5.08J Biological Chemistry II (2016)
Answers to PS 10

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

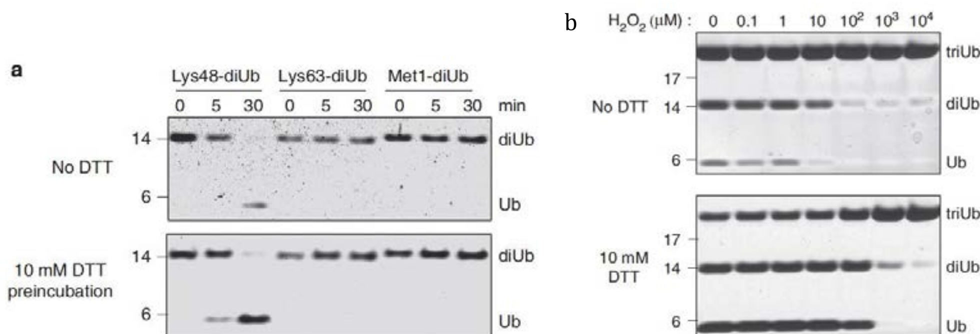

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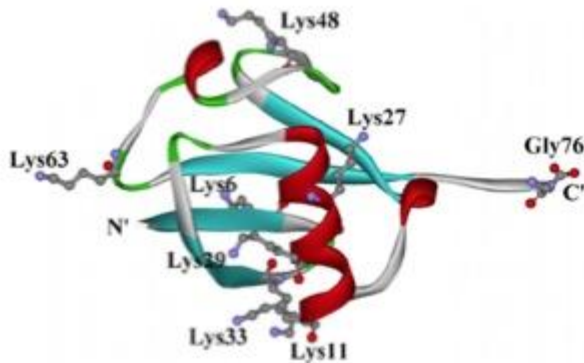
Figure 1. **a**. DUB assay of truncated A20 OUT with three different diUb substrates containing different Ub linkages. The DUB assay was carried out in the presence or absence of the reducing agent DTT. **b**. A20 was first incubated with indicated amounts of H_2O_2 for 15 min at 25°C and then with 100 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. This problem was taken from *Nature Communications* 2013 4, 1-9.



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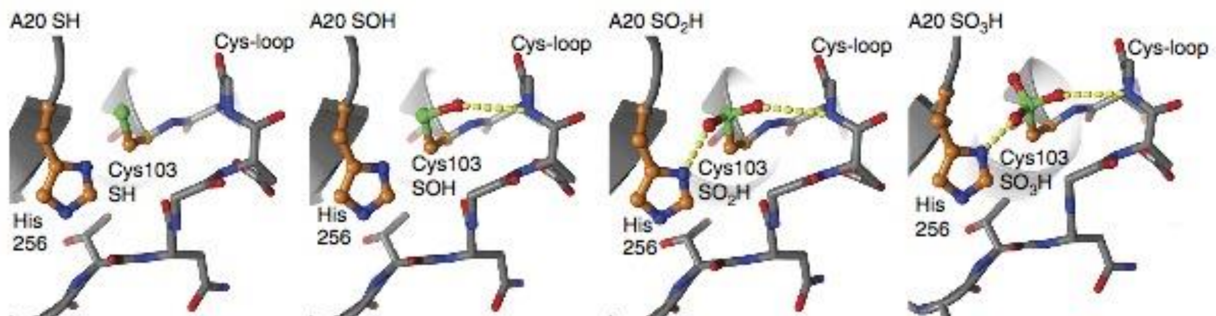
Above the diUb structure in which the linkage between the two Ubs is an isopeptide between lys48 (on the surface) and G76 at the C-terminus of Ub.

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?

Cysteine proteases have an active site cysteine and histidine. In general the pKa of the cysteine is perturbed and the cysteine is in the thiolate form and the histidine is protonated. Below is the active site of the DUB in this problem. On the far left you can see the His/Cys dyad and then to the right in the Figure below, you see different states of cysteine oxidation subsequent to treatment of the DUB with H_2O_2. Remember cysteine proteases are more chemically reactive relative to serine proteases because the cysteine is a better nucleophile (lower pKa). Thus one only needs a dyad to carry out peptide bond hydrolysis through tetrahedral intermediates or transition states (never detected experimentally) and the covalent acyl-enzyme intermediate. The latter is then hydrolyzed to give product.



