5.08J Biological Chemistry II (2016)
PS 10 Chemistry 5.08 (Spring 2016)
Topics PS10: PTM and MS analysis, fluorescence methods in assays and in cells

Problem 1. In Module 7 on ROS, you learned that for post-translational modifications (PTMs) such as sulfenylation and phosphorylation to be important in regulation, the modification must be reversible. Ub is another example of a PTM. Polyubiquitin (polyUb) signals differ in structure and function depending on the linkage type within the polyUb chain. In fact, thus far, eight distinct Ub chain linkages have been identified in cells and they appear to have distinct functions. While Lys48 and Lys11-linked Ub polymers target proteins for proteosomal degradation, Lys63- and amino terminal-Met1-linked polyUb regulate non-degradative functions such as activation of protein kinase cascades during activation of the transcription factor NF-κB. Ub modifications are reversed by five families of deubiquitinases (DUBs) in human cells. Four of these five families are cysteine proteases which contain a thiol with a low pKa in their active site. As you have seen with EGFR studies in the Carroll paper, kinase/phosphatase signaling can be altered by PTM by phosphorylation and oxidation. The data described below addresses the issue of whether DUBs, specifically the A20 OUT DUB proposed to be a key player in the NF-κB signaling pathway, can be regulated by ROS.

Initally two types of experiments were carried out on the A20 OUT domain, a truncated version of the DUB containing residues 1-366. The substrates used in these experiments to monitor the activity of this DUB, were diUb or triUb with different isopeptide linkages including Lys48, Lys63, or Met1-diUb.

Experiment 1

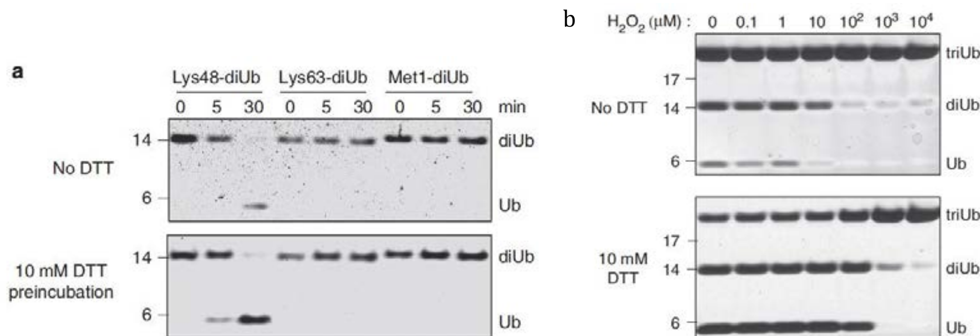

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Figure 1. **a**. DUB assay of truncated A20 OUT with three different diUb substrates containing different Ub linkages. The DUB assay was carried out in the presence or absence of the reducing agent DTT. **b**. A20 was first incubated with indicated amounts of H_2O_2 for 15 min at 25°C and then with 100 U of catalase to destroy the H_2O_2. The sample was split then into two aliquots and 10 mM DTT was added to one half for 15 min. The activity of A20 was then tested in an assay using Ly48-linked triUb as a substrate.

Questions:

1. Draw the structure of Lys48-diUb, focusing on the chemical linkage.
2. DUBs are cysteine proteases. Given your understanding of the chemical mechanisms of peptide bond hydrolysis by cysteine proteases in general, what residues are likely involved in catalysis and what is their role?

3. What is the purpose of Experiment 1? Describe how the results given in Figure 1a and 1b provide information about the biology/chemistry of this DUB.

Experiment 2. A second set of experiments was performed to determine the site of A20 modification by H_2O_2 using the methods discussed in recitation 12. The truncated A20 OUT domain (shown in Figure 2a) was incubated with dimethylsulfoxide (control) or DAz-2 during H_2O_2 treatment in vitro. DAz-2 is an azide-labeled version of the dimedone probe you saw in recitation. Excess probe was removed and DAz-2 modified protein was conjugated to a biotin tag using click chemistry.
To identify the site(s) of modification, A20 was subjected to MS analysis and the results are shown in Figure 2c. The predicted mass of sulfenylated A20 is 43452 Da.

