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Time window expansion for HDX analysis of an intrinsically disordered protein

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Abstract

Application of typical HDX methods to examine intrinsically disordered proteins (IDP), proteins that are natively unstructured and highly dynamic at physiological pH, is limited due to the rapid exchange of unprotected amide hydrogens with solvent. The exchange rates of these fast exchanging amides are usually faster than the shortest time scale (10s) employed in typical automated HDX-MS experiments. Considering the functional importance of IDPs and their association with many diseases, it is valuable to develop methods that allow the study of solution dynamics of these proteins as well as the ability to probe the interaction of IDPs with their wide range of binding partners. Here, we report the application of time window expansion to the millisecond range by altering the on-exchange pH of the HDX experiment to study a well characterized IDP; the activation domain of the nuclear receptor coactivator, peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1 α). This method enabled mapping the regions of PGC-1 α that are stabilized upon binding the ligand binding domain (LBD) of the nuclear receptor peroxisome proliferator-activated receptor gamma (PPAR γ). We further demonstrate the method's applicability to other binding partners of the IDP PGC-1 α and pave the way for characterizing many other biologically important ID proteins.

Keywords

Hydrogen deuterium exchange; automation; electrospray ionization; HPLC; mass spectrometry; nuclear receptors; intrinsically disorder; proteins; conformational mobility; protein dynamics

Introduction

Most proteins are globular in nature and are well-ordered at physiological pH. However, at physiological pH, a significant number of proteins have a high degree of conformational flexibility and are devoid of any detectable secondary structure. These proteins are called intrinsically disordered proteins or IDPs. Additionally, ordered proteins have been found to contain disordered regions or domains and these are referred to as intrinsically disordered regions or IDRs [1–5]. IDPs typically differ from ordered proteins in amino acid composition, isoelectric point, and hydrophobicity. IDPs are significantly devoid of bulky aromatic and hydrophobic core promoting amino acids (Leu, Ile, Val, Trp, Tyr, Phe), enriched with polar amino acids (Arg, Gln, Ser, Glu), and contain a higher frequency of structure disrupting amino acids (Gly and Pro) [1, 5, 6]. These unique features of IDPs have

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enabled the development of algorithms that can predict if a protein is likely to be intrinsically disordered or contains large regions that might be IDRs [7–10].

Despite the lack of defined structure, IDPs have been shown to play important and perhaps unique roles in biology. Well-structured proteins are mostly involved in enzymatic reactions, channels and transport mechanisms as well as cellular structure whereas IDPs are typically associated with molecular recognition, protein modification and molecular assembly [11]. The eukaryotic proteome has been shown to be enriched in IDPs, with a third of the proteins containing intrinsically disordered regions of 30 or more amino acids. The disordered nature and prevalence of IDPs strongly suggests a crucial role for them in molecular recognition [7, 12]. For an IDP to carry out function, the protein needs to transition towards a more stable structure. This transition could be transient, lasting only long enough to trigger a downstream effect or the more stable structure could be long lived. The mechanism for this transition is a process called coupled binding and folding where upon interaction with its target binding partner, the IDPs undergo a transition from the disordered (unstructured) to ordered (structured) state [3, 13, 14]. IDP interactions with their partner protein are typically characterized by low affinity but high specificity, which is critical for transient protein-protein interaction during molecular signaling. Uversky et al. [14] reported that a single IDP can specifically bind to a range of different protein partners, and these distinct interactions can lead to different protein folds in a binding partner-dependent manner.

