

## Video Article

# Amide Hydrogen/Deuterium Exchange & MALDI-TOF Mass Spectrometry Analysis of Pak2 Activation

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## Abstract

Amide hydrogen/deuterium exchange (H/D exchange) coupled with mass spectrometry has been widely used to analyze the interface of protein-protein interactions, protein conformational changes, protein dynamics and protein-ligand interactions. H/D exchange on the backbone amide positions has been utilized to measure the deuteration rates of the micro-regions in a protein by mass spectrometry<sup>1,2,3</sup>. The resolution of this method depends on pepsin digestion of the deuterated protein of interest into peptides that normally range from 3-20 residues. Although the resolution of H/D exchange measured by mass spectrometry is lower than the single residue resolution measured by the Heteronuclear Single Quantum Coherence (HSQC) method of NMR, the mass spectrometry measurement in H/D exchange is not restricted by the size of the protein<sup>4</sup>. H/D exchange is carried out in an aqueous solution which maintains protein conformation. We provide a method that utilizes the MALDI-TOF for detection<sup>2</sup>, instead of a HPLC/ESI (electrospray ionization)-MS system<sup>5,6</sup>. The MALDI-TOF provides accurate mass intensity data for the peptides of the digested protein, in this case protein kinase Pak2 (also called  $\gamma$ -Pak). Proteolysis of Pak 2 is carried out in an offline pepsin digestion. This alternative method, when the user does not have access to a HPLC and pepsin column connected to mass spectrometry, or when the pepsin column on HPLC does not result in an optimal digestion map, for example, the heavily disulfide-bonded secreted Phospholipase A<sub>2</sub> (sPLA<sub>2</sub>). Utilizing this method, we successfully monitored changes in the deuteration level during activation of Pak2 by caspase 3 cleavage and autophosphorylation<sup>7,8,9</sup>.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/3602/>

## Protocol

### 1. Pak2 Activation

1. Add the appropriate pre-tested amount of caspase 3 to 20  $\mu$ l of 12 mg/ml Pak2 in buffer A (50 mM Tris-HCl, 150 mM NaCl, and 2 mM DTT at pH 7.5) and incubate at 34 °C for 30 min to fully cleave and activate Pak2.
2. Analyze the cleavage products by SDS-PAGE (10 %) and Coomassie blue staining.
3. Add the cleaved Pak2 to activation buffer B (50 mM Tris-HCl, 150 mM NaCl, 15 mM MgCl<sub>2</sub>, 10 mM ATP) at pH 7.5, in 20  $\mu$ l to a final concentration of 10 mg/ml Pak2, and incubate at 34 °C for 30 min to fully activate Pak2 by autophosphorylation at 8 sites.
4. Confirm full cleavage of Pak2 by migration of the p34 and p27 fragments as determined by SDS-PAGE<sup>10</sup>. Proper verification of the autophosphorylation conditions is made via autoradiography in a separate experiment using (<sup>32</sup>P)ATP or mass spectrometry to detect phosphopeptides.

### 2. Identification of Pepsin-Digested Pak2 Fragments

1. Add 1 ml of 0.1 % Trifluoroacetic Acid (TFA) at pH 2.5 to wash the agarose beads twice prior to use. Then, centrifuge the samples for 15 sec at 2,000 x g to remove the beads.
2. Add Pak2 (20  $\mu$ g in 2  $\mu$ l of 150 mM NaCl, 50 mM Tris-HCl and 2 mM DTT at pH 7.5) to 100  $\mu$ l of 0.1 % TFA, and incubate the sample for 5 min with 30  $\mu$ l of pepsin-conjugated agarose beads.
3. Equilibrate a reverse phase HPLC C18 column with a primary solvent system of 0.1 % TFA at pH 2.5. The pepsin digest (50  $\mu$ l) is eluted using a linear 100-min gradient of 0 - 80 % acetonitrile containing 0.1 % TFA at a flow rate of 0.2 ml/min. Fractions are collected every 2 min.
4. Dry the HPLC fractions with a speed-vac (3 h) and resuspend the samples in a solution of acetonitrile (5  $\mu$ l) and 0.1 % TFA (5  $\mu$ l) at pH 2.5.
5. Initially, each fraction is screened for peptides using the MALDI-TOF PerSeptive Biosystems Voyager DE STR (PE Biosystems, Foster City, CA, USA).

