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응용 자료

Conformational Characterization of Calmodulin by Hydrogen Deuterium Exchange Mass Spectrometry

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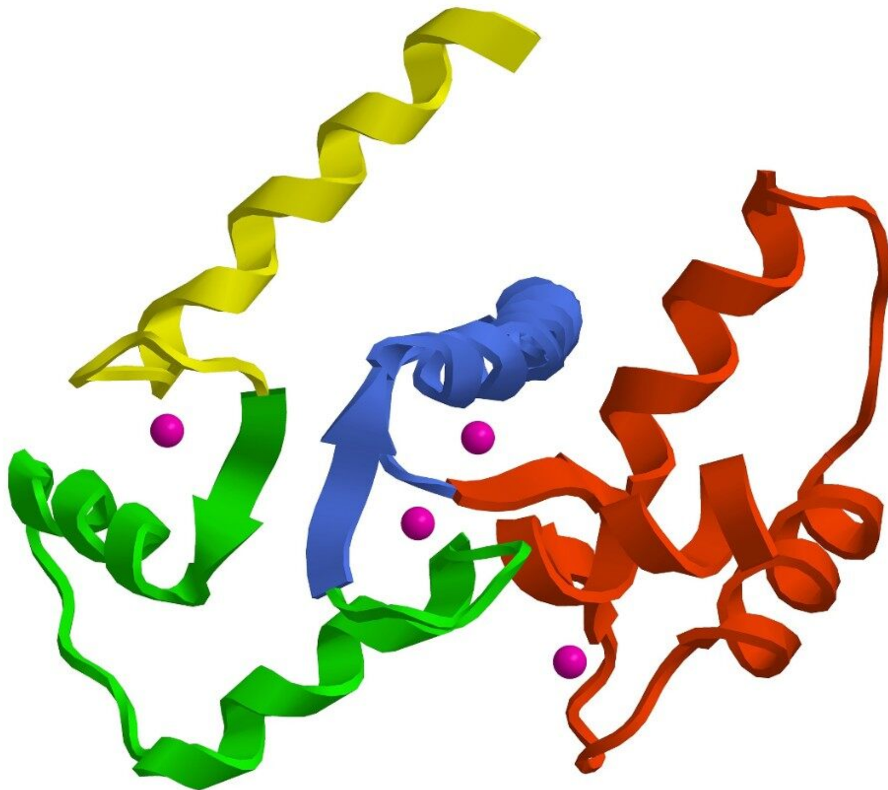

Abstract

This application note describes the HDX MS workflows using the nanoACQUITY UPLC System with HDX Technology to determine changes in conformation between apo and holo calmodulin, using hydrogen deuterium exchange combined with high resolution MS- HDX, or HXMS.

Benefits

To determine changes in conformation between apo- and holo-calmodulin using hydrogen deuterium exchange combined with high resolution MS.

Introduction

Correct protein conformation is essential for proper biological function of protein therapeutics. Changes in protein conformation are a major concern in the biopharmaceutical industry and conformational characterization is a difficult task. Many analytical tools such as circular dichroism (CD), differential scanning calorimetry (DSC), and analytical ultracentrifugation (AUC) are used to study the higher-order structure of proteins. These methods sample global conformation and cannot determine where conformational changes occur. Nuclear magnetic resonance (NMR) and X-ray crystallography determine protein structure with high spatial resolution, but both technologies require substantial amounts of sample and are not routinely applied to biopharmaceutical products due to the significant time and effort required.

Hydrogen deuterium exchange (HDX or HX MS) mass spectrometry has proven to be a useful analytical method for the study of protein dynamics and changes to protein conformation.[1] Successful HDX studies require an LC-MS system that can perform rapid chromatographic separations at 0 °C and make accurate mass measurements of small quantities of deuterium labeled proteins and peptides.[2]

When an HDX experiment is performed with UPLC separation technology and high-resolution mass spectrometry, subtle conformational changes can be revealed and the locations of these changes are determined at the peptide level. Such innovative technology with a commercially available system solution makes conformational analyses a more practical method. Waters HDX Technology has been adopted as an effective analytical tool for biotech researchers who are studying higher-order structure of protein drugs. Characterizing higher-order structure is one of the key factors in drug discovery and early development

phases and adopting this technology will offer a distinct advantage in understanding the safety and efficacy of biopharmaceuticals.

In this application note, we introduce the nanoACQUITY UPLC System with HDX Technology (Figure 1). This system consists of a nanoACQUITY UPLC Binary Solvent Manager (BSM), Auxiliary Solvent Manger (ASM), and HDX Manager. The HDX Manager is capable of rapid online protein digestion, desalting, and highly resolving chromatographic separations at 0 °C. Operating at 0 °C is required to reduce the deuterium loss during analyses. With this system, subtle differences in deuterium uptake can be adequately determined with very reproducible HDX conditions. A Xevo QTof MS, utilizing MS^E with ProteinLynx Global SERVER (PLGS) Software, provides an ideal tool for accurate mass analysis and reliable peptic peptide assignment.

