MS can be combined with computational methods in order to obtain a better understating about the binding interfaces

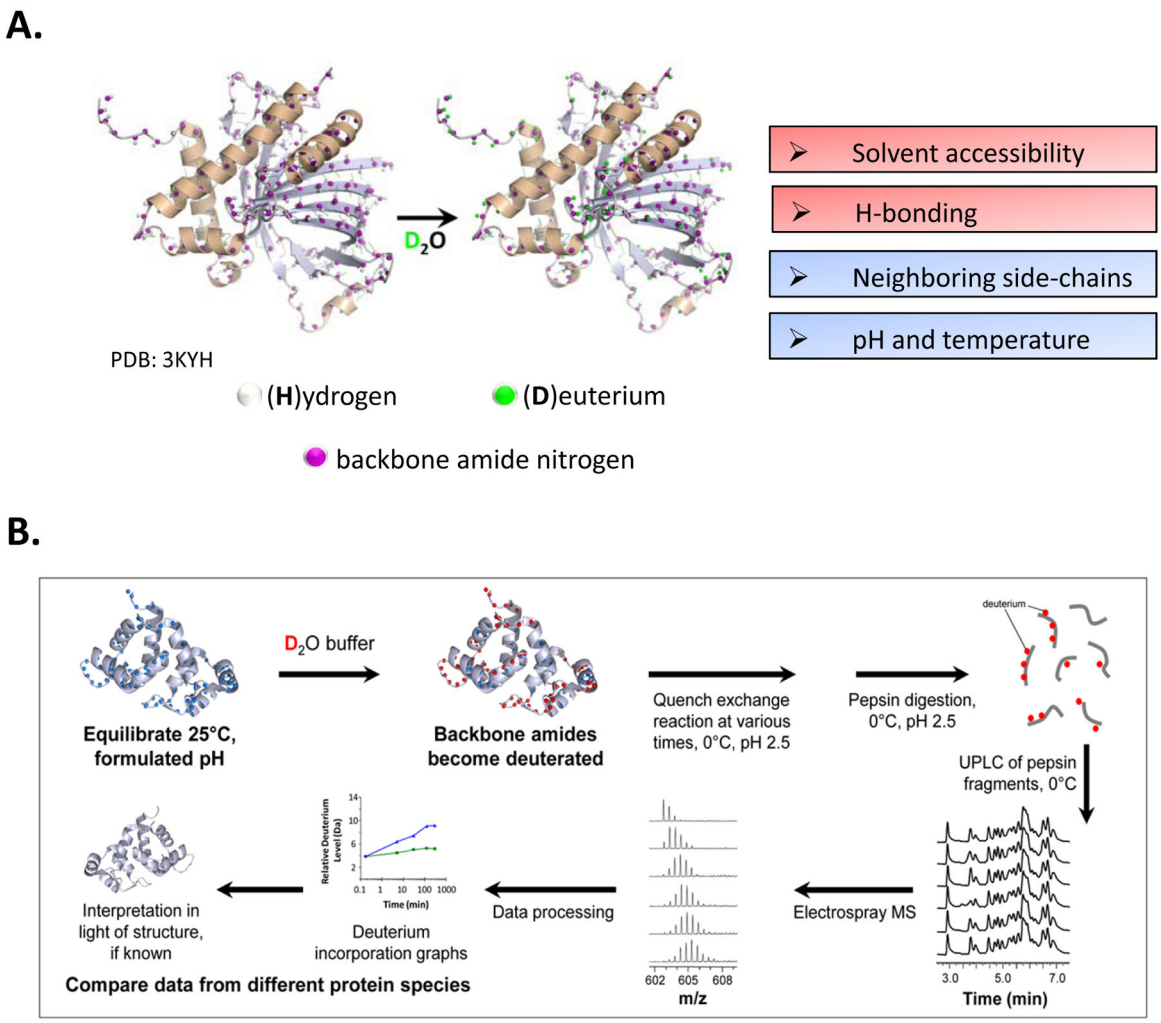
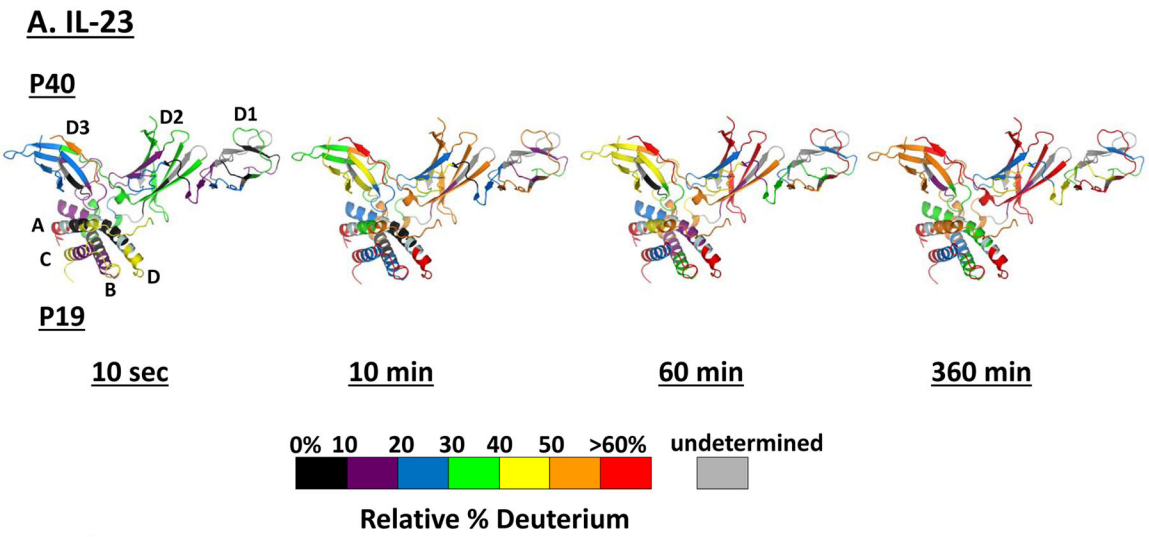