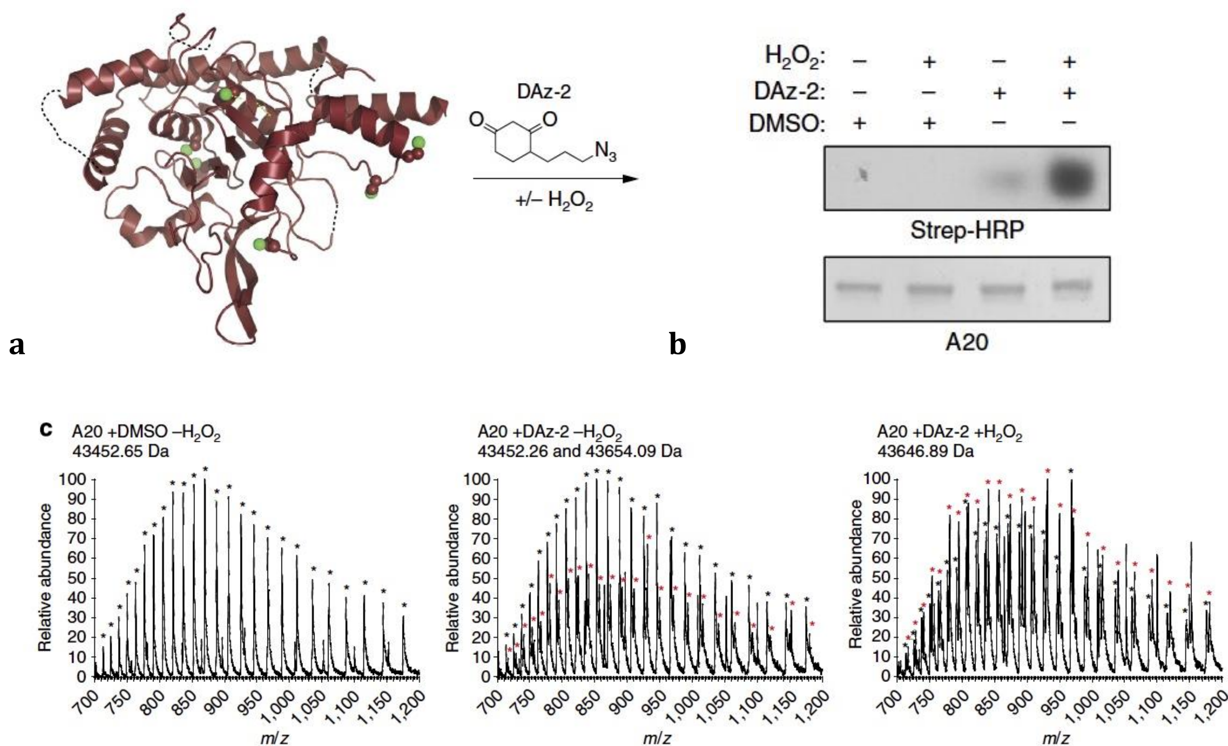

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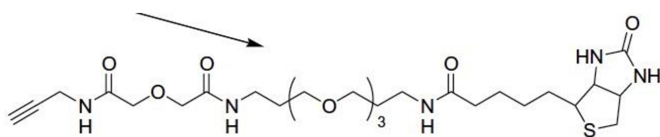
Figure 2. **a.** Structure of the truncated-A20 with the 6 detectable cysteines (green spheres). All of these studies have been done in vitro. **b.** A20 has been treated as indicated with H_2O_2 (50 μM for 60 min), DAz-2 (see structure) or DMSO (the control). The reagents are removed and the reaction mixture incubated with biotin conjugated to an acetylene (one example is shown below). Detection of the PTM was then carried out by incubation with Streptavidin-coupled horseradish peroxidase (Strep-HRP). Streptavidin binds to biotin. The A20 gel below the Strep-HRP blot indicates that loading of A20 was the same in all lanes. **c.** Left, mass-spectrum of intact A20 domain without H_2O_2 treatment. Middle, untreated A20 labeled with DAz-2. Right, A20 treated with 50 μM H_2O_2 for 1 h at room temperature and then labeled with DAz-2.



Questions:

4. Describe the method of detection in Figure 2b that was used to generate the blot labeled Strep-HRP. Show the steps involved and the chemical transformations.

5. Explain the differences in the MS data in Figure 2c (right, middle, and left). Why are their so many peaks in each spectrum and how do these peaks together provide confidence in the molecular weight of the A20? Explain the molecular basis for the red and black * peaks.
6. Describe an experiment to identify the site(s) of labeling in the sample on the right (Figure 2c).
7. What conclusions can you draw about the importance of PTM in the A20 DUB from these in vitro studies?
8. What additional controls could be carried out to make you more confident in your model?