Figure 1. nanoACQUITY UPLC System with HDX Technology. HDX Manager, Binary Solvent Manager (BSM), and Auxiliary Solvent Manager (ASM) are shown from top to bottom, respectively. The HDX Manager is a key component, which can perform the online pepsin digestion and maintain the low temperature at 0 °C required for HDX MS.

To demonstrate this technology, the conformational changes in bovine calmodulin (CaM) were studied. Calmodulin is an important intracellular calcium receptor and it is known that it undergoes a conformational change upon calcium binding.[3] This change regulates the biological activities of target proteins. Here we describe the HDX workflows using the nanoACQUITY UPLC System with HDX Technology at both whole protein and peptide levels to show how the conformational study is performed.

Experimental

Protein preparation:

Bovine calmodulin purchased from Sigma

Protein buffer solution:	20 mM HEPES in 100% H_2O pH 7.40 with and without 50 μM calcium[3]
D_2O labeling solution:	20 mM HEPES in 99.99% D_2O at pD 7.40 with and without calcium[3]
Quenching solution:	33 mM HCl 100 % H_2O pH 2.50

Method

LC conditions:

LC system:	Waters nanoACQUITY UPLC with HDX Technology including HDX Manager
Columns:	ACQUITY UPLC BEH C_{18} 1.7-μm 1.0 x 100 mm for peptides ACQUITY UPLC BEH C_4 1.7-μm 2.1 x 50 mm for intact proteins

LC conditions:

Trapping column:	ACQUITY VanGuard Pre-Column, BEH C_{18}, 2.1 x 5 mm
Desalting column:	MassPREPTM Desalting Column, 2.1 x 5 mm
Column temp.:	0 °C
Online digestion column:	Immobilized pepsin column 2.1 x 30 mm from Applied Biosystems
Analytical flow rate:	40 µL/min
Mobile phase A:	0.1 % formic acid in water, pH 2.1
Mobile phase B:	0.1 % formic acid in acetonitrile, pH 2.1
Gradient:	3 to 40 % B in 7 min
Auxiliary mobile phase A:	0.05 % formic acid in water pH 2.50 for digestion flow
Trapping condition:	3 min at 100 µL/min

MS conditions:

MS system:	Waters Xevo QTof MS
Ionization mode:	ESI +
Capillary voltage:	3.0 kV
Collision energy:	6 V

Sampling Cone voltage:	35 V
Source temp.:	80 °C
Desolvation temp.:	175 °C
Lock Mass:	Glufibrinopeptide, 100 fmol/µL in 50% acetonitrile, 0.1% formic acid at 5 µL/min

Data management:

MassLynx 4.1 Software, ProteinLynx Global SERVER (PLGS) Software

Results and Discussion

Sample

Holo and apo bovine calmodulin were prepared with and without calcium, respectively, in protein buffer solution. Both proteins were labeled with 20-fold dilution with D_2O labeling solution and incubated for 10 seconds, 1, 10, 60, and 240 minutes at room temperature. The labeling reaction was quenched by adding quenching solution to reduce the pH to 2.5. The quenched samples were immediately placed at 0 °C.

Global HDX analysis for intact protein

Global HDX analysis provides information about overall conformational changes by comparing the deuterium uptake of control vs. analyte proteins. The intact HDX workflow is illustrated in Figure 2. First, the protein is labeled with deuterium and incubated for selected amounts of time between 10 seconds and 4 hours.

