PROBING SITE-SPECIFIC INTERACTIONS BETWEEN EPIDERMAL GROWTH FACTOR RECEPTOR AND AN ADNECTIN USING HDX-ETD MS APPROACH

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INTRODUCTION

Epidermal growth factor receptor (EGFR) is a key cellular signaling protein. Blocking EGFR signaling is a validated strategy for cancer therapy. Adnectins are targeted therapeutic proteins derived from the tenth type III domain of human fibronectin, and their protein constructs can be optimized for high affinity binding to targets. The anti-EGFR adnectin was identified that specifically bound to EGFR and inhibited its intracellular signaling.

In this work, we applied hydrogen/deuterium exchange mass spectrometry (HDX-MS) coupled with electron transfer dissociation (ETD) to probe the binding contacts at the amino acid residue levels [1-2] in the EGFR/adnectin complex. Utilizing ETD on a targeted peptide of interest allowed us to obtain site-specific HDX information for the binding epitope in EGFR/adnectin. This study demonstrates the advantage of combining HDX-MS with ETD to achieve high spatial-resolution and site-specific structural analysis of binding interfaces.

METHODS

Samples: Human EGFR and Adnectin were prepared at 6.7 mg/mL and 2.3 mg/mL, respectively. EGFR and Adnectin were mixed in 1:2 ratio and incubated for 30 min at room temperature. Bound and unbound (EGFR only) were labeled in 20-fold of PBS in deuterium oxide, pH 7.2 and incubated for 1 hour (peptide level exp.) and 30 min (residue level exp.). Labeled samples were quenched with an equal volume of pre-chilled 200 mM phosphate buffer with 0.5 M TCEP, 4 M GnHCl to at pH 2.4. Undeuterated control and dueterated runs were run in triplicate (Figure 1). P1 peptide was purchased from AnaSpec Inc.

System: Quenched samples (denatured and reduced) were subjected to online pepsin digestion at 25 °C for 3.5 min. Waters nanoACQUITY UPLC system with HDX technology was used for rapid peptide separation at 0 °C. 6 min linear gradient (92-60% of 0.1% formic acid in water) was used. Waters Synapt G2-S HDMS system was used for collisional induced dissociation (CID) and ETD modes.

ETD parameters:
Source capillary =3.0 kV, cone=20, offset=10
Triwave trap waveheight=0.3 V, RF setting trap ETD=450 volts
Gas control Trap = 22.0 mL/min, Transfer=0.7 mL/min
MS method= MSMS (targeted ETD)
ETD reagent: 1,3-Dicyanobenzene

Software: Undeuterated control data were first processed in Waters ProteinLynx Global Server (**PLGS**). Both undeuterated and deuterated data were then processed in Waters **DynamX 3.0** for

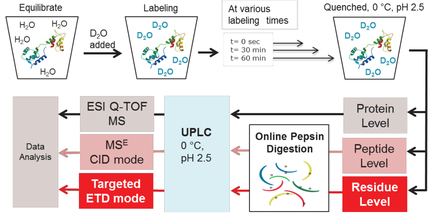


Figure 1. HDX workflow *with three HDX experimental pathways. Protein level with no digestion (gray), peptide level with online pepsin digestion on CID mode (pink), and individual residue level on ETD mode (red) are illustrated. In this study, the peptide HDX analysis was performed first to identify the peptide of interest followed by the targeted ETD analysis.*

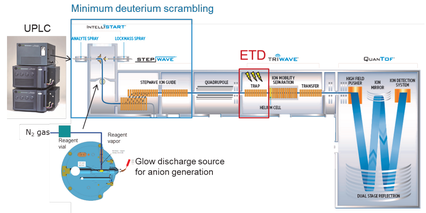


Figure 2. Synapt G2-S with ETD functionality*. The triwave trap region is the ETD reaction cell as highlighted in red box. The blue box illustrates the front-end that includes a glow discharge source and stepwave. A minimum deuterium scrambling was confirmed in the region of front-end during ETD (See Figure 5 for detail). The nanoUPLC system with HDX technology was used for an LC inlet.*

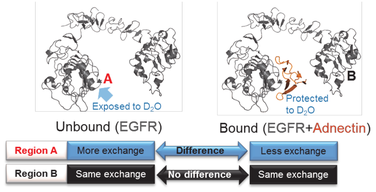


Figure 3. HDX data interpretation for binding interaction study. *When Adnectin (orange) binds to EGFR (gray), the interacting region A showed different deuterium uptakes between the bound and unbound forms. The similar deuterium uptake between the bound and unbound forms for the region B suggests that this location has little influence on binding interaction.*

RESULTS AND DISCUSSION

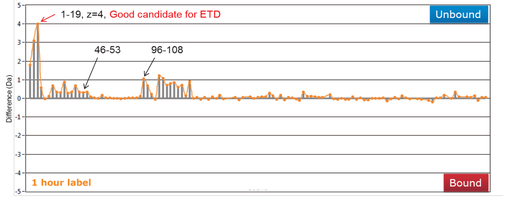
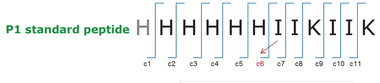


Figure 4. Peptide level of HDX analysis. *The deuterium difference of each peptide in EGFR was compared between the unbound (top) and bound (bottom) forms. Three different regions (1-19, 46-53, and 96-108) showed higher uptakes in the unbound form with multiple overlapping peptides. The quadruply charged peptide 1-19 (LEEKKVCQGTSNKLTQLGT) was selected for ETD/HDX MS analysis.*