An increasing number of IDPs have been shown to be associated with disease [5, 15, 16]. Given their role in molecular recognition and link to disease, detailed analysis of IDP-binding partner interactions is critical to understanding their mechanism of action. While NMR and crystallography have been applied to the study of the structural dynamics and folding properties of disordered proteins [17, 18], we sought to determine if solution phase hydrogen/deuterium exchange (HDX) coupled with mass spectrometry (HDX-MS) could be informative in such studies. HDX-MS has emerged as a sensitive and rapid technique to localize alterations in conformational dynamics in protein-protein interactions [19–21]. In a typical HDX experiment the target protein is diluted into excess deuterated (D_2O) buffer and on-exchange of solvent deuterons is allowed to proceed over a specified time window. The rate of H/D exchange of backbone amide hydrogens is determined by mass spectrometry following quenching of the exchange reaction and digestion of the target protein using acid stable protease. The exchange rate greatly depends on the temperature and pH of the reaction [22–24]. Low pH, typically 2.5, is required post HDX reaction to preserve on-exchanged deuterons in peptic peptides of the target protein. Regions of a protein protected from amide H/D exchange have high conformational stability and order whereas regions that lack protection from exchange possess a high degree of conformational flexibility and disorder. In a differential HDX experiment, on-exchange of the protein of interest is carried out in the presence and absence of a binding partner (where the binding partner can be a protein, DNA, small molecule, etc.). Regions of the protein that reveal perturbations in HDX kinetics upon binding often map to the region of binding partner interaction or can be attributed to allosteric effects.

Although there are several reports of the application of HDX towards the study of unstructured regions within folded proteins [25, 26], there are few reports of HDX analysis of IDPs [27–29]. This is not surprising as IDPs contain few protected amide hydrogens due to the lack of a stable structure. As a result, peptic peptides from such proteins are fully saturated with deuterium at the earliest on-exchange time point (typically ~10s) that can readily be measured on an automated HDX-MS platform. As a result, the window for detecting perturbation in HDX kinetics in an IDP is very small. One alternative of measuring HDX rates on millisecond timescale is a quenched-flow apparatus [30, 31]. The quenched-flow approach lacks versatility in that one can only measure short on-exchange time points,

therefore a separate apparatus must be used for the longer time points (>1s). Recently Coales et al. [24] showed that by altering the pH of the on-exchange reaction buffer, one can effectively expand the time window to measure accurately the HDX kinetics of very fast or very slow exchanging amide hydrogens.

PGC-1 is an important coregulator of several nuclear receptors and has been implicated in a wide range of disorders [32]. In this report, we examine the activation domain of PGC-1 (AA 2-220, called as PGC-1 220 hereafter), a well-characterized IDP. The HDX characteristics of PGC-1 220 were examined in the presence and absence of the nuclear receptor PPAR. At physiological pH, there was no detectable difference in the HDX kinetics in any of the peptic peptides that cover nearly the entire sequence of PGC-1. By lowering the pH of the on-exchange reaction to effectively expand the HDX time window into the millisecond range enabled the detection of differential HDX in regions of PGC-1 220 that transiently fold upon binding to PPARG. Comparison of deuterium on-exchange plots at low pH and physiological pH for a range of peptic peptides validates that the approach does not alter the protein structure in solution. Finally, we demonstrate the utility of this approach with other PGC-1 target proteins that are of high interest as drug targets for new therapies targeting a range of disorders and diseases [33, 34].

Experimental

Materials and reagents

Rosiglitazone, tris (2-carboxyethyl) phosphine (TCEP), Urea, 99.9% D₂O solution, Formic acid and Trifluoroacetic acid were purchased from Sigma-Aldrich.

Protein production

PGC-1 220 was prepared as reported earlier [35]. Production of the ligand binding domain (LBD) of PPAR [36], ERR [35] and ROR [37] are reported elsewhere.

Buffer and pH

For physiological pH buffer (pH 7.5), Tris-HCl 50mM, NaCl 150 mM and TCEP 10mM was used. For low pH buffer (pH 6.0), phosphate-citrate 50 mM, NaCl 150 mM and TCEP 10 mM was used. pH of all buffers were measured with a Oakton pH meter.