Figure 1.

A. Protein amide hydrogen/deuterium exchange in solution. B. Typical workflow for HDX MS experiment that was followed for the IL-23 labeling (reprinted with permission from the Chimica Oggi / Chemistry Today).



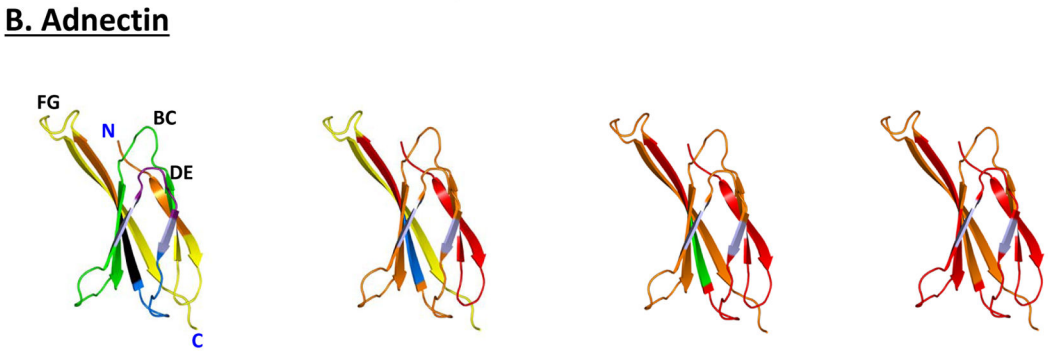


Figure 2.

Summary of HDX MS data for (A) IL-23 free in solution and (B) Adnectin 2 free in solution. IL-23 used in this study was expressed in Sf9 cells and the Adnectin 2 was expressed as previously described [29]. All comparison experiments were conducted under identical conditions and deuterium uptake levels were not corrected for back-exchange [40]. The error of measuring the mass of each peptide was ± 0.20 Da in this experimental setup, consistent with previously obtained values [68–70].

