TECHNICAL NOTE 65887

Hydrogen deuterium exchange mass spectrometry for protein structural characterization

Authors: Terry Zhang and Rosa Viner
Thermo Fisher Scientific, San Jose, CA, USA

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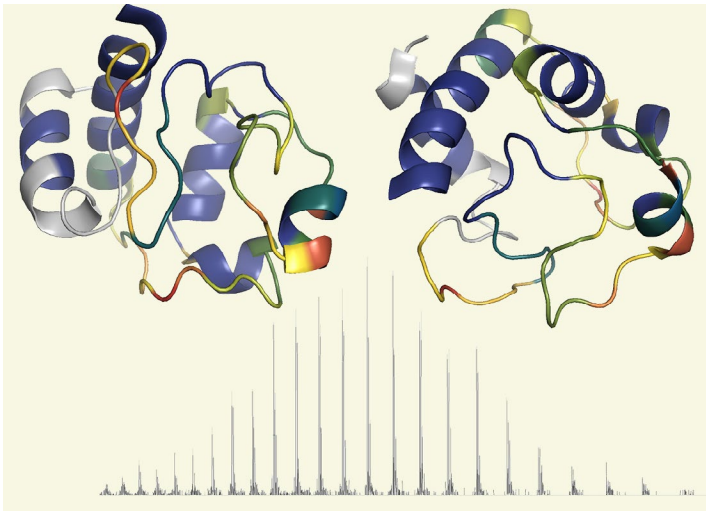

Goal

The aim of this work is to demonstrate a fully automated hydrogen deuterium exchange mass spectrometry workflow to study protein conformation.

Introduction

The characterization of protein structures and protein complexes is essential for understanding protein function and the mechanisms of action in a biological system. These proteins, the complexes, the networks they form, the various interactions that occur during complex formation, and the folding they undertake all govern biological activities. Traditional techniques for studying protein complex structures, folding and protein-protein interactions include X-ray crystallography, cryo-electron microscopy (cryo-EM), and nuclear magnetic resonance (NMR). However, these techniques require large amounts of highly purified proteins, may not allow for the analysis of proteins in their native conditions, and are limited to examining structures in their static states. These traditional techniques are also limited by the size of the biomolecule that is being examined, and the fact that many proteins are simply not amenable to these types of analysis limits their applicability.

Recently, hydrogen deuterium exchange mass spectrometry (HDX-MS) has emerged as a powerful tool to fill the analytical gap.[1,2] In this technical note, a fully automated HDX-MS workflow highlighted with the Thermo Scientific™ Orbitrap Exploris™ 480 mass spectrometer is described and applied to study the conformation of standard protein cytochrome c.

Experimental

Methods and materials

Materials	
Protein	Cytochrome c (Sigma, 9007-43-6), 40 µm in water
Columns	Thermo Scientific™ Acclaim™ PepMap™ 100 C18, 1.0 mm × 5 cm, 3 µm, 100A (P/N 164454)
Trapping column	Thermo Scientific™ Acclaim™ PepMap™ 300 C18 5 µm, (P/N 163593), Holder (P/N 164650)
Digestion column	Immobilized protease III/pepsin, NovaBioAssays (NSA2014002)
Mobile phase A	0.1% formic acid in water
Mobile phase B	0.1% formic acid in 80% acetonitrile, 20% water
Quenching buffer	2 M GuHCL, 100 mM citric acid, pH 2.2
Labeling buffer	20 mM hepes in deuterated water, pD 7.4
Mass spectrometer	Orbitrap Exploris 480 MS
Liquid chromatography	Thermo Scientific™ UltiMate™ NCS-3500RS

LC conditions

Separation conditions		
Retention [min]	**Flow [µL/min]**	**%B**
0.9	40.00	3.0
1.0	40.00	10.0
11.0	40.00	35.0
12.0	40.00	95.0
14.0	40.00	95.0
15.0	40.00	3.0
16.0	40.00	95.0
17.0	40.00	95.0
18.0	40.00	3.0

Loading conditions		
Retention [min]	**Flow [µL/min]**	**%B**
2.0	150	0
2.1	100	0
5.0	100	0
5.1	100	2
10.0	100	2
11.0	100	0
18.0	100	0
19.0	150	0

Mass spectrometry parameters

ESI source conditions	MS
Spray voltage (V)	3600
Sheath gas	20
Aux gas	2
Capillary temperature (°C)	220

MS conditions

Parameter	MS	MS/MS
MS full resolution	60K	60K
AGC	100%	100%
MS mass range	300–1300	200–2000
RF lens	40	
Isolation		2
Dynamic exclusion		Top 15
Collision energy (%)		27
Charge state	2–6	2–6
Intensity threshold		5e4
Max injection time (ms)	100	150

HDX workstation

An HDX platform comprised an H/D-X PAL™ (Trajan, LEAP-HD-Automation) and a Thermo Scientific™ UltiMate™ 3000 pump system coupled to an Orbitrap Exploris 480 mass spectrometer capable of being operated in a fully automated fashion was developed (Figure 1). The HDX workstation includes: 1) the H/D-X PAL autosampler composed of individually temperature-controlled sample plate and labeling plate, 2) independently temperature-controlled syringe head and pepsin column, and 3) independently temperature-controlled cooling chamber. Figure 2A shows the three valve configurations at different stages of the experiment inside the cooling chamber. The flexibility allows for maximum productivity. Figure 2B shows the dual heads on the single high-pressure mixing pump on the left and ternary loading pump on the right. Flow rates and solvents can be changed on the fly during the experiment. The execution of the whole experiment is completely automated and controlled by Chronos™ software. Chronos software is fully integrated with Thermo Scientific™ Xcalibur™ software. Figure 3 is the HDX experimental flow chart.