Hydrogen deuterium exchange and data analysis

Solution-phase amide HDX was carried out with a fully automated system as described previously [38] with slight modifications. The automation system (CTC HTS PAL, LEAP Technologies, Carrboro, NC) was housed inside a chromatography cabinet held at 4°C. PGC-1 2-220 and rosiglitazone bound PPAR was mixed at 1:1 molar ratio and incubated for 1.5h at 4°C for complex formation before subjecting to HDX. For HDX reaction, 5 µl of 10 µM apo PGC-1 2-220 or the complex (1:1 molar mixture of PGC-1 2-220 and PPAR) was diluted to 25 µl with D₂O-containing HDX buffer (either pH 7.5 or pH 6) and incubated at 4 °C for 10s, 30s, 60s, 900s or 3,600s. Following on exchange, unwanted forward or back exchange was minimized and the protein was denatured by dilution to 50 µl with 0.1% (v/v) TFA in 3 M urea and 50 mM TCEP. Samples were then passed across an immobilized pepsin column (prepared in house; [39]) at 50 µl min⁻¹ (0.1% v/v TFA, 15 °C); the resulting peptides were trapped on a C₈ trap cartridge (Hypersil Gold, Thermo Fisher). Peptides were then gradient-eluted (4% (w/v) CH₃CN to 40% (w/v) CH₃CN, 0.3% (w/v) formic acid over 5 min, at 4 °C) across a 1 mm × 50 mm C₁₈ HPLC column (Hypersil Gold, Thermo Fisher) and subjected to electrospray ionization directly coupled to a high resolution (60,000) Orbitrap mass spectrometer (LTQ Orbitrap XL with ETD, Thermo Fisher). MS/MS data were acquired in separate experiments with a 60 minute gradient. Data-dependent MSMS

was performed in the absence of exposure to deuterium and the amino acid sequence of each peptide used in the HDX peptide set were confirmed if they had a MASCOT score of 20 or greater and had no ambiguous hits using a decoy (reverse) database. For on-exchange experiments, the intensity weighted average m/z value (centroid) of each peptide's isotopic envelope was calculated using software developed in-house [40]. For back exchange correction, full deuterium control was run as reported previously [41] and an average of 70% recovery (ranging from 60% to 80%) was estimated. To measure the difference in exchange rates between experiments, we calculated the average percentage deuterium uptake for native PGC-1 220 following 10, 30, 60, 900 and 3,600 s of on exchange. From this value, we subtracted the average percent deuterium uptake measured for PGC-1 220 bound with either LBDs of PPAR , ROR , or ERR .

Temperature and pH dependence of amide hydrogen exchange rate

The calculation is performed with equation 1 (below) as shown in Coales et al. [24]

$$K_{\text{ch}} \sim k_{\text{OH}} [\text{OH}^-] = A \exp(-E_a/RT) [\text{OH}^-] \quad (1)$$

A is the frequency factor and E_a is the activation energy of the base-catalyzed amide hydrogen exchange reaction. Using this equation, intrinsic exchange rate (K_{ch}) at pH 6.0 is converted to pH 7.5. The temperature is constant at 4°C.

Results and discussion

Apo dynamics of intrinsically disordered PGC-1 α 2-220 at physiological pH

PGC-1 is an important transcription coactivator which has key roles in metabolic processes and has been implicated in the development of type II diabetes. It interacts with many transcription factors, including nuclear receptors [32], and was first identified as a ligand-independent transcriptional coactivator of PPAR [42]. The interaction between PGC-1 and nuclear receptors is mediated at least in part by the characteristic nuclear receptor boxes or LXXLL motifs (marked as NR box 1, 2 and 3 in Fig 1A) that are located within the activation domain of PGC-1 and within the LBDs of NRs. Most structural studies of these NR box interaction studies have been limited to small peptide fragments containing either one or two NR box motifs [43, 44]. Though the crystal structure of PPAR LBD in complex with PGC-1 (AA 101-220) was solved by the Xu group [44], only NR box 2 was resolved. A previous report describes the characterization of the interaction between ERR LBD and the entire activation domain of PGC-1 220 using a range of biophysical techniques including HDX [35]. In this report, protection from H/D exchange was observed on ERR LBD upon binding PGC-1 220, clearly identifying the binding site. However, in the same experiments it was not possible to detect protection from H/D exchange in PGC-1 220. This experiment demonstrated the interaction between these two proteins, and mapped the PGC-1 binding site on ERR LBD, but due to the highly dynamic nature of IDPs the converse was not possible [35]. This observation highlights the difficulty in analyzing IDPs.

