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Protein Dynamics Revealed by Hydrogen Deuterium Exchange Mass Spectrometry: Correlation between Experiments and Simulation

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Abstract

Hydrogen deuterium exchange mass spectrometry (HDX-MS) is a powerful technique for studying protein dynamics, which is an important factor governing protein functions. However, the process of hydrogen deuterium exchange (HDX) of proteins is highly complex and the underlying mechanism has not yet been fully understood. Meanwhile, molecular dynamics (MD) simulation is a computational technique that can be used to elucidate HDX behaviour on proteins and facilitate interpretation of HDX-MS data. This article aims to summarize the current understandings on the mechanism of HDX and its correlation with MD simulation, and to discuss the recent development in the techniques of HDX-MS and MD simulation and the perspectives of these two techniques in protein dynamics study.

Keywords: Protein dynamics; hydrogen deuterium exchange; mass spectrometry; molecular dynamics simulation; nuclear magnetic resonance.

Introduction

The dynamics properties of biomolecules are essential to their functions, and are often complementary to their static structures.[1] Protein dynamics covers a broad range of molecular transitions and fluctuations reflected in multiple time scales. The structure of protein is often considered as the specific configuration at minimum free energy while the dynamics is more concerned about transient conformations with higher energy. The conformational dynamics reflects both the thermodynamic and kinetic aspects of the transition processes. Thus, the interplays between structure and dynamics are critical for the biofunctions, i.e., catalysis,[2] biomolecular recognition[3] and cooperative complexation,[4] of proteins.

Various techniques, such as nuclear magnetic resonance (NMR) spectroscopy, hydrogen deuterium exchange mass spectrometry (HDX-MS) and molecular dynamics (MD) simulation,[5] have been applied to investigate protein dynamics. NMR can obtain structural and dynamic properties of proteins at the atomic level while MD simulation reveals explicit protein conformations at varying time scales by trajectory analysis. In recent years, HDX-MS has become increasingly important in protein dynamics studies.[6] In this technique, proteins are incubated in deuterated buffers and the isotopic exchanges between the solvent deuterium and protein amide hydrogens (NH) are monitored by mass spectrometry. The exchanges cause little interferences to the proteins and allow the proteins to remain their original states. The exchange kinetics can reflect the accessibility of amide hydrogens towards solvent, thus indicating the flexibility of proteins. Compared with other techniques such as NMR applied in the monitoring of HDX of proteins, mass spectrometry has the distinct advantages of low sample consumption, less limitation in protein size, and the capability of detecting co-existing protein conformers.

On the other hand, molecular dynamics (MD) simulation is an important technique in elucidating HDX behaviour on a computational basis,[7,8] and the combination of HDX-MS and MD simulation is a powerful approach to study protein dynamics.[9] This article aims to summarize the mechanisms and applications of HDX-MS in studying protein dynamics and the use of MD in elucidating HDX-MS results. The existing limitations and further perspectives of these two techniques will also be discussed.

Development of HDX-MS

HDX was first applied for the structural study of proteins by Linderstrøm-Lang in the 1950s.[10] In 1972, Englander et al. presented a comprehensive review on the principles and knowledge of HDX gained in the previous twenty years.[11] In the early stage, HDX in combination with infrared spectroscopy or proton magnetic resonance was applied to study protein dynamics. The "breathing" concept proposed at that time has been evolved into the most accepted theory nowadays.[12] Several factors, e.g. temperature, solvent, isotope effects, should be taken into considerations when studying HDX, because these parameters could affect the exchange, leading to variations in the HDX rates.