- The 16 fractions containing peptides are subjected to tandem mass spectrometry using a Q-TOF mass spectrometer and a QSTAR XL oMALDI MS/MS to identify the fragments.
- The sequence of each peptide is verified by the product ions generated by the tandem mass spectrometry with the theoretical m/z ratios based on the primary sequence of Pak2 using the Protein Prospector website (<http://prospector.ucsf.edu/>)<sup>11</sup>.

### 3. Measurement of Amide H/D Exchange

- Hold all solutions and reagents needed for H/D exchange at 25 °C in a water bath. ATP is dissolved in water and the pH is adjusted to 7.0 prior to use. The additional 4mM ATP in the D<sub>2</sub>O buffer is to prevent ATP dissociated from Pak2 after dilution.
- Initiate H/D exchange by addition of 18 µl of buffered D<sub>2</sub>O (50 mM MOPS pH 6.98, 125 mM NaCl) to 2 µl (20 µg) of Pak2 in a buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5) and 2 mM DTT, resulting in a Pak2 level of 10 mg/ml and a pH of 7.0 at 0 °C.
- Incubate the samples in triplicate in the H/D exchange for 0, 0.5, 1, 3, 5, and 10 min, or 24 h.
- Quench the H/D exchange process by adding 180 µl of ice-cold 0.1 % TFA at pH 2.2, which brings the pH to 2.5 in a final volume of 200 µl. For the samples containing ATP, 180 µl of ice-cold 0.11 % TFA at pH 2.2 is used to maintain the pH at 2.5 in the quenched condition.
- Add an aliquot (100 µl) of each quenched sample to 30 µl of activated pepsin-conjugated agarose beads. The pepsin digestion is allowed to proceed on ice for five min and the samples are vortexed every 30 sec.
- Terminate the digestion by centrifugation of the sample for 15 sec at 5,000 x g at 4 °C to remove the pepsin.
- Rapidly freeze the samples in liquid N<sub>2</sub> and store at -80 °C for no more than 2 days prior to MALDI-TOF analysis.
- The matrix for MALDI is 5 mg/ml of α-cyano-4-hydroxycinnamic acid (CHCA) dissolved in a solution (1:1:1) of acetonitrile, ethanol and 0.1 % TFA at pH 2.5 and held at 0 °C.
- Partially thaw each sample quickly and mix 1 µl of each sample with 1 µl of matrix and spot on a 4 °C MALDI target plate.
- Apply a moderate vacuum to the plate to dry the spots in 30 sec prior to MALDI-TOF analysis. The time between thawing of the sample and spectrum retrieval is about 3 min.
- Acquire MALDI-TOF mass spectra with a PerSeptive Biosystems Voyager DE STR. Acquire data at a 2-GHz sampling rate at 100,000 data channels, with a 20,000-V accelerating voltage, and a 65 % grid voltage using delayed extraction with a 180-ns pulse delay. One hundred scans are averaged in 2 min. The MALDI-TOF instrument is not restricted to any brand or any particular software.
- Use the Data Explorer 4.0 computer program to calculate the deuteration of each fragment.
- Calculate the back exchange based on the ratio of the actual incorporated deuterium number at 24 hr of H/D exchange divided by all possible exchangeable sites, which are the backbone amides except the N-terminal amide (**Figure 1**).

### 4. Representative Results:

The caspase cleavage and autophosphorylation are verified by SDS-PAGE Commassie staining. Inactive Pak2 is a single band of 58 kDa. Caspase cleavage of Pak2 is complete, and produces two fragments, p27 and p34 which migrate separately. The migration of autophosphorylated p27 and p34 shows an overlap on the gel due to the retarded migration of the fully phosphorylated p27 fragment (**Figure 2**).