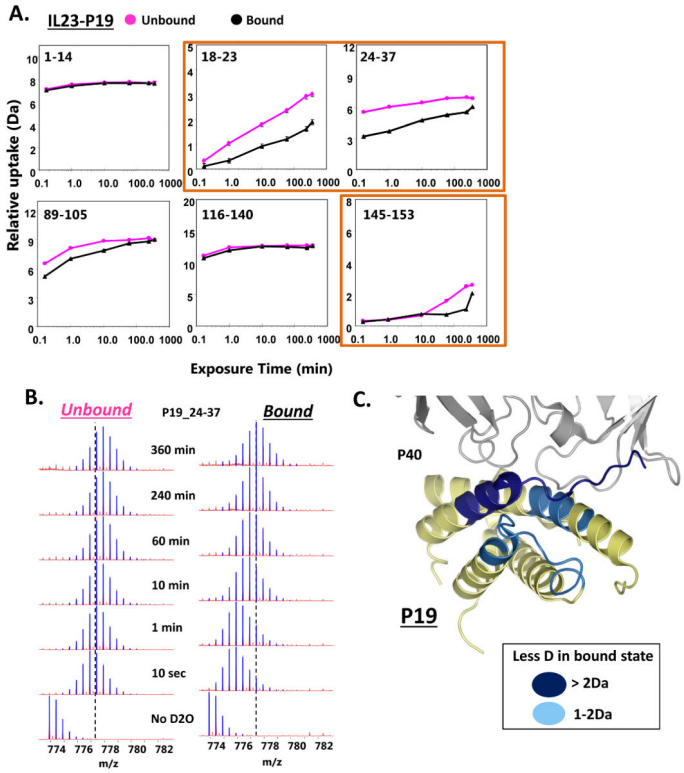


Figure 3.

A. Deuterium uptake curves in HDX for peptides in IL-23 p19 subunit between bound and unbound states. B. Mass spectra between the bound and unbound states of peptide 24–37 from p19 subunit. C. Location of the IL-23 p19 peptides exhibiting less deuterium uptake upon Adnectin 2 binding mapped onto the structure of IL-23 p19 subunit in the IL-23:Adnectin 2 co-crystal (PDB entry 3QWR; ref. [29]). Changes in deuterium uptake levels upon binding were colored: dark blue (2 Da or more at least one time point), light blue (1–2 Da), and no changes pale yellow (0–0.5 Da).

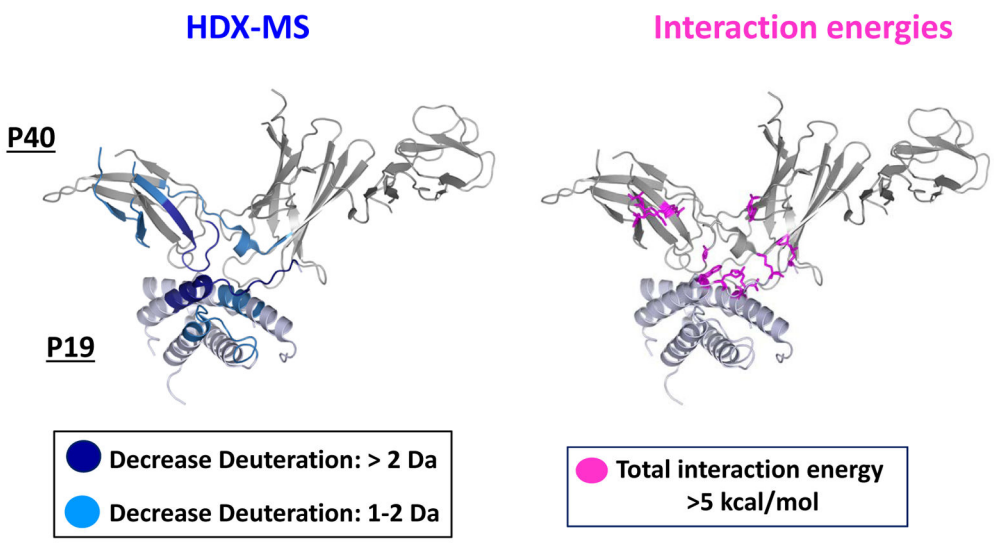


Figure 4.