Figure 1A shows the deuterium uptake level in different segments of PGC-1 220 as a function of time. The values within each peptide represent the average deuterium level across six time points for three replicates (values in parentheses are the standard deviation where n=3). It is evident from the selected build up curves that most of the backbone amides are highly exchanged (>80%) within the earliest time point (10s) typically used on an automated HDX platform (Fig. 1B). This is consistent with the intrinsically disordered nature of PGC-1 220 as reported earlier [35]. All the peptic peptide fragments generated that include and flank the three NR boxes of PGC-1 220 are highly dynamic and become 80% exchanged within 10s and 100% exchanged within 1 hr of incubation time (Fig. 1B).

HDX analysis of PGC-1 α in the presence and absence of PPAR γ at pH 7.5 and pH 6.0

In our initial experiments, we performed differential HDX analysis of PGC-1 220 in the presence and absence of rosiglitazone bound PPAR LBD at physiological pH (7.5). Li et al. [44] has reported the strong interaction between NR box 2 of PGC-1 (referred to as ID1 in [40]) and PPAR and the non-essential role of NR box 3. However, differential HDX analysis carried out at physiological pH showed no significant changes in the deuterium incorporation level of PGC-1 220 in the presence of PPAR (Figure 2A). It is important to note that at the shortest time point measured (10s), most exchangeable amides were saturated with deuterium. Thus, it is possible that the transient helical structure formed within the IDR rich PGC-1 220 upon interaction with PPAR interconverts to unstructured conformers at the time scale faster than 10s. If this assumption is valid, an HDX experiment carried out on the millisecond time scale might be able to detect changes in the deuterium incorporation between free and PPAR bound PGC-1 220. Coales et al. [24] reported that by lowering the reaction pH one can effectively expand the on-exchange time window to the millisecond level without changing the experimental HDX on-exchange time. To this end, we carried out differential HDX experiment of PGC-1 220 in the presence of PPAR at pH 6.0 (Fig. 2B). HDX experiments performed at pH 6.0 result in a 100-fold reduction in the on-exchange rate of amide hydrogens as compared to that at pH 8. This reduction in rate and the observation that the dynamic behavior of many proteins remain similar between pH 6–8 [24] encouraged us to examine the HDX characteristics of PGC-1 220 -NR interactions at pH 6.0.

As shown in Figure 2B, the HDX perturbation map of PGC-1 220 at pH 6.0 shows that NR box 2 is highly protected from exchange which could arise from the formation of secondary structure upon interaction with PPAR. In agreement with an earlier report NR box 2 showed strongest interaction with PPAR whereas NR box 3 was not involved in the protein-protein interaction [44]. The NR box 2 segment forms structure upon PPAR LBD interaction via both intermolecular and intramolecular interactions [44]. Interestingly, the dynamics of the peptide region near NR box 1 (AA 86-90) showed some protection from exchange upon interaction with PPAR LBD, although the magnitude of protection is lower compared to the region containing NR box 2. This region (AA 86-90) was not present in the PGC-1 construct used for the PGC-1 /PPAR LBD co-crystal structure. Figure 3 (also, supplementary figure 1) shows the deuterium build up curves of selected regions of PGC-1 220. It is clear that the greatest protection from solvent exchange is observed for the peptides containing NR box 2 (AA 142-154) while peptides that contain NR box 3 were not protected from exchange. Interestingly, peptides that are near or contain NR box 1 (AA 90-94) showed weak but statistically significant solvent exchange protection, most evident at longer on-exchange time points.