H/D exchange experiments are carried out multiple times between 0 and 10 min (**Figure 3**). As an example, the time course of deuterium incorporation into the m/z peak 1697.85 of inactive Pak2 is composed of multiple isotopic peaks as shown by the shift of the mass envelope over time.

## Deuteration of Proteolytic Fragments

$$D(t) = \frac{[m(t) - m(std)]}{(BackExchangeRatio)} - D(4.5\%SideChain)$$

***D(t)***: The number of deuterons at time t

***m(t)***: The observed mass at time t

***m(std)***: The non-deuterated mass

***Back Exchange Ratio***: The deuteron number in the fully deuterated sample divided by total exchangeable sites.

Figure 1. Equation for the calculation of deuteration.

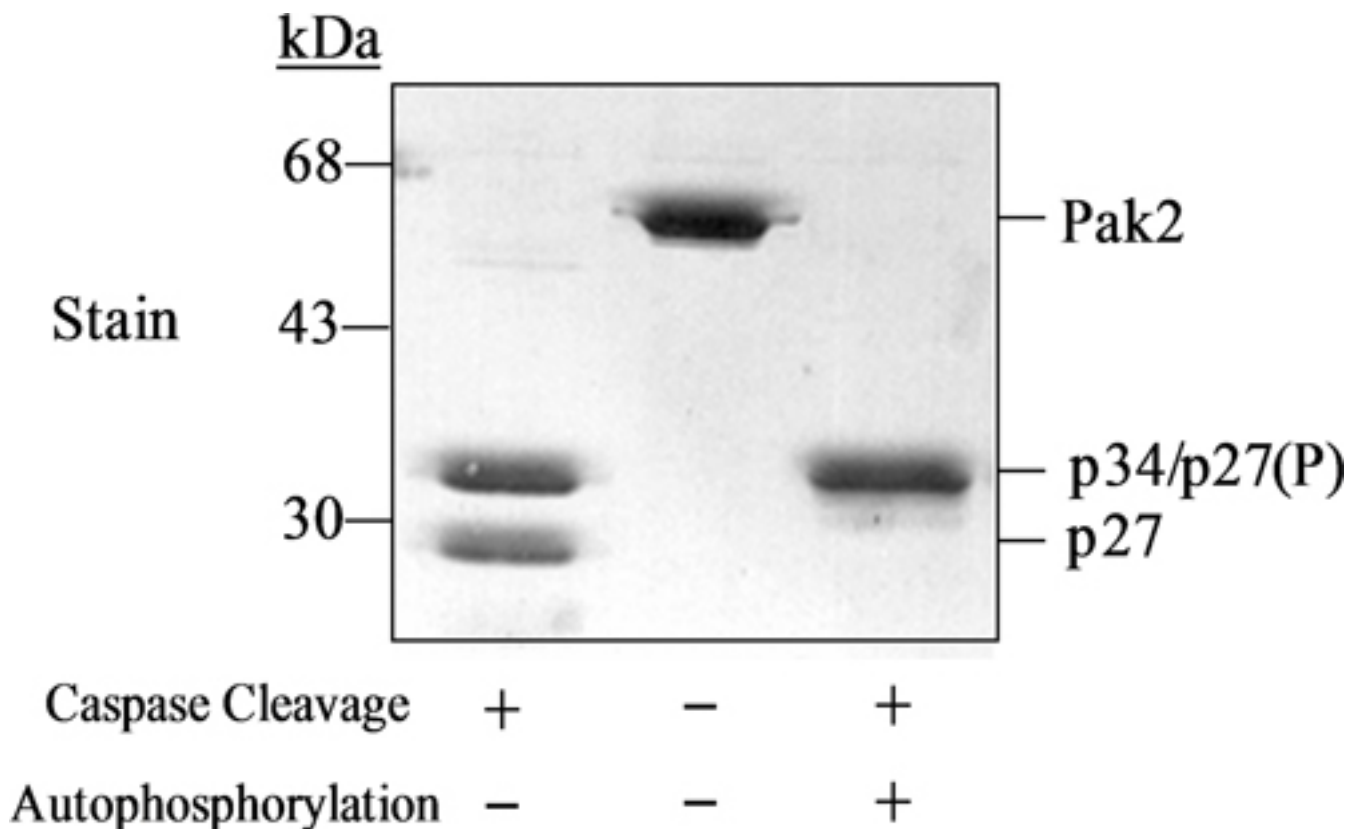
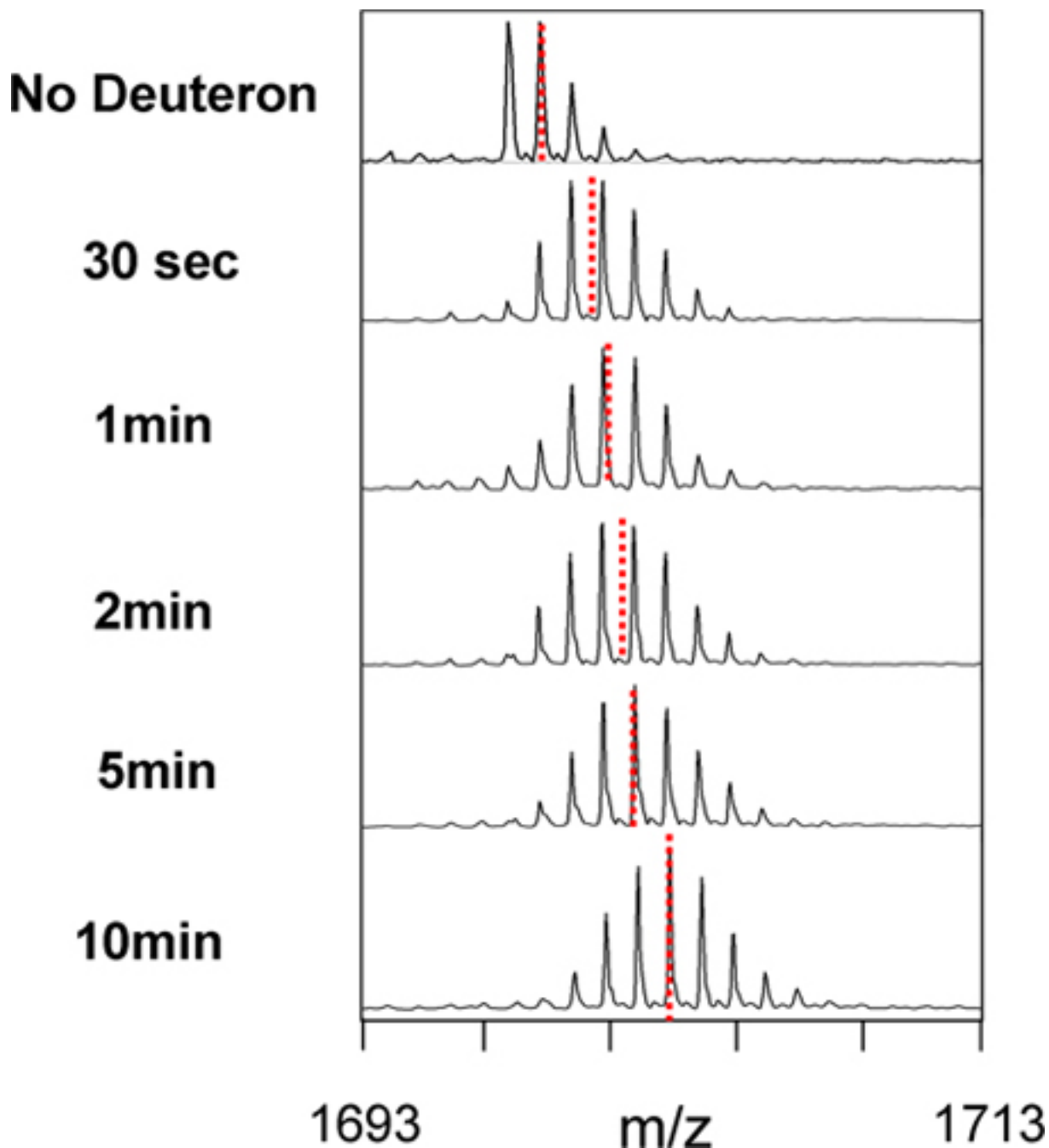