Figure 1. HDX-MS workstation

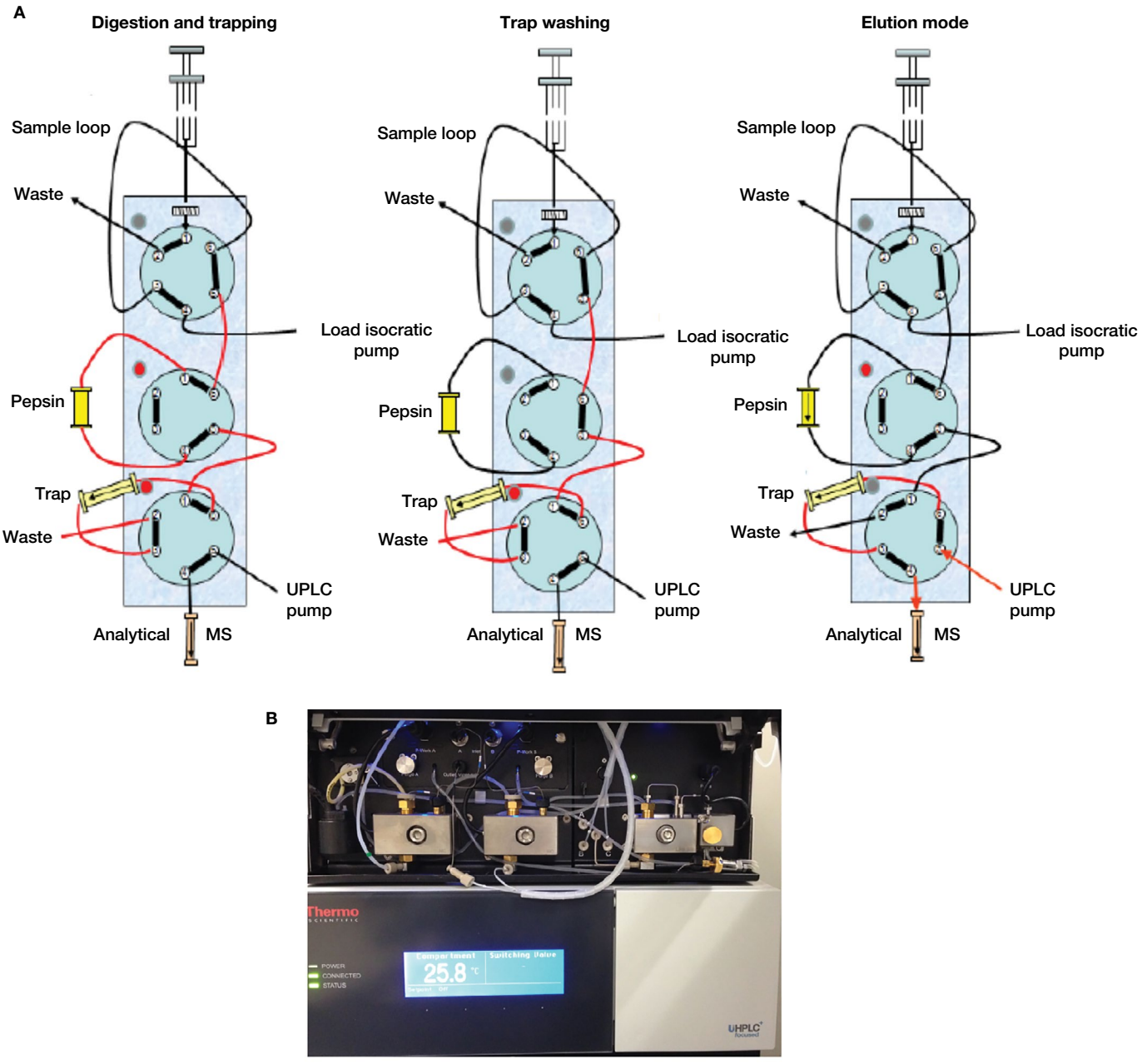

Figure 2. (A) HDX workstation sample delivery path: cooling chamber; (B) UltiMate NCS-3500RS LC system with binary rapid separation micro flow pump and quaternary loading pump