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

To carry out studies on DUBs, one first needs to establish an assay. Assay development requires a knowledge of the substrate and then a method to monitor product formation. To learn about substrate specificity the authors investigated the importance of the linkage between the Ubs (Figure 1a). They investigated linkages between G76 (the C-terminus of Ub) to Lys48, Lys63 or the N-terminal methionine. They stopped the reaction and then monitored cleavage of diUbs to monoUb using SDS PAGE analysis. They carried out these experiments in the presence of absence

of DTT. The results revealed that under the conditions examined only the diUb with the Lys48 linkage was cleaved. They further revealed that if DUB was treated with DTT, presumably to reduce any oxidized active site cysteine, that the cleavage was much more efficient, relative to its absence. Thus this experiment gave them insight about substrate specificity and supported the proposal that a cysteine may be important in peptide bond cleavage. In the part b, they used triUb substrates with Lys48 linkages. The studies on the top gel (sample without any DTT) revealed that the extent of cleavage to diUb and monoUb decreased as the concentration of H_2O_2 increases. This results suggests that the active site is modified and the enzyme becomes inactive. When the same sample is treated with DTT, which can reduce the sulfenic acid of cysteine back to cysteine, then the enzyme becomes more active and the cleavage to mono and diUb products is greatly increased. This data thus supports a model where sulfenylation could be an important regulatory mechanism for DUBs. However, the key is the kinetics of this process, its occurrence in vivo and the relationship to a biological response.

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 11. 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 **(should be unmodified A20) not sulfenylated A20** is 43452 Da.