In 1991, Katta and Chait first combined HDX and electrospray ionization mass spectrometry (ESI-MS) to investigate the conformational changes of bovine ubiquitin under different conditions.[13] In 1993, Zhang and Smith developed high-performance liquid chromatography coupled with mass spectrometry to monitor the isotopic exchange of horse heart cytochrome *c* with a bottom-up strategy.[14] Resolution of HDX was improved with the protein digested into peptides by pepsin, the acid-stable proteases, allowing acquisition of local conformational dynamics information, e.g.,

different HDX extents of the segment 95-104 of cytochrome *c* corresponding to its native and denatured conditions (see Fig. 1). Other MS detection approaches, such as nano-electrospray ionization[15] and matrix-assisted laser desorption ionization (MALDI),[16] were also applied to monitor HDX without the use of chromatographic separation. Compared with other techniques, e.g., IR, UV and NMR, MS could be the most natural one for detection of HDX since it was originally invented for isotopic analysis in the 1910s.[17] The improved sensitivity and resolution of MS in recent years have enhanced the robustness and capability of HDX-MS and allowed the technique to be applied in various fields.

Classical mechanistic model of HDX

The two-step model (Scheme 1) as proposed by Linderstrøm-Lang[10] is widely accepted for the reaction of HDX. This is based on the hypothesis that amide hydrogens undergo exchange when the hydrogens are accessible to solvent and are not protected by hydrogen bonding. Since the equilibrium state is dynamic, namely there is fluctuation between closed form and open form, the exchange rate is determined by this equilibrium state ($K_{op} = k_{op} / k_{clo}$) and the intrinsic exchange rate (k_{int}, i.e., the exchange rate of unprotected amide hydrogens). Thus, the exchange rate k_{ex} can be defined as shown in scheme 2. If $k_{clo} >> k_{int}$, the exchange rate could be simplified to $k_{ex} = K_{op} \times k_{int}$, which is known as EX2 kinetics. To the other extreme, if $k_{clo} << k_{int}$, then $k_{ex} = k_{op}$, which represents EX1 kinetics. This two-step model is widely fitted into the study of protein folding and conformational dynamics.[18] HDX can probe the solvent accessibility and hydrogen bonding of local residues, reflecting the conformations and dynamics of proteins.

Since the exchange is mediated by acid, base and water, the exchange rate is dependent on pH/pD in solution. The equation given by Bai et al. in 1993 is shown in scheme 3.[19] This exchange rate is a function of pH/pD in the solution, and the exchange rate is minimum at ~pH 2.5. During the chromatographic separation, the pH of mobile phase is typically maintained at around 2.5 to minimize the back exchange.

[1]

$$k_{ex} = (k_{op} \times k_{int}) / (k_{clo} + k_{int}) \quad [2]$$

$$k_{ex} = k_A 10^{-pD} + k_B 10^{pD-pK_D} + k_W \quad [3]$$

Englander's group determined the amide hydrogen exchange for oxidized equine cytochrome *c* using NMR in order to explore the context of HDX.[20] Various structural parameters, including hydrogen bonding, rigidity and solvent accessibility, were examined for a large number of amide hydrogens. It was found that the amide hydrogens highly protected by hydrogen bonding were allowed to isotopic exchange when the H-bonds were broken. However, the protection factors and the HDX rates were poorly correlated with the H-bond strength calculated from the X-ray and NMR structures (see Fig. 2A & B). For the hydrogens involved in hydrogen bonds in rigid structures, i.e., helices, the protection of HDX showed good correlation with the burial depth, indicating the distance of amide hydrogen to the protein surface (Fig. 2C & D). In a later study, Englander group examined the relationship between protein dynamics and HDX again with Staphylococcal nuclease.[21,22] This study further stressed the critical role of hydrogen bonding and solvent exposure. Dynamic motions at various scales, including global unfolding, cooperative unfolding of secondary structure and local fluctuations, could be dominant factors of the exchange

process. Most importantly, the dynamics revealed by HDX can span a wide time scale from millisecond to second, which could cover protein motions like folding/unfolding,[18,23,24] biomolecular interactions,[25] catalysis[2] and conformational transition states.[26]