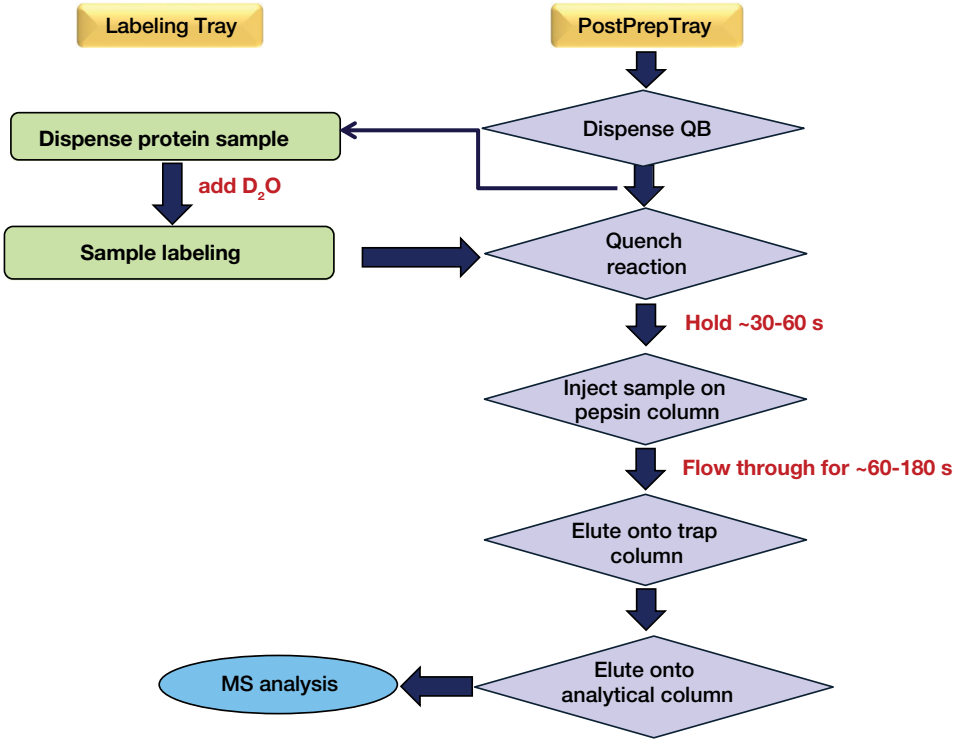

Figure 3. HDX-MS workflow experimental flow chart

Experimental design

Cytochrome c (40 uM) was diluted (1 to 9 ratio) with labeling buffer and incubated over multiple time points at 4 °C. The samples were then quenched and subjected to online proteolitic digestion (NovaBioassay Immobilized protease III/pepsin, 2.1 x 3 mm) at 2 °C. The digested peptides were injected onto a C18 trap column (0.5 mm × 15 mm) and eluted to a C18 reversed phase column (1 mm × 50 mm). The separated peptides were analyzed with the Orbitrap Exploris 480 mass spectrometer. Figure 4 shows the flowchart of the experimental steps. The HDX-MS experiment was performed with data-dependent MS^2 for peptide identification first, followed by MS full scan spectra in triplicate.

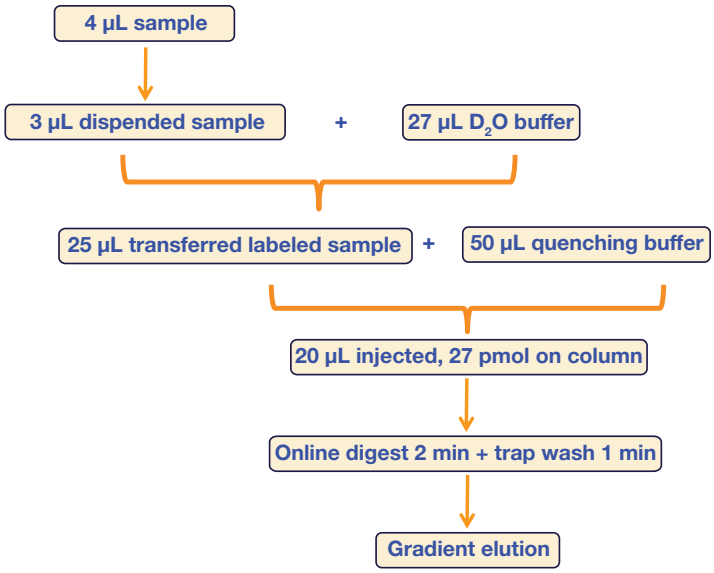

Figure 4. HDX-MS experimental steps

Data analysis

All data analysis was performed using Thermo Scientific™ BioPharma Finder™ 3.2 integrated software, including the protection factor plots based on the experimental deuterium incorporation. HDX experimental data were also analyzed with HDExaminer™ 2.5 software (Sierra Analytics).