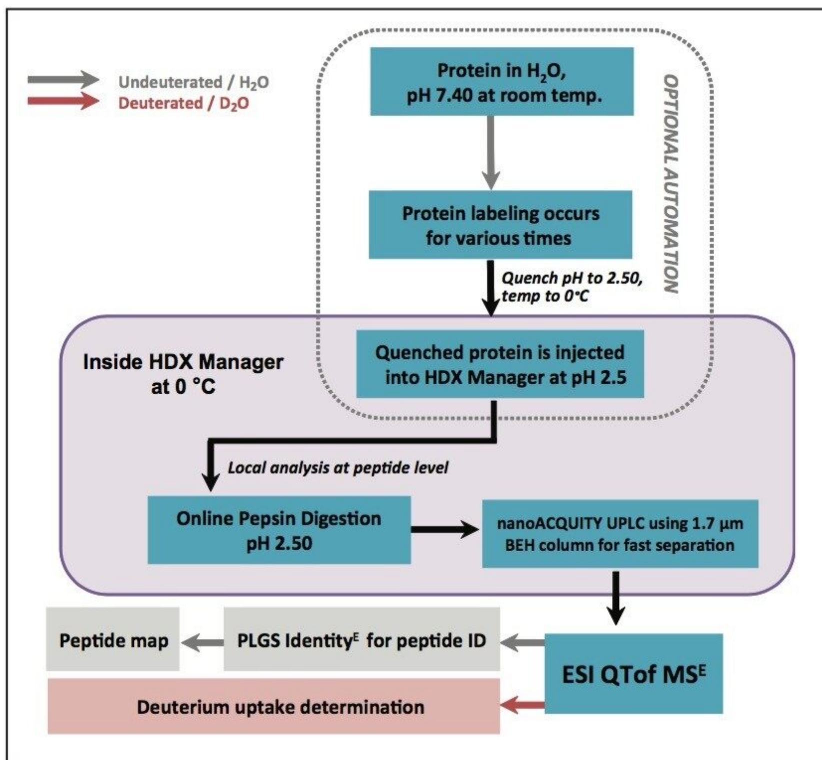

Figure 2. Global HDX workflow for intact protein analyses. The labeled protein is quenched to pH 2.50, and directly injected into the HDX manager, where online desalting and separation is performed at 0 °C.

During the exchange reaction in solution, amide hydrogens on the protein backbone exchange at a rate that depends on both solvent accessibility and hydrogen bonding. The deuterium uptake as a function of time will be different between control and analyte if there is a difference in higher-order structure. The exchange reaction is quenched with cold acidic (pH 2.50) buffer and then quickly injected into the HDX Manager. The temperature inside the HDX Manager is set at 0 °C to minimize back-exchange. Back-exchange must be controlled because deuterium can exchange back to hydrogen in the 100 % H_2O LC solvents. Therefore, low temperature, acidic pH, and a rapid run time are needed to minimize back-exchange. The HDX Manager in conjunction with the nanoACQUITY UPLC System is designed to satisfy specific HDX requirements such as fast 0 °C operation.

The Waters HDX System can be fully automated for sample preparation (Figure 2, highlighted in dashed line). The laborious preparation steps such as labeling, time measurement, quenching, and injection are handled by robotics. This system configuration saves a significant amount of time for laboratories performing high-throughput analyses.

Apo-calmodulin is known to be an open structure without bound calcium (Figure 3, left top panel). When calcium ions are bound to calmodulin (holo-), the protein adopts a more compact conformation (Figure 3, left bottom). The deuterium uptake curves display the relative deuterium level and compare the difference of apo and holo at each labeling time point (Figure 3, right panel). After structural conversion from apo to holo, a lower amount of deuteration was observed in holo calmodulin, indicating that fewer amide hydrogens were protected from exchange compared to the apo state.