MD simulation for elucidation of HDX behaviour

MD simulation can provide information at the atomic level for protein dynamics[27] and serve as a powerful tool to evaluate HDX behaviour.[28,29] Garcia and Hummer investigated the conformational dynamics of cytochrome *c* in an effort to correlate HDX kinetics and MD simulation in aqueous solution.[7] The system was examined at four temperatures ranging from 300 K to 550 K for at least 1.5 ns during which global motions and local hydrogen bonds were analysed. The hydration and hydrogen bonding of backbone were evaluated, and multi-basin dynamics of cytochrome *c* was characterized to reflect a hierarchical energy landscape where multiple energy-minima basins were sampled within a few hundred picoseconds. It was interesting to see that large fluctuations were not adequate to break the hydrogen bonds in some rigid regions to allow HDX. More recently, Craig et al. simulated the HDX for ubiquitin, chymotrypsin inhibitor 2 and staphylococcal nuclease, based on funnelled structure models.[30] The simulation using coarse-grained model examined large-scale unfolding transitions or unfolded sub-states which could dominate the HDX process. The open and closed conformations were characterized and protection factors at the residual level were predicted and compared with experimental values obtained by NMR. Their results emphasized the dominant role of the local environment in disturbing the stability of hydrogen bonds. Alternatively, the topology of protein could be determinant for the exchange of amide hydrogens. The coarse-grained conformational sampling method was also found to improve the correlation of predictive and experimental HDX data.[31]

Further understanding of HDX could be achieved by increasing the computing power for MD simulation.[32] A 100-ns-long simulation of ERK2 in four different states was conducted by Petruk et al. to obtain insight into the HDX experiments.[9] The averaged solvent accessible surface area (SASA) and the number of water molecules in the first NH solvation shell correlated well with the number of amide hydrogens exchanged per peptide, suggesting that the measured exchanges due to solvent exposure or fluctuations involved the breaking of hydrogen bonds. Persson and Halle analyzed a 0.262-ms MD trajectory of the protein BPTI to define the open state (O state) where more than two water molecules coordinated with exchangeable hydrogens for all the tested amide hydrogens (Fig. 3A-C).[29] Interestingly, a mean 100-ps residence of O state was identified, indicating that the HDX rates were governed by the reclosed frequency (Fig. 3D). The protection factors computed from this model correlated well with the experiments. Despite the demonstrated simulating power of molecular dynamics, a comparison with the HDX experiment for this protein G variant showed the demand for improvement of force fields to describe hydrogen bonding and backbone hydration.[8]

A recent study by McAllister and Konermann discussed the challenges in interpreting HDX data due to the complexity and ambiguous understanding of the HDX process.[33] From a 1-μs all-atom simulation, they explored the correlation between experiments and simulation where 57 out of 72 amide exchanges could be explained based on hydrogen bonding and solvent accessibility. Discrepancies were observed for some exposed amide hydrogens. These amide hydrogens are not hydrogen bonded and are solvent-accessible, but were found to be protected from exchange, as labelled by blue asterisks in Figure 4e. Electrostatic factors could account for this unexpected

surface amide protection, but it is challenging to include this into consideration for simulations. The authors also criticized the idea that hydrogen bonding to crystallographic waters might protect surface amide hydrogens from exchange because these solvent molecules immobilize not only in the crystal, but also in bulk solution. Nevertheless, it should not be neglected that water molecules play an essential role in maintaining the high-ordered structures of proteins as hydrogen-bonding is a fundamental intramolecular interaction.[34] The importance of water molecules in protein-ligand binding is increasingly recognized in recent years because displacement of water molecules would influence the binding affinity between proteins and ligands.[35] HDX, which makes use of water to examine the dynamics of protein molecules, could be an important technique in studying the effect of water molecules on protein structure/dynamics and functions.

Essentially, all-atom MD simulation does not exactly compute hydrogen exchange at ambient conditions, so it is not surprising that discrepancies can be found between these two techniques. Basically, HDX monitors the interplay between proteins and water molecules while MD simulation captures the intrinsic motions of atoms in proteins. There could be correlations between them, but such correlations would be in a non-linear manner, leading to the difficulties encountered in interpreting these data. Perhaps further modifications of the force fields and criteria for defining hydrogen bonding and solvent accessibility are required for more reliable and comprehensive simulation of HDX behaviour.