Results and discussion

In these experiments, more than 100 peptides were identified enabling 99% protein sequence coverage, and of these, 93 of the most reproducible peptides were selected for deuterium incorporation calculation. The average mass difference between undeuterated peptides with deuterated peptides was calculated and used for protein conformation characterization. Figure 5 shows high reproducibility of the LC separation, which is a very important factor for accurate deuterium uptake calculation. Figure 6 shows an example of the peptide labeling dynamic over 2 hours. The isotopic distributions shifted from hydrogen to deuterium over the time course as expected. The deuterium uptake values were calculated by the HDExaminer software. Overall, the results were very reproducible, with small *t*-test error bars observed for the deuterium uptake measurements across multiple runs. The peptides deuterium uptake information from different protein states could be compared as shown in Figure 7. Figure 7A is the mirror plot of the average of the triplicate runs vs. single run peptides deuterium uptake measurement. The plots show that some peptides have more deuterium uptake and some peptides have less deuterium uptake depending on the protein conformation along with the sequence. The less deuterium uptake area has less solvent accessibility, meaning more structured, while the less structured area would have more deuterium uptake. To compare the differences of the analyzed protein samples, the residual plot could be built by subtracting the peptide deuterium uptake as shown in Figure 7B. The plot demonstrates that the peptide's deuterium uptake differences were very small, less than 2% on average among the two compared protein samples with only couple of outliers.