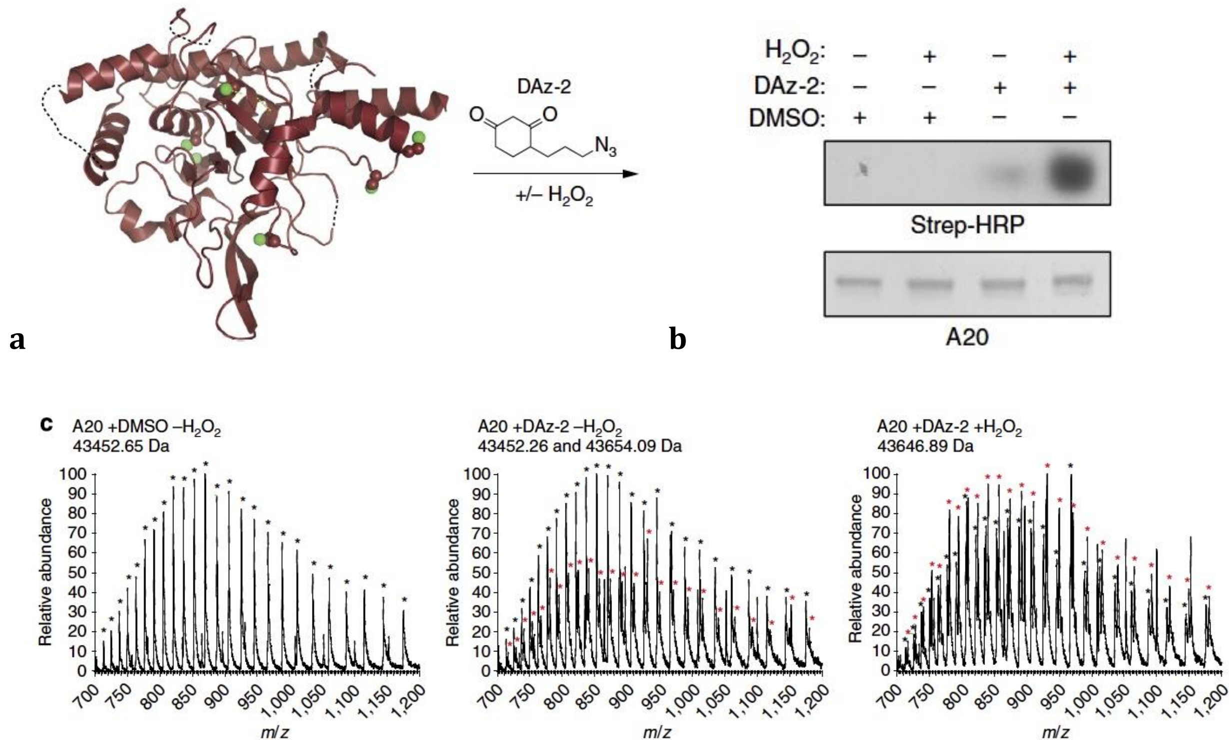

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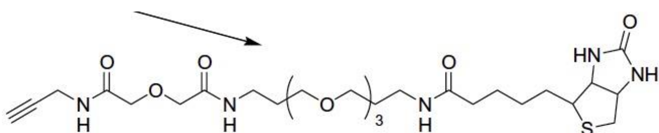
Figure 2. **a**. Structure of the truncated-A20 with the 6 detectable cysteines (green spheres). All of these studies have been done in vitro. **b**. A20 has been treated as indicated with H_2O_2 (50 μM for 60 min), DAz-2 (see structure) or DMSO (the control). The reagents are removed and the reaction 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.



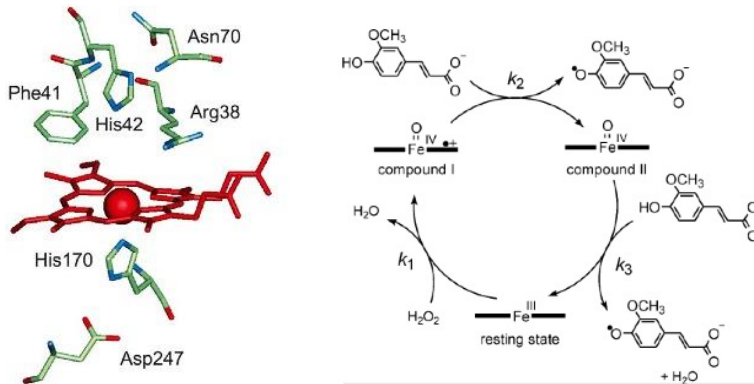
Questions:

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.

In this experiment, A20 is purified and studies are in vitro. In Figure 2b one observes a small amount of dimedone labeled-A20 even in the absence of H_2O_2. This suggests that the active site cysteine (or an allosteric site cysteine) is susceptible to oxidation (lane 3). However, after treatment with H_2O_2 (50 μM, 60 min) the band is much more intense. Two points: first the reaction with the oxidant is slow. In vivo the concentrations of H_2O_2 might spike to this concentration (high effective molarity), but would be rapidly reduced to less toxic levels. DAz-2 is able to access the cysteine (presumably the active site) and modify it, suggesting the location of the linker allows the dimedone reaction to still proceed. The observed signal (Figure 2b) is associated with the DAz-2 reacting with the acetylenic biotin above via click chemistry that forms a stable covalent linkage. The conjugate is analyzed by SDS PAGE. Detection of the biotin and hence A20 occurs using the protein Steptavidin, which forms a tight complex with biotin. Streptavidin is conjugated to horseradish peroxidase which in the presence of an oxidizable substrate such as diaminobenzidine generates a polymeric brown material which is detectable.

Digression HRP is analogous to MPO discussed in the ROS lecture 1. The substrate is either H_2O_2 or an organic peroxide (ROOH) which reacts with the Fe^{3+}-porphryin to generate a $Fe^{4+}=O$ porphryin π cation radical (compound I intermediate) which is highly oxidizing. This intermediate can then be reduced back to the ground state by two successive one electron transfers via a substrate such as a phenol or diaminobenzidine. The one electron oxidized species can polymerize (latter case) or be detected by chemiluminescence (see recitation 2/3).

Insert a mechanism (not required but is similar to the one electron chemistry with myloperoxidase.