Perspectives

Recent advancements in the technique of HDX-MS could significantly enhance the capability and applicability of this technique in the study of protein dynamics in the future. Recent development in gas-phase HDX using ND_3 revealed new applications of HDX-MS, which enabled sub-millisecond monitoring of hydrogen deuterium exchange.[36] The binding interface of protein complexes could be well defined using HDX in the gas phase inside the mass spectrometer. It should be noted that this HDX technique explores the protein conformations in the gas phase and its mechanism of exchange might be different from that of in-solution HDX. More recently, fast HDX in liquid droplets determined by nanoESI-MS using a theta-capillary also realized sub-millisecond HDX.[37] The peptides and D_2O from two channels of the capillary reacted during traveling between the ESI emitter and MS inlet. Thus, the HDX could vary from ten to several hundred microseconds according to the configuration of the emitter and inlet. However, the measured HDX rate in droplets was found accelerated compared with that in bulk solution. These labelling methods can improve the temporal resolution of HDX, leading to broader applications in dynamics study.

The approaches to monitor the HDX process have also significantly improved during the past decades. To achieve high sequence coverage in HDX-MS study, several enzymes including type XIII protease[38] and Nepenthesin[39] were demonstrated to deliver high digestion efficiency under exchange-quenched conditions (pH 2.5, 0 ^{o}C). These proteases provided alternatives to the conventionally used pepsin and allowed acquisition of more complete sequence coverage by combining the results obtained by different proteases. Following the protease digestion, liquid chromatography separation was usually performed at 0 ^{o}C to minimize back exchange. Recently, a technique of sub-zero ultrahigh performance liquid chromatography was developed to retain

separation power even under low temperature.[40] In addition, a nano-ESI HDX-MS configuration was also developed for structural interrogation of large protein systems with significantly less sample consumption.[41] The improvement of resolution has been regarded as the central issue of HDX and can be achieved by peptide fragmentation in the gas phase. Recent development of gas-phase dissociation methods with low-degree deuterium scrambling, e.g., electron transfer dissociation (ETD),[42] could further improve the capability of HDX-MS in studying protein dynamics down to the single residue level. Novel fragmentation techniques, such as UV Photodissociation (UVPD), were developed to also reduce the deuterium scrambling.[43] A recent study demonstrated the determination of sub-localization of deuterium even for ETD data with partial scrambling.[44] Computation methods were also developed to solve the problem of scrambling for HDX-MS dataset with a high degree of scrambling.[45] The development of ion mobility mass spectrometry further advanced HDX-MS by facilitating separation of peptides in complex mixtures.[46] In recent years, HDX-MS has been developed as a tool for quality control of biopharmaceutical molecules, such as mono-antibodies and antibody-drug conjugates, which are becoming an essential part of the drug industry.[47] Furthermore, HDX-MS has been extensively used in drug screening in the field of drug development,[48] probably due to the development of robust automated HDX-MS platforms.

The future of protein dynamics research is promising with synergetic orthogonal approaches, including NMR, cryo-EM, HDX-MS and MD simulation. Currently, MD simulation generally can reach up to milliseconds trajectory of protein molecule, while HDX can be performed on the range of up to hours or days. It is extremely challenging to simulate the exchange in comparable time scale. However, with improving understanding of the factors governing HDX and advancing

computing technologies, it is possible to achieve closer correlations between the dynamics information obtained by MD simulation and HDX, and the combination of HDX-MS with MD simulation can be a powerful approach to interpret some complicated results and facilitate protein research.[28]