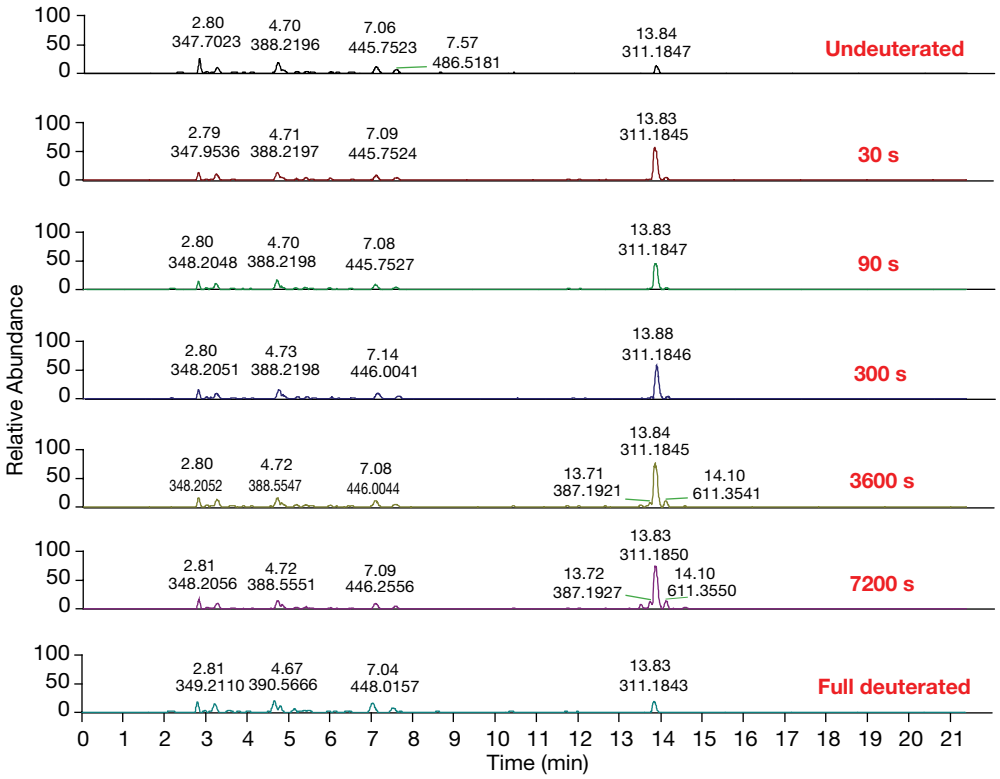

Figure 5. LC-MS base peak chromatograms of the HDX-MS experiment

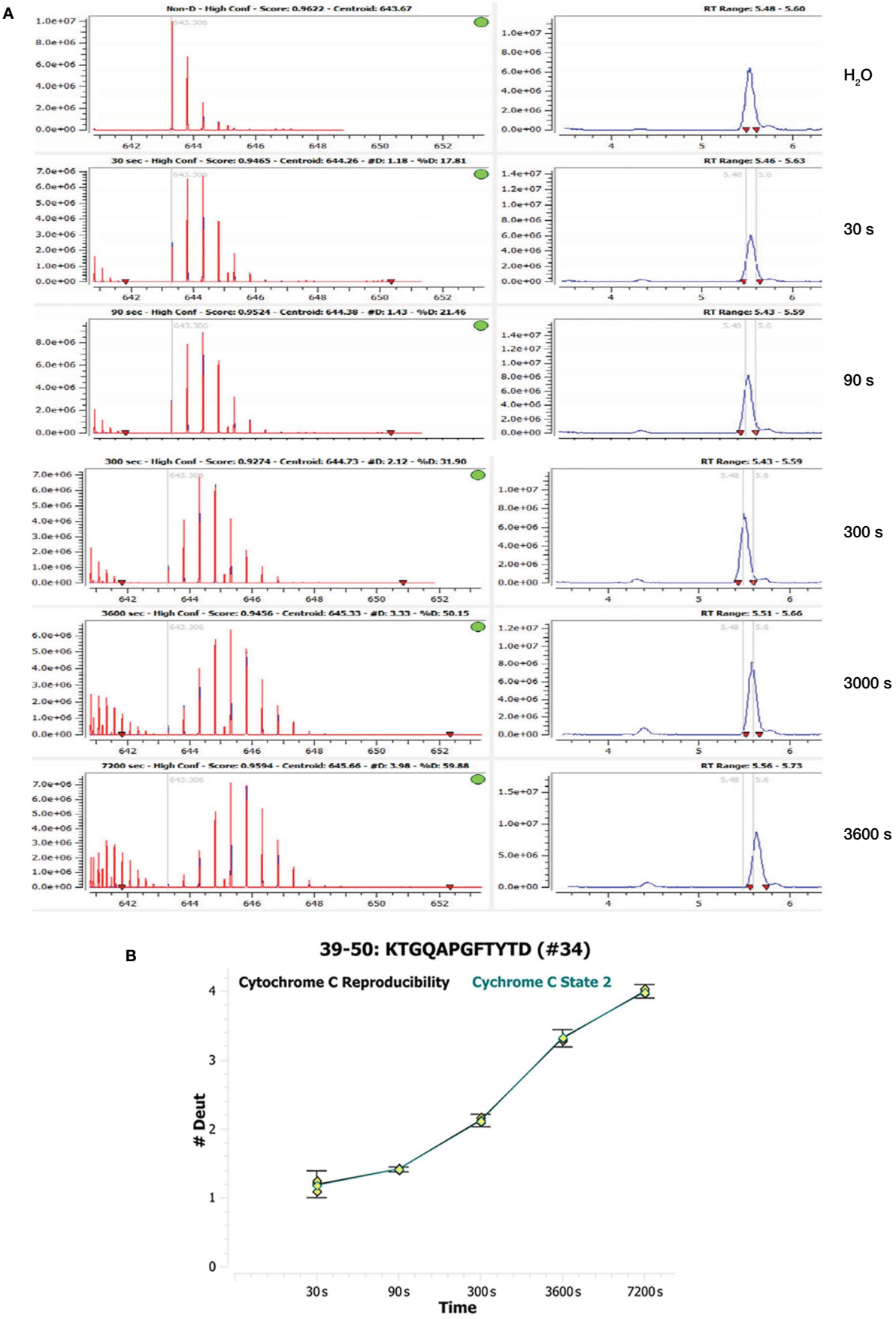

Figure 6. HDX-MS of 39–50 Cytochrome c peptide KTGQAPGFTYTD. (A) Left panel: labeling profile of over 2-hour time course isotopic cluster distribution. Right panel: the peptide base peak chromatogram over 2-hour time course. (B): Reproducibility of deuterium uptake plots