Problem 2. One of the strategies for the chemoprevention of degenerative diseases involves upregulation of antioxidant and free radical detoxification gene products by increasing intracellular concentrations of the transcription factor erythroid2-related factor 2 (Nrf2). The proposal has been made recently that this can be done by disruption of the interaction between Nrf2 and the Kelch-like ECH associated protein 1 (Keap1), a substrate adaptor protein for a Cul3-dependent E3 ubiquitin ligase. Under basal conditions Keap1 represses Nrf2 by targeting the transcription factor for ubiquitination and degradation by the proteasome. This problem is focused on a recent assay using FRET methods to find inhibitors of the Nrf2-Keap1 interaction.

The first step in the assay development was to design fluorescently labeled domains of both Keap1 and Nrf2. The construct design shown in Figure 3a is based on the identification of a "high affinity" motif peptide ETGE (residues contained within a 16-mer peptide) of Nrf2. This Nrf2 construct also has a hexaHis tag at the N-terminus followed by a cyan-fluorescent protein (CFP) and is called CFP-Nrf. A TEV protease cleavage site has also been inserted in the construct (labeled TEV). The Keap construct is composed of a YFP (yellow fluorescent protein) conjugated to a Kelch domain and is called YFP-Kelch.

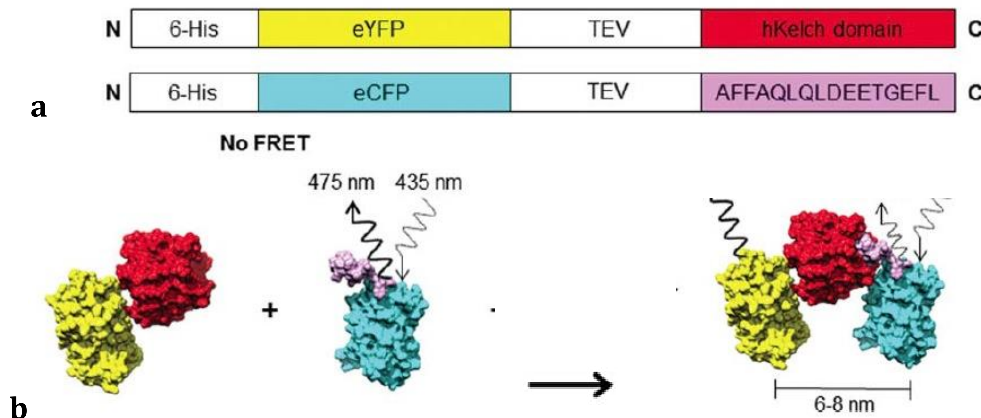


Figure 3 **a**. Schematic representation of the domains of YFP-Kelch (top) and CFP-Nrf2 (bottom). **b**. Molecular model of the YFP-Kelch and the CFP-Nrf2 FRET pair constructed from the human Keap1 Kelch domain (red) with YFP (yellow) and the 16mer-Nrf2 peptide (pink) and CFP (blue) construct. The separation between the CFP and the YFP chromophore residues in the complex of these proteins was estimated to be 6 to 8 nm, a distance suitable for this FRET pair.

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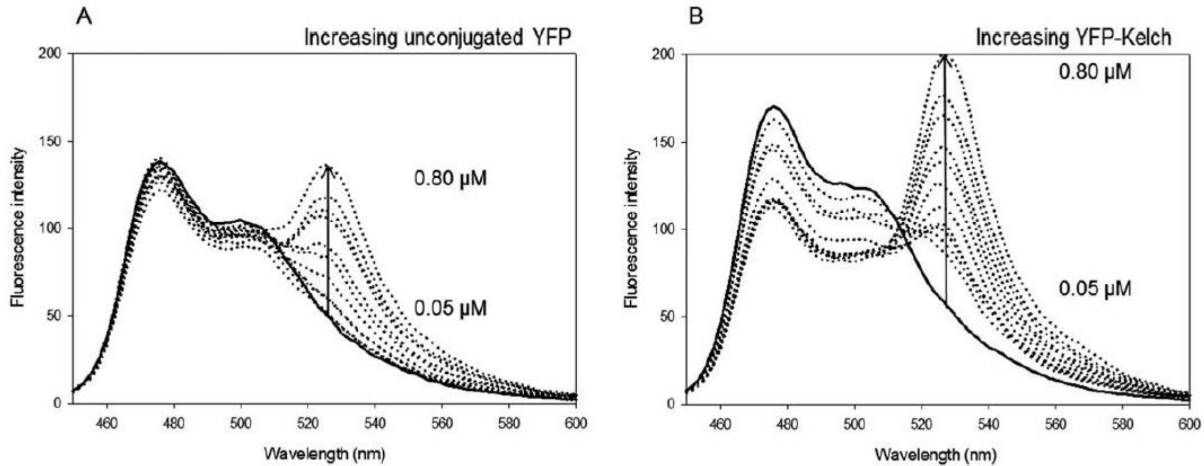