Time window expansion changes deuterium exchange kinetics but not dynamics of PGC-1 α 220

The amide hydrogens in proteins are proposed to exchange as follow [45],

Closed $\rightarrow$ Open $\rightarrow$ exchanged

While k_{op} is defined as the rate at which amide hydrogen converts from closed state to open state, k_{cl} is defined as the rate at which amide hydrogen converts from open state to closed state. K_{op} is defined as the open equilibrium constant ($K_{op}=k_{op}/k_{cl}$), given HDX occurs under EX2 mechanism where $k_{cl} \gg k_{op}$ (in most cases). Constant K_{op} means constant protein dynamics and it is essential to check that the protein dynamics do not vary within the experimental pH range employed for the HDX study (pH 6.0 – pH 7.5). If the K_{op} is unchanged at two different pH values, the deuterium build up curves, after converting the

exchange time to standard condition (pH 7.5), should overlay on top of each other. Coales et al. [24] showed that the deuterium build up curves for cytochrome c do in fact overlay for a number different pH values tested. However, the analysis of another protein (human growth hormone) showed that the build up curves overlaid at certain segments, but would deviate at other regions of the protein.

Converting the time window of our HDX experiment according to equation 1, we find that the 10 sec exchange time at pH 6.0 is equivalent to 0.316 sec at pH 7.5 (temperature constant at 4° C). The corresponding exchange times of other time points are tabulated in Table 1. Figure 4 (and supplementary figure 2) shows the deuterium build up curves for selected regions of PGC-1 220 where all the time points are converted to standard condition (pH 7.5 at 4°C). Overlapping of the curves (brown colored pH 7.5 and blue colored pH 6.0) indicated that the protein dynamics did not alter between these two pH values. It is important to monitor the overlapping build up curves near the NR box regions (AA 90-94, AA 142-155, AA 142-153, AA 210-217 and AA 209-217) because these regions selectively make contacts (and hence form structure) during PGC-1 220-NR LBD interactions. Therefore, we can conclude that the protection from deuterium exchange observed in the differential HDX experiment (Fig. 2B) was due to the transient structure formation in PGC-1 220 NR box regions that would otherwise only be detectable at millisecond time scale (pH 7.4).

To obviate the possibility that the affinity between PGC-1 220 and PPAR increased markedly at pH 6 and in turn increased protection from solvent exchange, we performed a melting temperature (T_m) measurement of the binary complex at pH 6.0 and pH 7.5. As evident in supplementary figure 3, there is no statistically significant difference in the calculated T_m 's. Thus, we conclude that the affinity of interaction between PGC-1 220 and PPAR is unchanged as a result of lowering the pH.

The reduction of the rate of exchange, and hence the effective expansion of time window is clearly evident when we compare the deuterium uptake level of apo PGC-1 220 at different time points for pH 6.0 and pH 7.5 (Table 2). The low level of deuterium in the 10s time point at pH 6.0 corresponds to only 0.316 s at pH 7.5. At this millisecond time point, the deuterium level was well below the saturation level and allowed us to detect if there is any protection from exchange upon structure formation.

Expansion of time window applicable to other PGC-1 α 220-NR LBD interactions

To investigate the applicability of this HDX time-expansion method to other PGC-1 -NR interactions, we performed differential HDX of PGC-1 220 in the presence of the LBDs of ERR and ROR at pH 6.0. In the presence of ROR LBD, all three NR boxes showed protection from deuterium exchange while in the presence of ERR LBD, only NR box 2 and NR box 3 showed protection from exchange (Fig 5A and B). This observation is in agreement with a previous report [35].

Conclusion

Although HDX MS is used to localize highly dynamic unstructured regions within a folded protein, the application of HDX MS to study the dynamics and interaction of IDPs is limited to a few reports [28, 29]. The major obstacle is the rapid H/D exchange rates of IDP amides which often get fully saturated (>80%) within the shortest time frame frequently used in most of the laboratories (10s). Here we demonstrate the applicability of time window expansion by lowering pH to measure the (effective) millisecond exchange rates of IDP amides. This method is easy to perform and incorporate in the previously established HDX workflow in reproducible manner. We were able to map the regions involved in interaction

with PPAR LBD, within the intrinsically disordered protein, PGC-1 220. This method can be readily applied for the analysis of other PGC-1 220-NR LBD interactions and opens up the possibility to study other IDPs or IDRs of otherwise folded proteins, for example, the N-terminal activation domain (AF-1) of full-length nuclear receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Apo dynamics of PGC-1 220 at physiological pH (7.5)

(A) NR boxes are indicated in the sequence with purple boxes. The bars below the sequence represent the peptide fragments resolved by mass spectrometry and the bar color represents the relative deuterium/hydrogen exchange (color code on top). Number within each bar is the average % of deuterium incorporated over six time points (10,30,60,300,900 and 3600 sec). (B) Deuterium build-up curves of selected peptides. NR box amino acid sequences are mentioned in the inset.