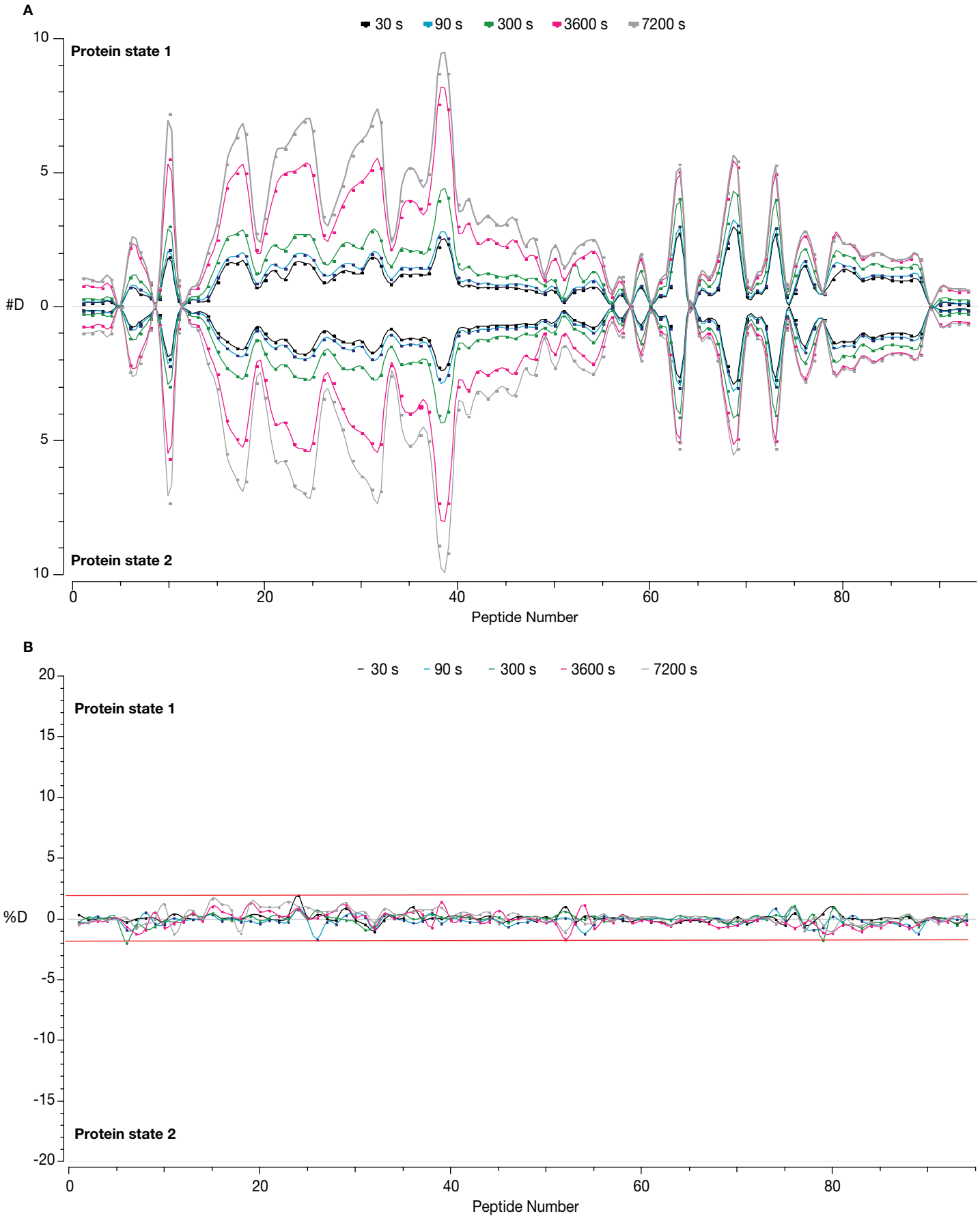

Figure 7. (A) Peptide deuterium uptake mirror plots of two protein states; (B) peptide's deuterium uptake residual plots of two protein states

Based on the peptide's deuterium uptake information, a protein heat map was constructed as shown in Figure 8. The missing sequence coverage on the protein deuterium incorporation heat map was due to heme binding to cytochrome c. The color from dark blue to red represents the deuterium incorporation from low to high, indicating the protein conformation from less structured to more structured. There are five regions with very low deuterium uptake and some regions with higher deuterium uptake. The deuterium uptake increased as expected over time for less structured regions. It is highly desirable to have as many sequentially overlapping peptides as possible identified for unlabeled protein to achieve high structural resolution in HDX experiments.[3,4] The high sensitivity of the Orbitrap Exploris 480 mass spectrometer is ideal for this purpose. For example, by using the overlapped peptides, 40–54, 40–55 and 49–61, 49–62, single residue level resolution could be obtained for 55 and 62 residues as shown in Figure 9. Several other regions with close to single residue level resolution were observed in the heat map. Utilizing the unique feature of the BioPharma Finder software, protection factor plots were calculated to review the protein solvent accessibility on the single residue level, as shown in Figure 10.

The deuterium uptake heat map can be directly mapped to the available crystal structure of cytochrome c (PDB 1hrc), as shown in Figure 11. The cytochrome c structure has five α-helices, and the five low deuterium uptake regions precisely reside in the α-helices. The short helix ring, tied to the unstructured regions including 49–55 residues, produced higher deuterium uptake rates compared to other helix regions. These results demonstrate the HDX-MS utility to study protein conformation dynamics. Accuracy and overall resolution of such fully automated experiments directly depends upon MS performance, sample preparation, and LC separation reproducibility.