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Figure captions

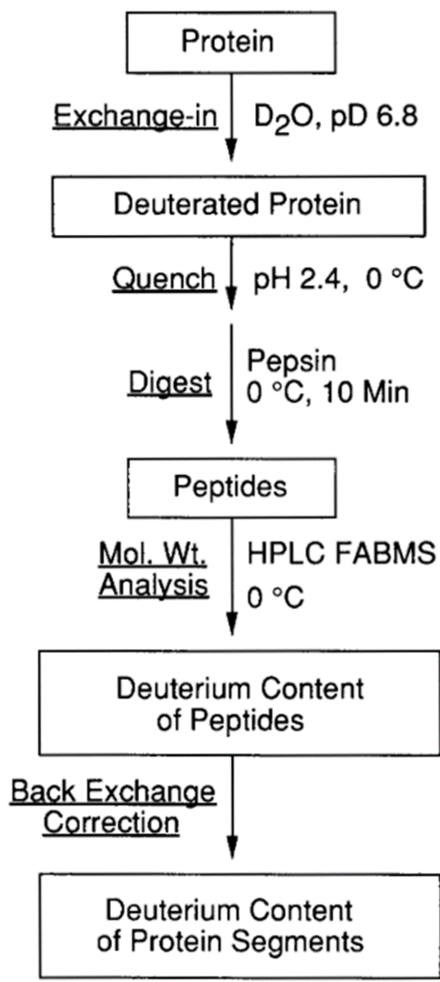
Fig. 1. Left panel: Flowchart of local HDX-MS. Right panel: Mass spectra of the molecular ion region 95-104 (*m/z* 1151) of cytochrome c incubated in H_2O (A), in D_2O (B) for 1h at 25 ℃, in D_2O for 3h at 75 ℃ (C). Reproduced with permission from Ref.14.

Fig. 2. Correlation plot for HDX protection and H-bond length computed from NMR (A) and X-ray (B) structures. Correlation plot for HDX protection and distance of NH to molecular surface for all measured NH (C) and 60s helices NH (D). Reproduced with permission from Ref. 20.

Fig. 3. Structure of the O state for the amides in BPTI. (A) Primary water–oxygen coordination number, N_w, of amide hydrogen for amides in O (open symbols) and C (solid symbols) states. (B) Probability that an amide hydrogen with $N_w \geq 2$ has a polar protein atom from a non-neighbor residue within 2.6 Å. (C) O/C r.m.s.d. for atoms within 7 Å of amide N (circles) and crystallographic root-mean-square fluctuation for same set of atoms (triangles). In A–C, the background shading indicates helix (beige) and β-sheet (light blue) structure. (D) Mean residence time for amides in O (open circles) and C (solid circles) state. Reproduced with permission from Ref. 29.

Fig. 4. Overview of experimental HDX data, and various properties extracted from a 1 μs simulation. (a) Experimental protection factors compiled by Craig et al. are highlighted as solid circles. Also shown are individual data sets as open triangles, open circles, and open squares. (b) Average distance between the backbone NH of each residue and the closest possible main chain

carbonyl acceptor. Values below 2.5 Å (red line) reflect the presence of a H-bond. (c) Same as in panel (b), but including side chains. (d) Average NH SASA values. The red line represents the SASA threshold of 0.23 $Å^2$. (e) Average bulk interaction rate, describing how fast bulk water diffuses to an NH site. Red asterisks represent exposed amides that are unprotected. Blue asterisks represent "problem cases", where exposed amides are strongly protected. Error bars shown in all panels represent standard deviations. Reprinted with permission from Ref. 33.