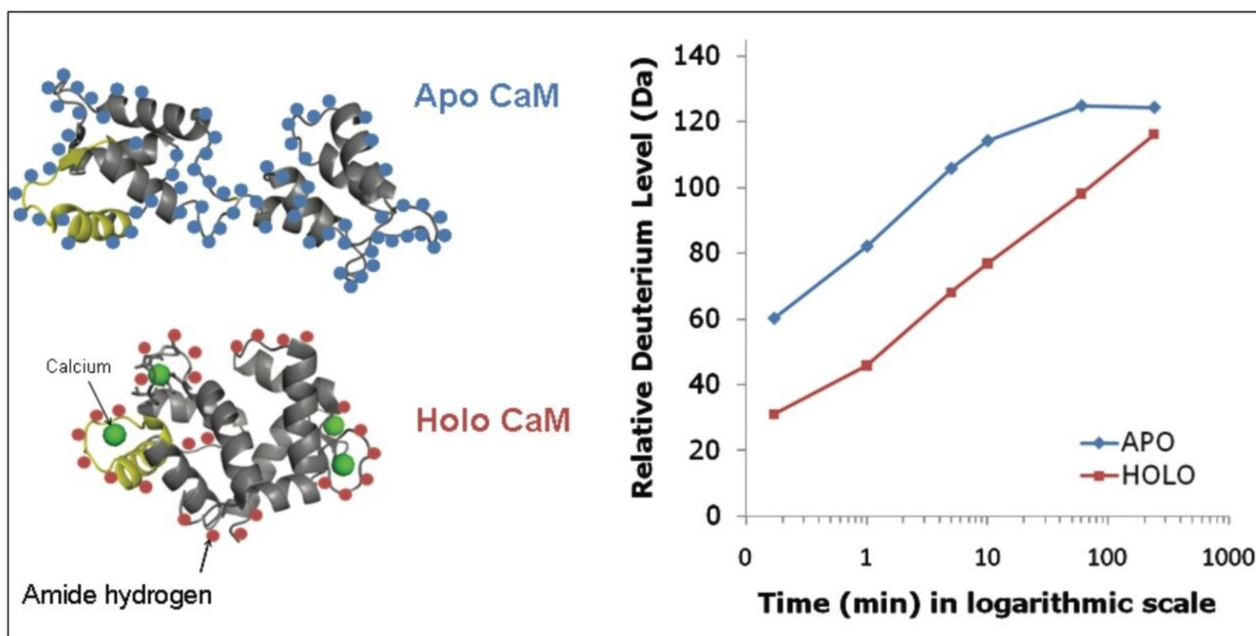

Figure 3. Deuterium uptake curves for intact apo and holo calmodulin with corresponding tertiary structures known from crystallography or NMR. The blue and red dots represent amide hydrogens that are available to exchange with deuterium in apo and holo, respectively. Lower deuterium uptake was observed in holo calmodulin due to its closed conformation.

Local HDX analysis for peptides

To locate where deuterium has been incorporated in a protein, the labeled and quenched protein is digested into peptides with an acidic protease (pepsin). Pepsin digestion of an undeuterated sample is performed to create a peptide map. In this peptide workflow (Figure 4), the HDX System performs an efficient pepsin online digestion and separates the peptides via UPLC with MS spectra collected in MS^E mode from the Xevo QTof MS. Finally, ProteinLynx Global SERVER (PLGS) Software processes the MS^E data to identify the peptides generated by pepsin. This UPLC-MS^E analysis offers several unique advantages compared to typical HPLC-MS.

- Robust online pepsin digestion
- Rapid peptide separation at 0 °C in less than 5 to 6 minutes
- UPLC resolution to be able to separate complex peptide digest of large proteins
- Independent temperature control for digestion and separation
- MS^E and PLGS for reliable peptic peptide ID

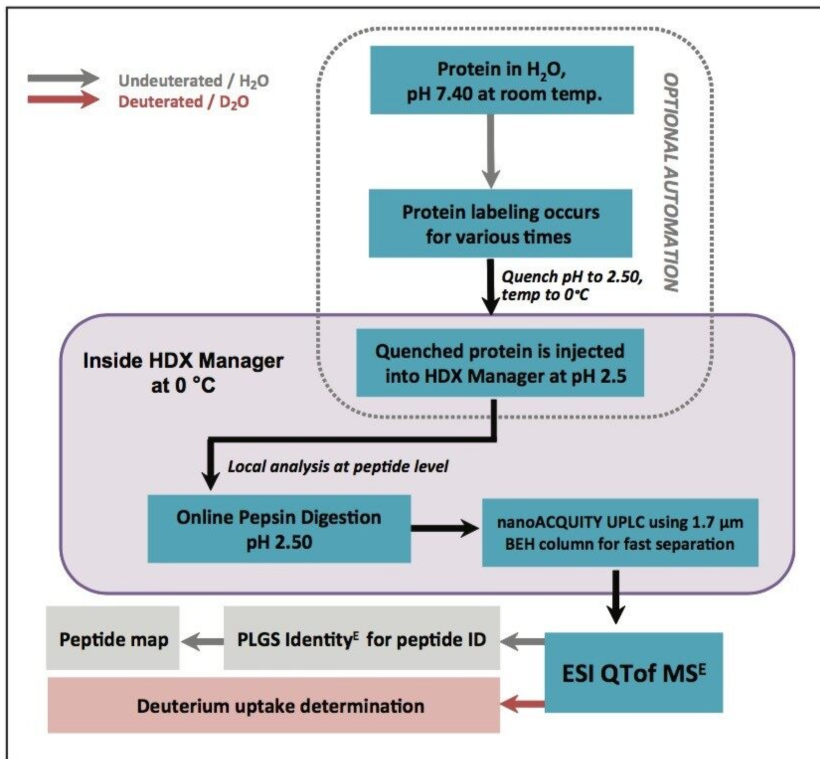

Figure 4. Peptide ID workflow. The peptide ID step is in gray and the HDX experiment is in red. The peptide ID step is needed to identify what peptides are produced during online digestion and to construct the coverage map.

Linear sequence coverage of 94% was observed in this study. Pepsin is active at low pH and maintains activity at low temperatures, which are ideal for HDX quench conditions. Pepsin also generates many overlapping peptides and short peptides helpful for localizing deuterium incorporation. The reproducible peptides of calmodulin by online pepsin digestion were used to construct a peptide map. These peptides shown in Figure 5 were found in all digestions in apo and holo samples. Identifying reproducible peptides is necessary because more robust analysis is possible when the deuterium uptake of the same peptide is

compared in deuterated apo and holo samples.