Comparison between HDX MS data and computational method. The peptides that were found to exhibit significant protection from deuteration upon Adnectin 2 binding are shown in dark blue and the ones with moderate protection from deuteration are shown in light blue. The residues that were found to be important for Adnectin 2 binding as identified by the computational method are represented in magenta. The cutoff for significance was set at $\Delta G > 5$ kcal/mol.

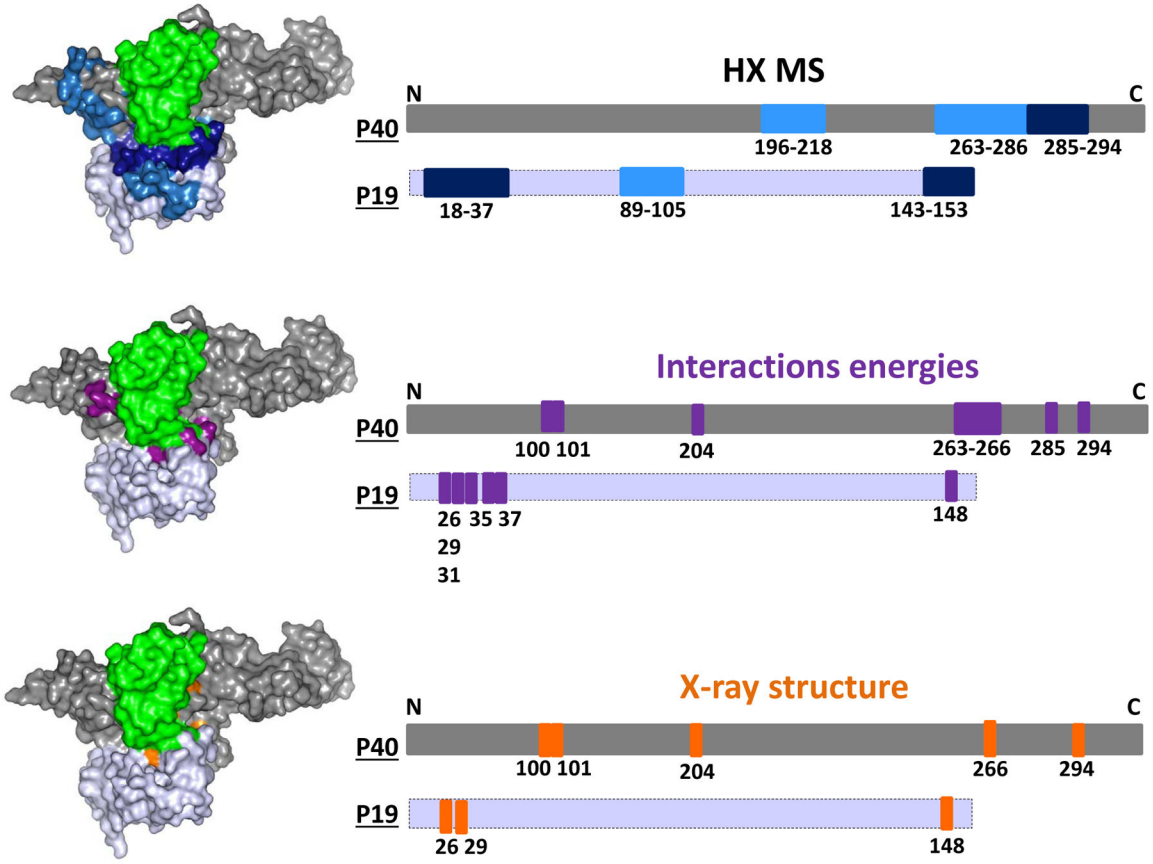


Figure 5.

Summary of the regions of IL-23 with differences in deuterium uptake as determined by HDX MS (top), by computational method (middle) and by the X- ray crystal structure [29] (bottom). A surface view of the complex (left) and linear (right) representations of IL-23 regions that are involved in Adnectin 2 binding are illustrated. Adnectin 2 is shown in green. The residues participating in complex formation that were identified by each method have been indicated in both views: dark blue and light blue for the HDX MS data, purple for the interactions energies and orange for the X-ray crystal structure.