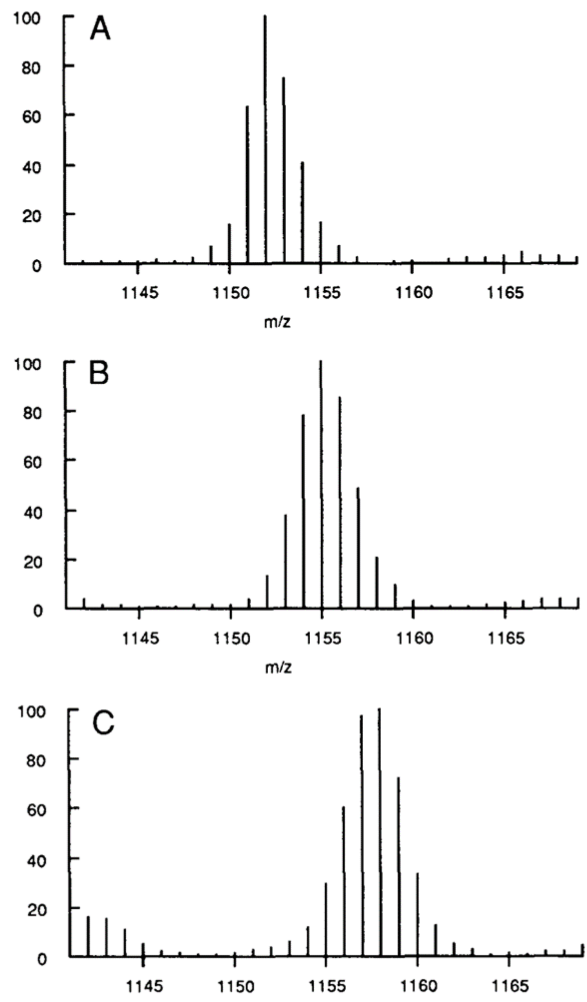


Figure 1