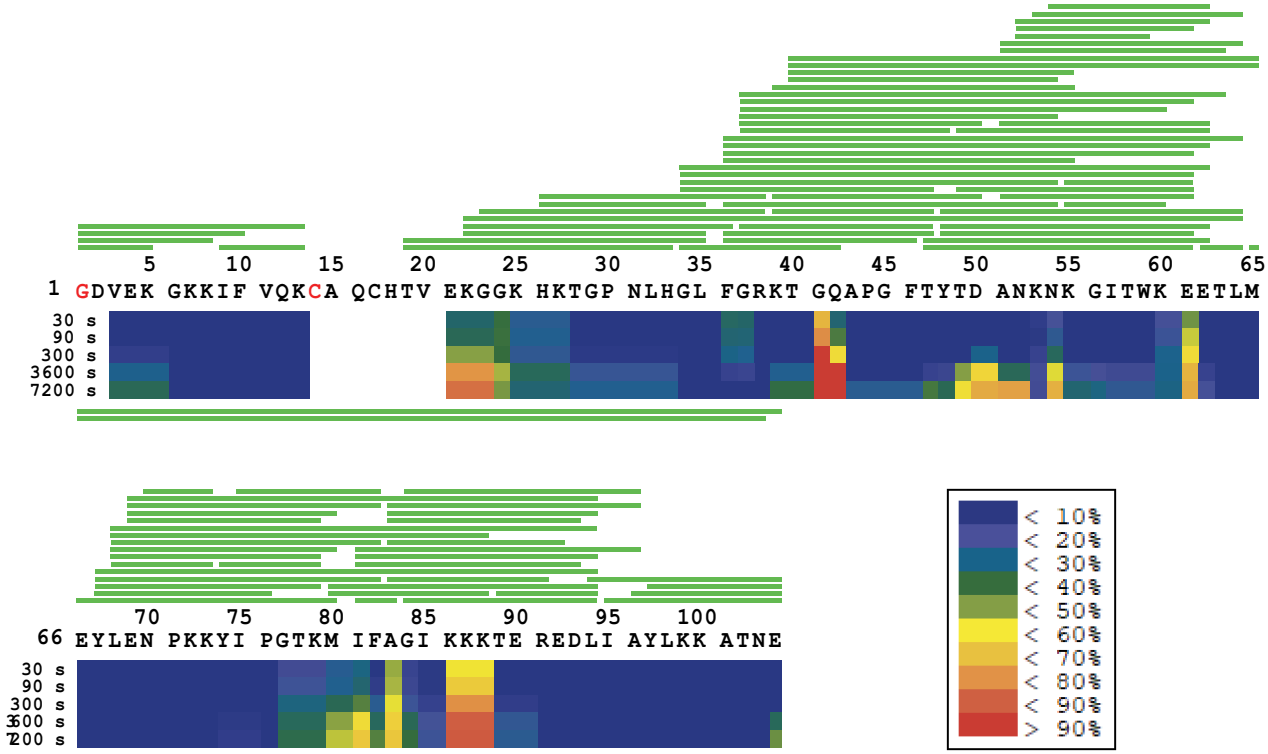

Figure 8. The protein deuterium incorporation heat map

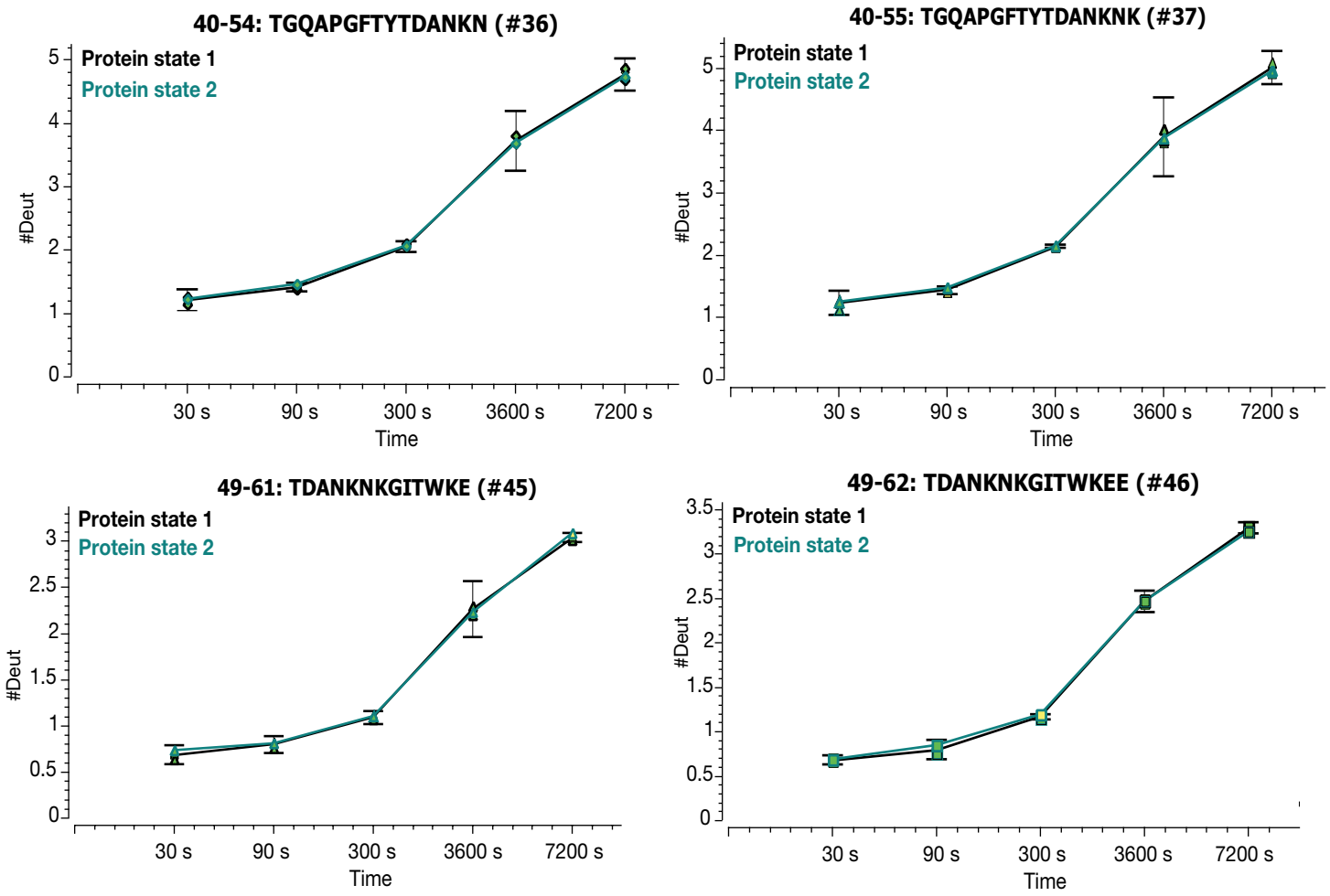

Figure 9. Single residue resolution for residues 55 and 62 could be obtained by subtracting overlapped peptide's deuterium uptake values