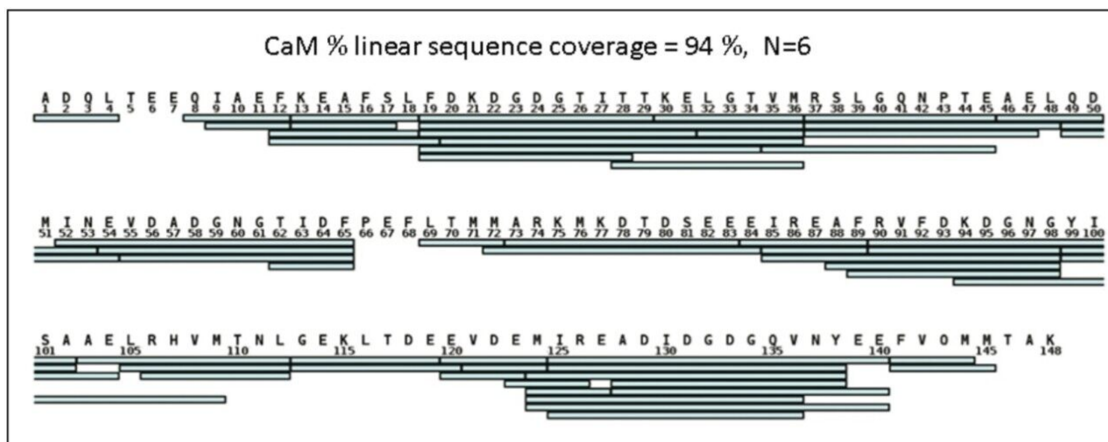

Figure 5. Calmodulin online pepsin digestion coverage map. Each bar under the sequence represents an identified peptide. These peptides were reproducibly observed (N=6).

The deuterated protein was digested and separated at 0 °C in same way as undeuterated protein (Figure 4, red arrows). Reproducible chromatographic separations were obtained throughout the labeling time-course (Figure 6, left panel). MS spectra showed the expected mass increase in *m/z* as a result of longer exposure to D_2O (Figure 6, right panel). The intensity weighted average mass of each isotopic envelope was found and compared to that of the undeuterated protein.

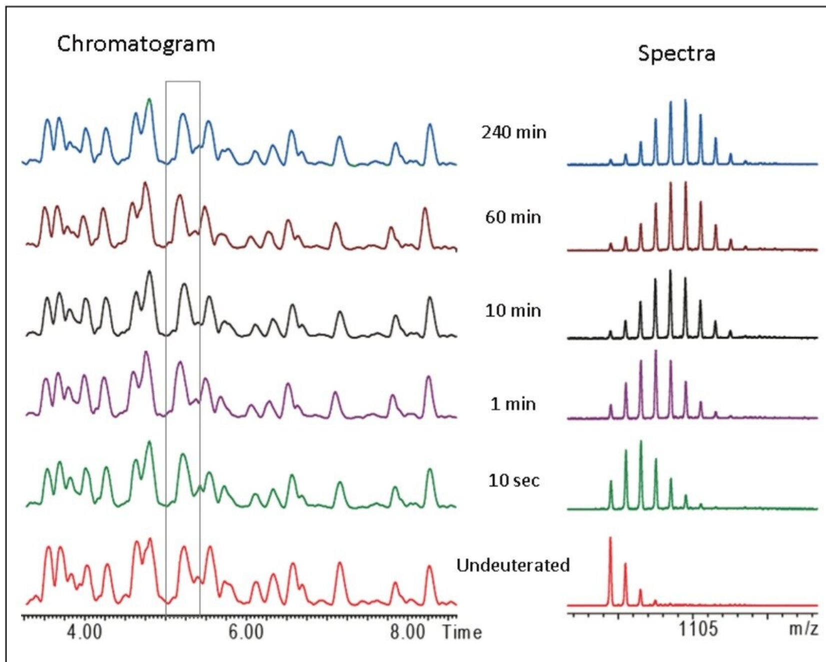

Figure 6. Reproducible chromatographic separations of calmodulin peptides following online pepsin digestion (left panel). The right panel shows the spectra of peptide (ADIDGDGQVNY) labeled from 10 seconds to 240 minutes. The m/z increases as a result of longer exposure to D_2O.

Peptide HDX data interpretation

Deuterium uptake into each peptic peptide was compared for apo and holo calmodulin. These data indicate that there was a change in deuterium uptake for certain peptides from apo to holo form. An example is shown in Figure 7. The highlighted arrows point out the different deuterium uptake in one peptide after 10 minutes of labeling.