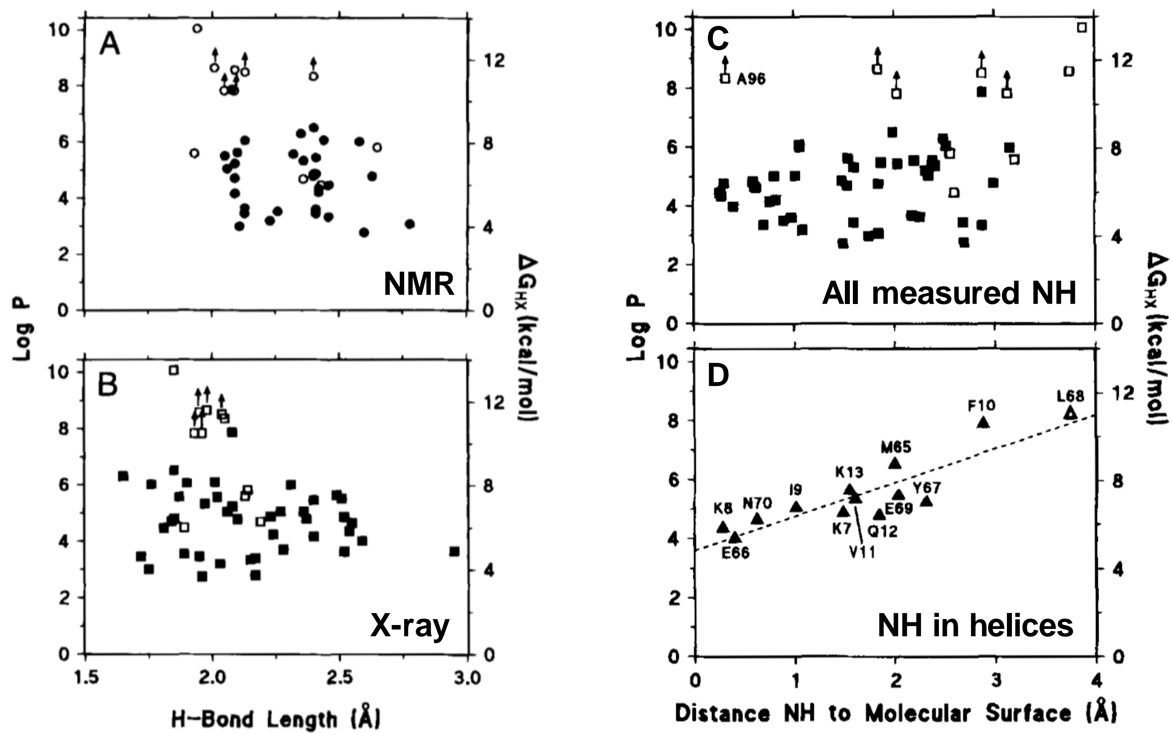


Figure 2

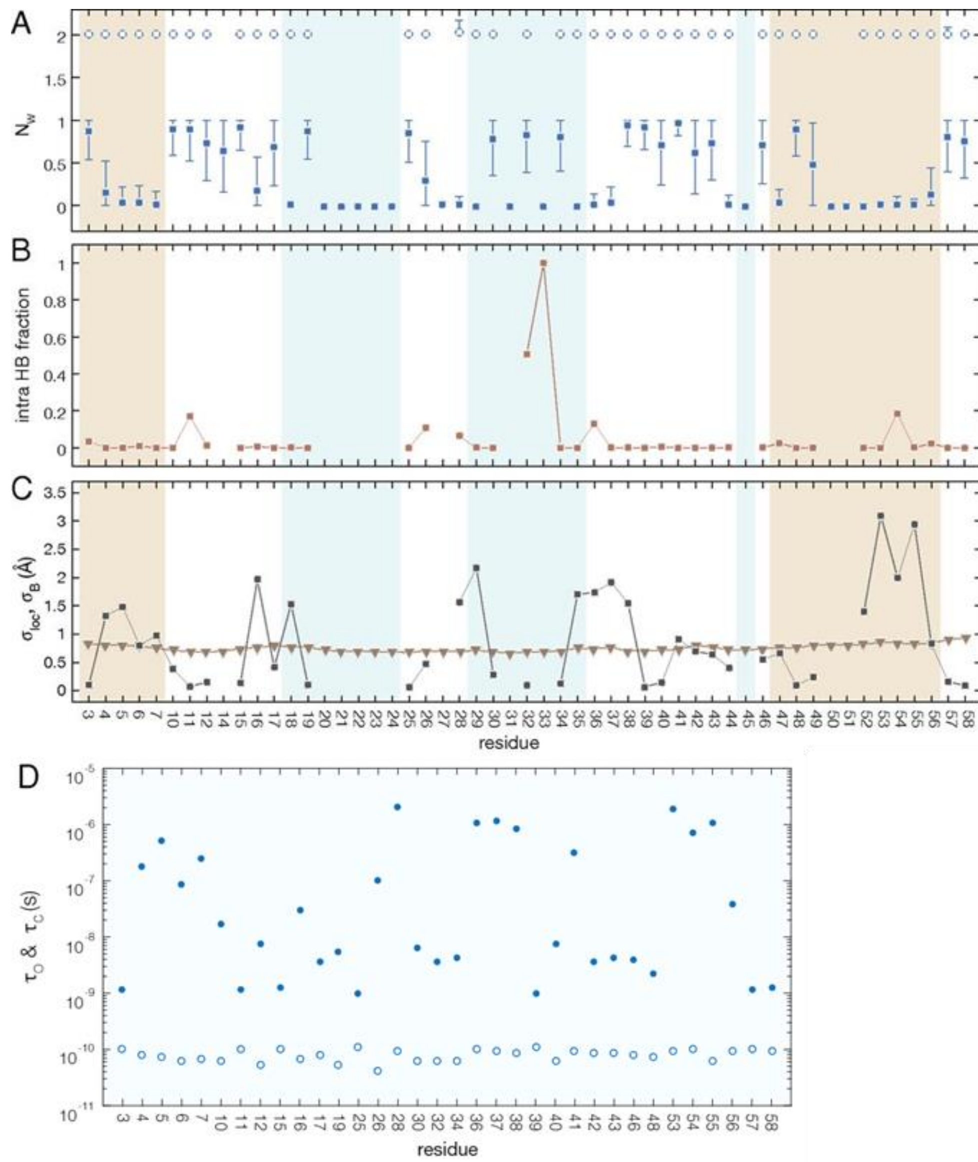