Figure 2. Differential HDX data of PGC-1 220 upon binding of PPAR LBD at pH 7.5 (A) and at pH 6.0 (B)

The bars below the sequence represent the peptide fragments resolved by mass spectrometry and the color of the bars the relative change in HDX protection between unbound and PPAR -Rosiglitazone bound PGC-1 220. Three NR boxes are marked in the sequence. Color key is drawn on top of the figure. ns-no significant changes.

Figure 3. Deuterium build up curves of the NR box regions of PGC-1 220

The differential HDX experiment was carried out with PGC-1 220 in the presence and absence of PPAR LBD-Rosiglitazone at pH 6.0.

Figure 4. Deuterium uptake in NR box regions of PGC-1 220 at pH 6.0 and pH 7.5

All time points are converted to pH 7.5 using equation 1 (also see table 1). All build up curves show overlap within the experimental errors indicating the unchanged dynamics of apo PGC-1 220 at pH 6.0 and 7.5.

Figure 5. Differential HDX data of PGC-1 220 upon binding of ROR LBD (A) and ERR LBD (B) at pH 6.0

The bars below the sequence represent the peptide fragments resolved by mass spectrometry and the color of the bars the relative change in HDX protection between unbound and ROR or ERR bound PGC-1 220. Three NR boxes are marked in the sequence. Color key is drawn on top of the figure. ns-no significant changes.

HDX reaction condition and exchange time corrected to standard HDX condition of pH 7.5 and 4°C according to equation (1).

Table 1

Exchange time corrected to standard condition of pH 7.5 and 4°C (Second)	HDX reaction condition	
	pH 6.0 (4°C)	pH 7.5 (4°C)
0.316 s	10 s	
0.949 s	30 s	
1.89 s	60 s	
9.49 s	300 s	
10 s		10 s
28.48 s	900 s	
30 s		30 s
60 s		60 s
113.92 s	3600 s	
300 s		300 s
900 s		900 s
3600 s		3600 s

Table 2
Deuterium uptake comparison view of selected PGC-1 220 peptides at pH 6.0 and pH 7.5

The color is based on color code on top of the table. 10 s time point at pH 6 corresponds to 0.316 s at pH 7.5, 60 s at pH 6.0 corresponds to 1.89 s at pH 7.5, 300 s at pH 6 corresponds to 9.49 s at pH 7.5 and 3600 s at pH 6.0 corresponds to 113.9 s at pH 7.5. SD is standard deviation of three replicates.

Start	End	Peptide	Charge	10s time point		60s time point		300s time point	
				pH 6	SD	pH 6	SD	pH 6	SD
-2	12	GSHMAWDMCSQDSVW	2	27	5	62	3	64	2
19	36	ALVGEDQPLCPDLPELDL	2	11	2	42	3	71	5
39	49	LDVNDLDTDSF	2	13	5	59	5	87	4
49	61	FLGGLKWCSDQSE	2	31	6	82	4	92	4
90	94	LTETL	1	7	6	68	5	90	5
95	107	DSLPEDEGLPSF	2	9	1	36	4	62	6
142	153	LKKLLAPANTQ	2	31	7	83	6	100	10
142	154	LKKLLAPANTQL	2	33	5	83	6	105	7
142	155	LKKLLAPANTQLS	2	33	6	80	5	97	5
209	217	LLKYLTTND	2	25	7	69	6	89	10
210	217	LKYLTTND	1	29	5	77	5	92	5