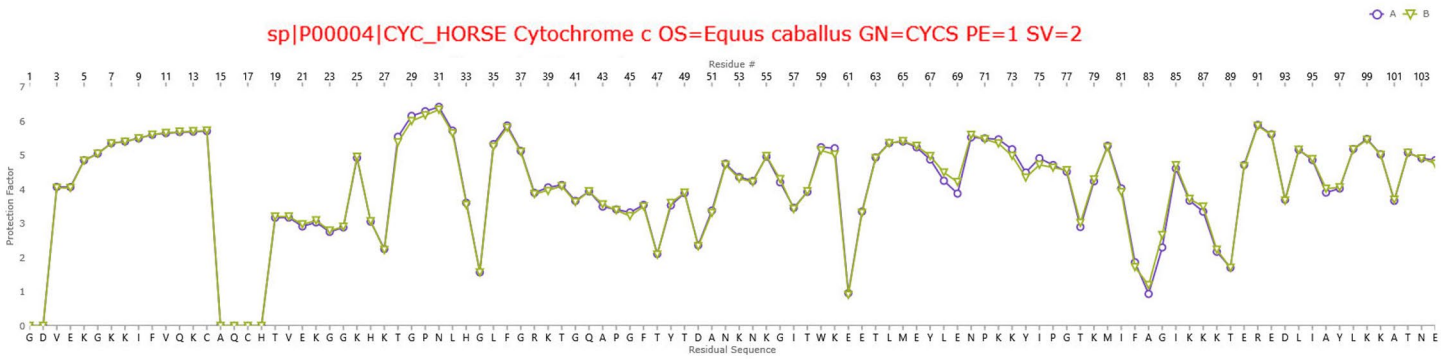

Figure 10. Protection factor plots

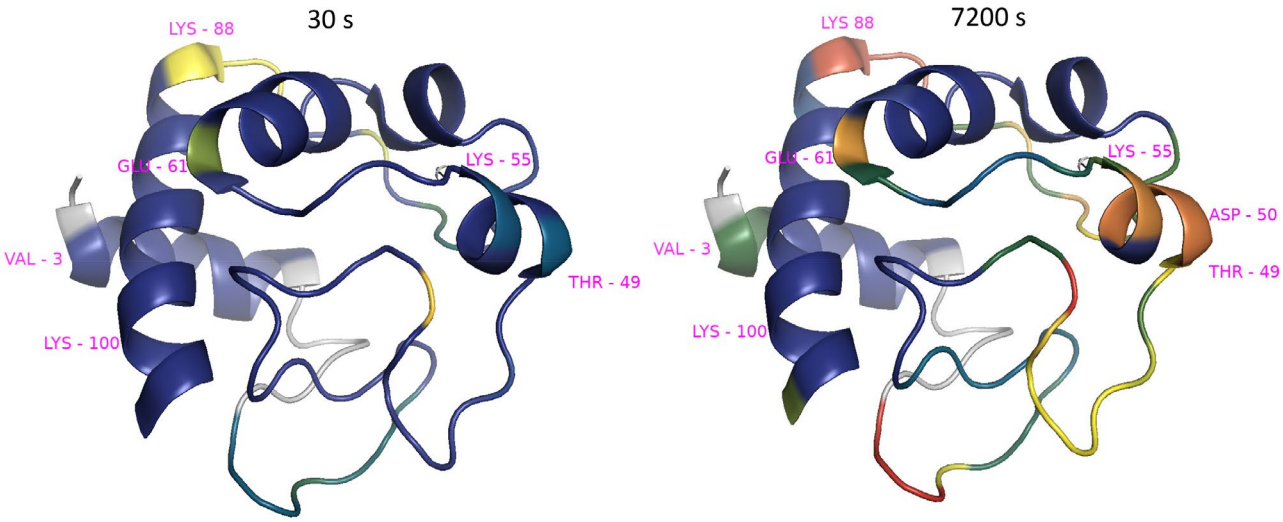

Figure 11. Deuterium uptake mapping (A) 30 s and (B) 7200 s within cytochrome c crystal structure (PDB:1hrc)

Conclusions

- The fully automatic HDX workstation consisting of the H/D-X PAL autosampler and the Orbitrap Exploris 480 mass spectrometer was described and used in this protein conformation study.
- Very robust and reproducible data were obtained.
- BioPharma Finder software supports all HDX-MS data analysis including peptide identification and HDX unique protection factor plots at the single reside level.

References

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