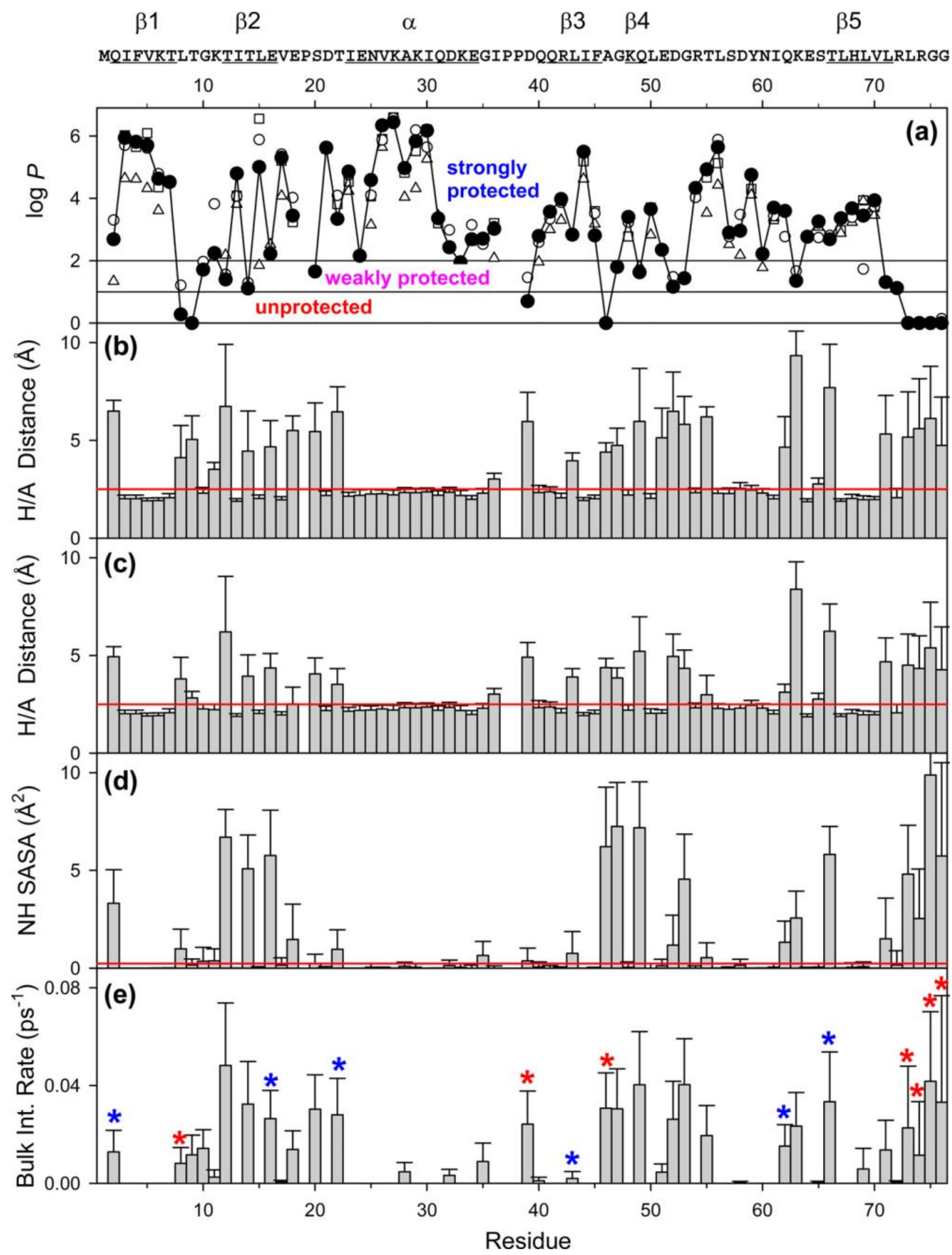


Figure 3



Fugure 4