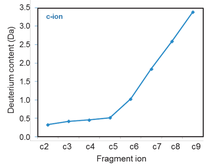


Figure 5. Confirmation of a minimum scrambling phenomenon with ETD. *P1 peptide sequence and its c-ion fragments are shown in the top panel. The red arrow highlights the amide hydrogen responsible for deuteration at Isoleucine7 is located at c_6 ion after ETD fragmentation. As an increased deuterium incorporation was observed, the location of deuteration from c_6 to c_9 was confirmed. However Histidine residues from c_2 to c_5 showed a minimum deuteration, which confirmed that there was no scrambling phenomenon with ETD. [ref 1]*

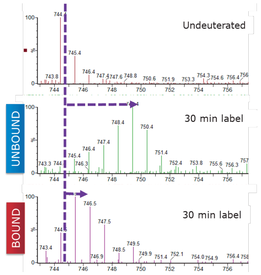


Figure 6. Uptake comparison of ETD fragment ion. *z_6 ion of EGFR peptide (residue 1-19) is shown as an example of undeuterated (top), 30 min deuterated unbound (middle), and 30 min deuterated bound (bottom). Higher uptake was found in the unbound form.*

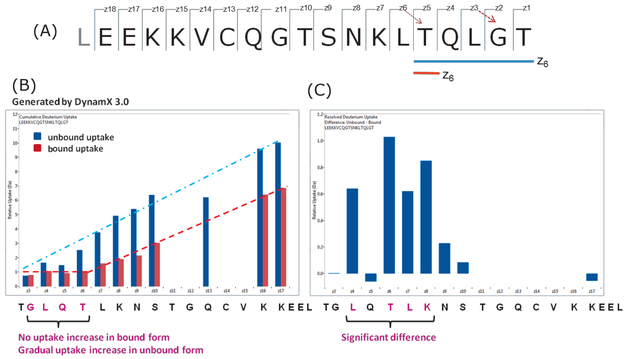


Figure 7. Deuterium content and difference at residue level. *The peptide sequence of 1-19 of EGFR (A). The blue line underneath of the sequence indicates the deuterium content from z1 to z6 ions and the deuterium content of unbound and bound was generated using DynamX 3.0 and plotted in B. The orange line indicates the deuterium difference between unbound and bound forms at the specified residue. This information was plotted in C. In plot B, the constant deuterium content was seen from z_3 to z_6 in bound form (corresponding T15, Q16, L17, and G18, in red) whereas the gradual uptake increases except for Q16 was observed in the same region (in blue dotted line). This suggests that these residues of EGFR are involved in binding with adnectin. In plot C, the deuterium difference between unbound and bound was calculated per individual residue. Residues at L17 and T15 in pink showed significant difference. Interestingly, the neighboring residues, L14 and K13, also showed significant difference in uptake. This observation suggests a partial deuterium protection at L14 and K13 caused by binding at the neighboring residues.*

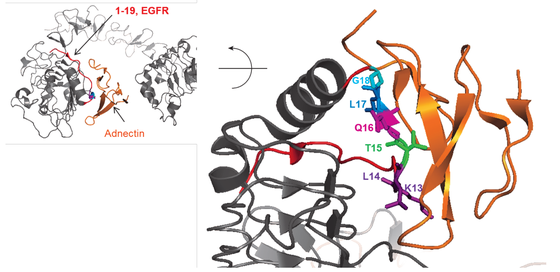


Figure 8. Locating difference in deuterium at residue level on a 3D structure. *The crystal structure of EGFR and adnectin (1NQL.pdb) confirmed that T15, Q16, L17, and G18 were directly involved in binding with Adnectin [ref 3]. Contact information from x-ray crystallography genuinely agrees with ETD/HDX data. Also ETD/HDX MS analysis found an additional piece information: partial deuterium protection at L14 and K13 (in purple) could be involved in interaction with adnectin since both residues are spatially close to adnectin.*

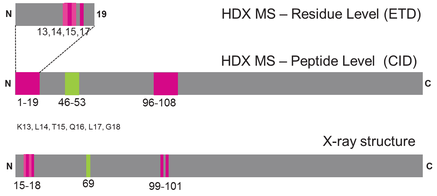


Figure 9. HDX MS and x-ray structure. Both HDX MS and X-ray structure agreed on the binding interface of EGFR and adnectin. Contact residue at 69 was not found in HDX MS at the peptide level, however, another peptide, 46-53, from the neighboring region showed a small protection in deuterium incorporation upon binding. ETD/HDX MS analysis confirmed the binding residues (at 15 and 17) from the crystal structure. ETD/HDX MS data also suggests additional residues at 13 and 14 involved in binding interface, which was not observed in the crystal structure.

CONCLUSIONS

- **HDX MS analysis is an enabling tool to probe the protein-protein interaction and their binding contacts.**
- **HDX MS analysis with CID rapidly determined the interaction location of EGFR/Adnectin at the peptide level.**
- **ETD/HDX MS analysis confirmed the binding sites of EGFR at the amino acid residue level.**
- **This study demonstrated the utility of combining HDX-MS with ETD to achieve high spatial-resolution at single amino acid level and site-specific structural analysis of binding interfaces.**
- **DynamX software 3.0 supports ETD-HDX data.**

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