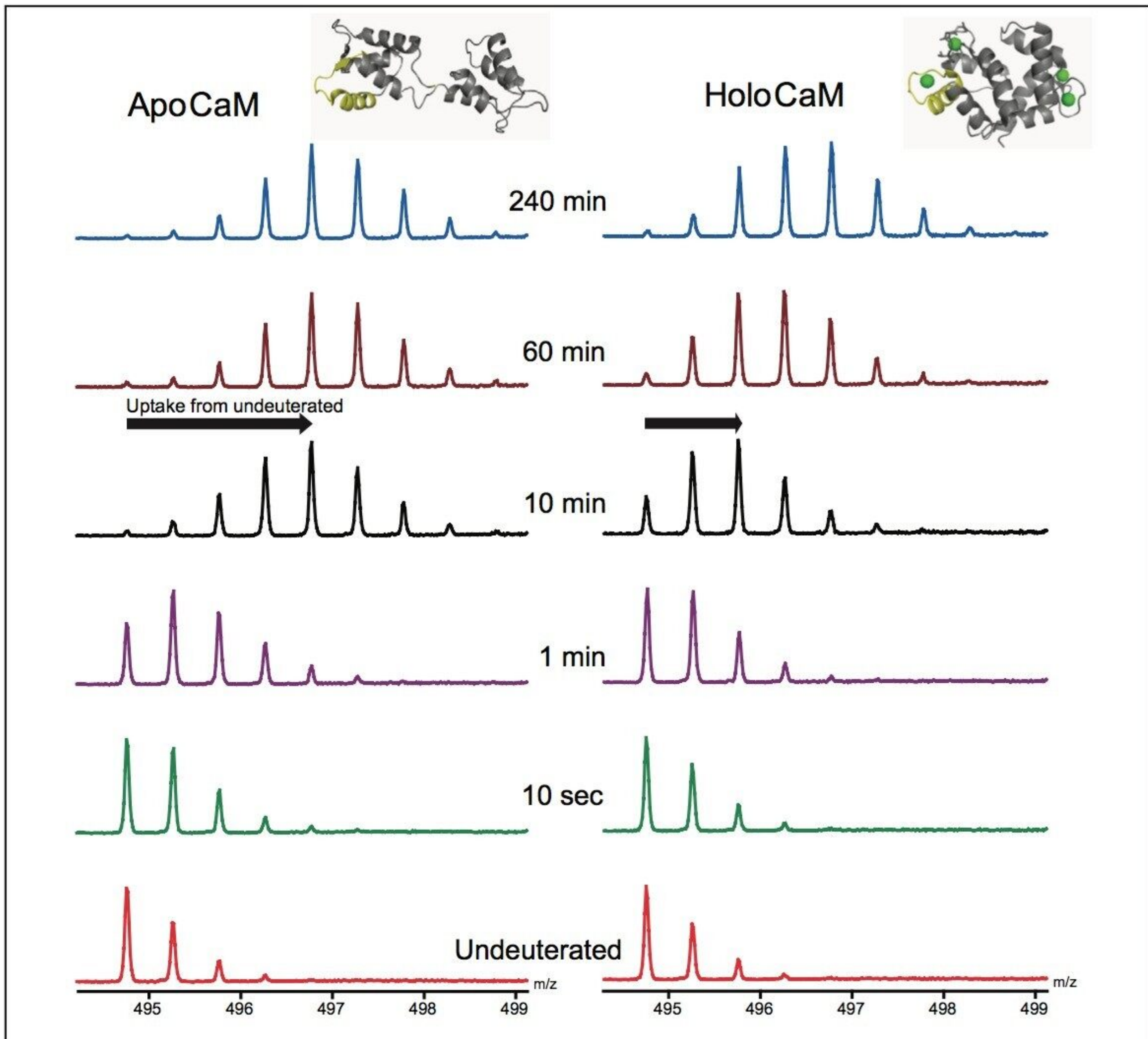

Figure 7. Different deuterium uptake of the same peptide found in Apo and Holo CaM. At 10-minute labeling, this peptide (FKEAFSLF, 12-19) showed a higher deuterium uptake in Apo (left panel) than in Holo (right panel).

Peptide HDX analysis typically generates hundreds of peptic peptides depending on the size of protein, therefore manually determining the deuterium uptake of each peptide is a time-consuming process. With Waters software, data interpretation is automated, and the data processing time is significantly reduced.

The measured deuterium incorporation can be plotted as deuterium uptake curves for each peptide (Figure 8). These charts show where the difference /no differences in deuterium uptake occur for all peptides. Figure 9 is one of the representations of HDX results. The relative percent deuterium uptake was color coded on 3D

structure, thus overall dynamics of apo and holo calmodulin can be easily compared over time. Three dimensional (3D) structures confirmed that those peptides with significant differences in deuterium uptake were located primarily the calcium binding region.