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Figure 4 Fluorescent emission spectra of direct titration of 0.11 μM CFP-Nrf2 with **A** the unconjugated YFP or with **B**, the YFP-Kelch at increasing concentrations from 0.05 to 0.8 μM. The solid line represents the donor alone before titration.

Questions:

1. Using the information from the control and experiment given in Figure 4, describe the results from each titration (A and B). Fill in the missing λ in Figure 3B (right). Explain whether this data suggests that a high throughput screen monitoring FRET to look for inhibitors of the Nrf2-Keap1 interaction has a chance to be successful.
2. Explain why the titrations in Figure 4 should be repeated at high salt concentration (ex, 150 mM NaCl) and in the presence of DMSO, a solvent often used to dissolve many small molecule drug candidates that are not very soluble in aqueous solution.
3. How might the small molecule drug candidates affect similar titrations to those described in Figure 4? Draw a fluorescence intensity vs λ graph for a successful effect of these additives.

Once the setup has been optimized, one would like to adopt this methodology to a multiwell plate format so that 10^5 small molecules can be investigated for their ability to disrupt the CFP-Nrf2•YFP-Kelch interaction. As a prelude to such experiments the investigators carried out a similar experiment in the presence or absence of a decameric peptide known to inhibit the Keap1-Nrf2 interactions. The results are shown in Figure 5 A and B.

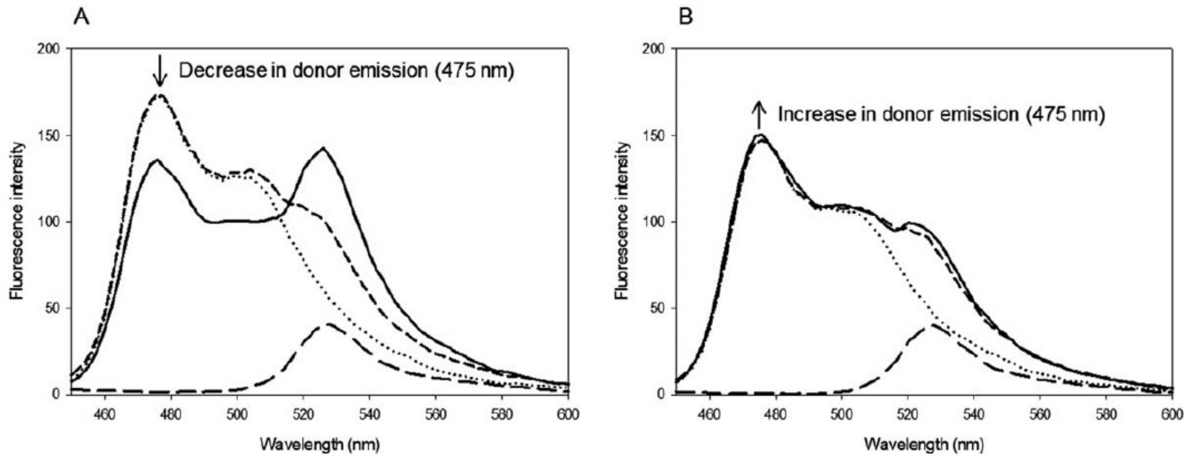

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Figure 5 Fluorescence emission spectra of YFP-Kelch and CFP-Nrf2 in the absence (A) or the presence (B) of a 10 μM unlabeled Nrf2-derived peptide inhibitor. Shown are the emission spectra of the FRET pair (solid line), donor alone (dotted line), acceptor alone (long dashed line), the sum of the donor and acceptor (short-dashed line).

Question:

4. Explain the results shown in Figure 5.

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