Figure 2. Autophosphorylation and caspase cleavage of Pak2. Caspase-cleaved Pak2 (left lane), intact inactive Pak2 (middle lane) and caspase-cleaved autophosphorylated Pak2 (right lane) were analyzed by SDS-PAGE and Coomassie blue staining. Adapted from Hsu, YH et al.<sup>2</sup>.



**Figure 3.** Mass spectrum of a time course of deuterium incorporation for inactive Pak2. Inactive Pak2 was subjected to H/D exchange for 0-10 min and analyzed by MALDI-TOF. An example of the expanded isotopic distribution of MALDI-TOF of peak  $m/z$  1697.85 during the time course of H/D exchange is shown. The red dotted line indicates the average of the mass envelope and the shift of the mass envelope shows the deuteration of a peptide.

## Discussion

Identification of the Pepsin-Digested Fragments is a critical step of the H/D exchange experiment. Incorrect identification of the peptide can lead to a wrong conclusion. Pepsin-digested Pak2 is eluted through a reverse phase HPLC C18 column to obtain a clean background. In our experiments, a total of 40 fractions were collected and subjected to MALDI-TOF. Multiple peptides could be identified in each of the fractions. The peptides were subjected to tandem mass spectrometry (Q-TOF MS/MS and oMALDI MS/MS) to identify their sequences. The MS/MS spectra of a peptide should have at least three product ions to confirm the identification of the peptide.

The Data Explorer 4.0 computer program (Applied Biosystems) performs the initial baseline correction and noise filtration. Each spectrum must be calibrated based on two sequenced peaks. We calibrate our spectrum by the theoretical mass of the undeuterated  $m/z$  which are 923.45 and 1697.84. The mass accuracy after calibration can reach 10 ppm. Upon deuteration, the mono-isotope may shift to a higher mass. Unitary

step increases to these  $m/z$  ratios yielded the enhanced masses associated with deuteration. The peak intensities of the mass envelope will not affect the deuteration level. However, the peak intensities of the isotopic peaks in a particular mass envelope are critical for the calculation of the average mass. The first step of the calculation of the incorporated deuterium is subtracting the peptide mass of the non-deuterated sample from the deuterated sample. Three other factors, the  $D_2O$  dilution, the residual deuterons in the side chains and the back exchange, will need to be further considered in the calculation (**Figure 1**). The  $D_2O$  dilution is the dilution factor of  $D_2O$  after mixing the sample and  $D_2O$  buffer to initiate the H/D exchange. The residual deuterium (4.5%) in the side chains of the pepsin-digested peptides needs to be subtracted. The back-exchange is the inevitable loss of deuteration in all deuterated and pepsin-digested peptides in the mass measurement process.

The most solvent accessible peptide ( $m/z = 1105.60$ ) after 24-hour of H/D exchange represents a full deuteration region. The incorporated deuterium number for each of the peptides is the difference between the centroid of the deuterated and non-deuterated Pak2 peptic peptides. The most highly deuterated peptide  $m/z$  1105 was selected to represent full deuteration when Pak2 was at 24 hr of H/D exchange. The Back Exchange Ratio was calculated by the actual incorporated deuterium number at 24 hr of H/D exchange divided by all possible exchangeable sites at peptide  $m/z$  1105.

## Disclosures

No conflicts of interest declared.

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