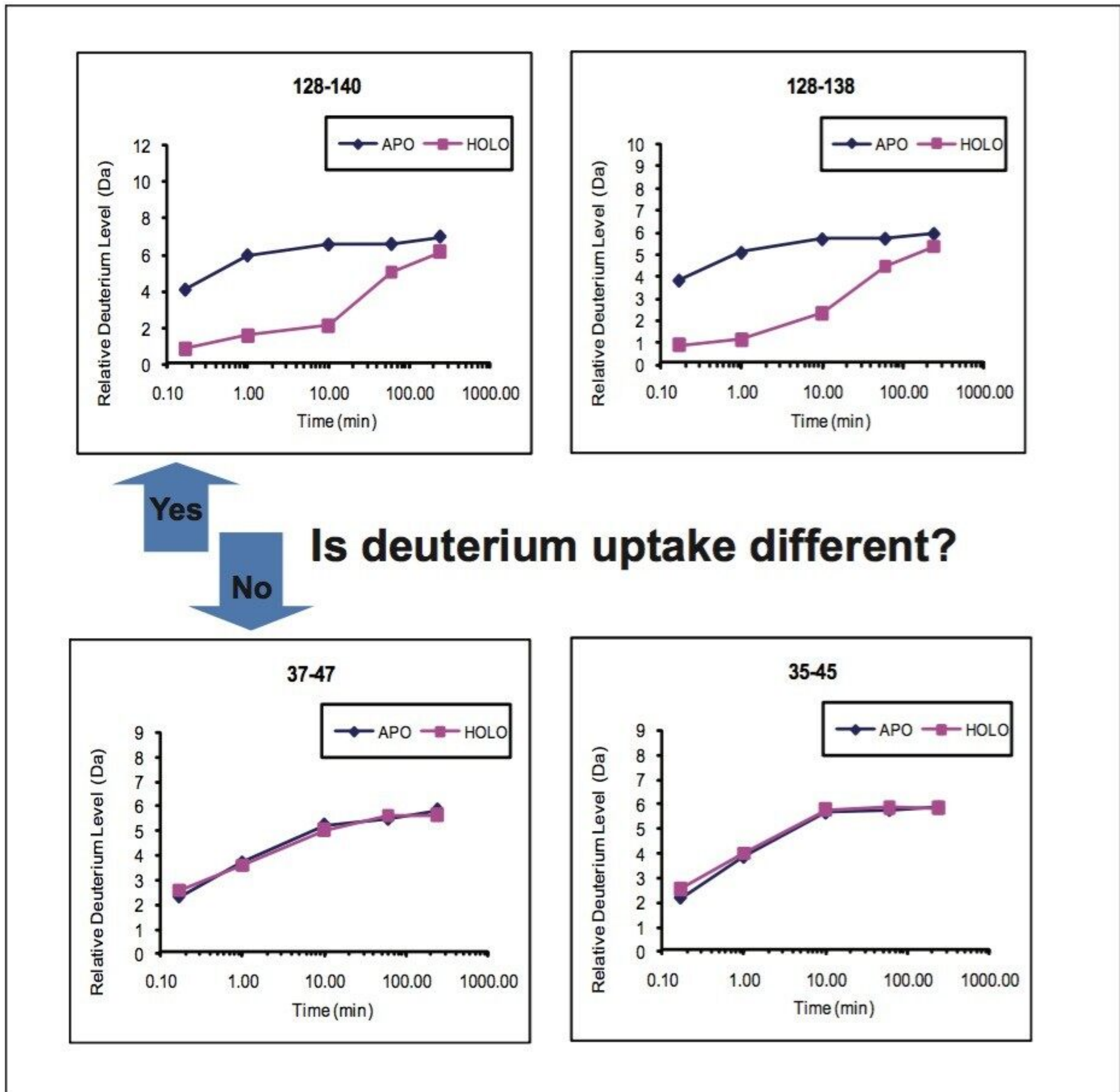

Figure 8. The Example of deuterium uptake curves for apo and holo calmodulin. Upper curves (overlapping peptides covering 128-140 and 128-138) illustrate that there was distinct change in deuterium uptake between apo and holo calmodulin. The lower curves (peptides 37-47 and 35-45) showed no change in deuterium uptake between apo and holo calmodulin. Every peptide represented in Figure 5 has its own uptake curve.

For example, the highlighted box on the left panel in Figure 9 indicated 32% deuterium uptake for apo calmodulin whereas the same peptide region in holo form only showed 8% deuterium uptake at 10 seconds of labeling time. Figure 10 is another example of data representation with corresponding 3D structures. This HDX heat map overlays the deuteration on the sequence. 3D structures obtained from X-ray crystallography or NMR are complementary to the HDX results if available.

Figure 9. HDX heat map display for assessing conformational difference in localized region (46 - 65). Two sets of colored bands of the heat map represent the apo (top band) and holo (bottom band) calmodulin of % deuterium uptake. Cooler color represents less exchange, thus greater protection from exchange. The 3D structures with black arrows are the corresponding regions of the displayed heat map (46-65). Represented holo region shows much cooler color in mostly purple and dark blue in early time points (for example at 10 seconds) than in apo calmodulin.

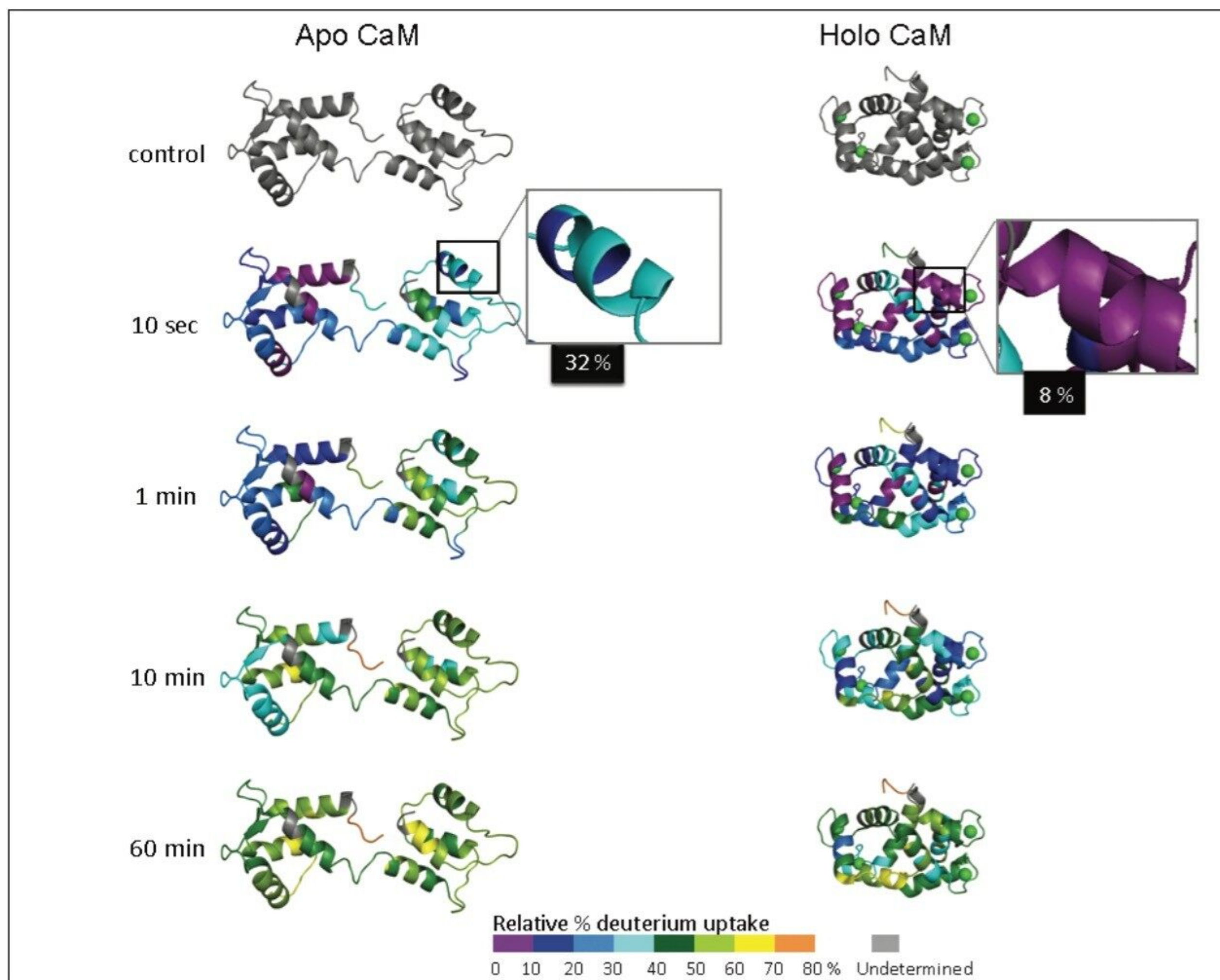

Figure 10. Deuterium uptake information represented on calmodulin apo and holo 3D structures (apo = 1CFD.pdb, holo = 1PRW.pdb). At 10 seconds labeling, two regions of calmodulin were compared in highlighted boxes. Overall, the apo structure shows more rapid exchange due to greater protein flexibility without calcium binding

Conclusion

The Waters nanoACQUITY UPLC System with HDX Technology, when combined with high-resolution MS, was effectively used to determine changes in calmodulin conformation upon calcium binding. This system provides unique features that improve the quality of HDX analysis.

- HDX workflow for global and local HDX analyses.
- Robust online pepsin digestion
- Rapid reproducible UPLC separation at 0 °C
- Accurate identification of peptic peptides by MS^E and PLGS
- Automated HDX data processing

Waters HDX Technology facilitates the study of protein conformation and protein dynamics at global and local levels. The information obtained helps researchers understand protein function and related biological activity. The HDX System is the informative analytical tool for biopharmaceutical drug discovery and development.

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Solutions in practice

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