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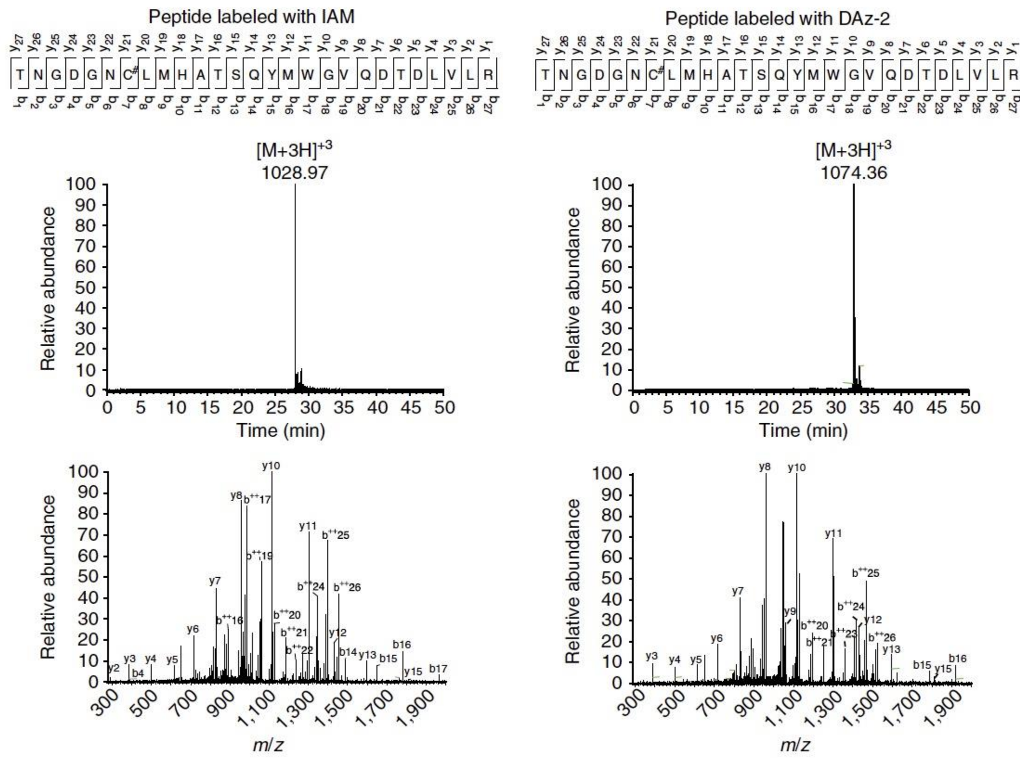
5. Explain the differences in the MS data in Figure 2c (right, middle, and left). Why are their so many peaks in each spectrum and how do these peaks together provide confidence in the molecular weight of the A20? Explain the molecular basis for the red and black * peaks.

The MS on the far left is the wt protein. You are looking at m/z that must be in the 1000 to 2000 da mass range for detection. From the different m/z peaks observed and a knowledge of the z, one can use all the information to calculate the MW of the protein. When the protein is reacted with DAz-2 one now sees an additional set of peaks (red star). You are given that the calculated mass is 202 different from the unmodified protein. This suggests that some of the cysteine in the wt protein has been modified by sulfenylation already and is detected by the dimedone reagent. Once the system is treated with H_2O_2, the extent of modification increases and is greater than the unmodified protein. The mass difference is 194. This number is very close to the expected mass of the protein modified with DAz-2 (195.2 da).

6. Describe an experiment to identify the site(s) of labeling in the sample on the right (Figure 2c).

To identify the site of labeling, the dimedone-modified protein was modified further with iodoacetamide to block the unreacted cysteines and prevent their oxidation during workup. The protein is then digested with trypsin to generate peptides. The peptides can then be examined by MS/MS analysis. We know the expected molecular weight of all the peptides given the protease specificity and the known cysteine modifications. See below but the appropriate m/z (+3) of the peptide of interest was identified and then sequenced. The sequencing allowed identification of the acetamide modified peptide in the case of the untreated A20 and of the dimedone modified protein in the case of the peroxide treated proteins.



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7. What conclusions can you draw about the importance of PTM in the A20 DUB from these in vitro studies?

The results suggest that this A20 DUB is readily sulfenylated, whether this modification plays an important role in vivo requires much more extensive characterization both in vivo and in vitro.

8. What additional controls could be carried out to make you more confident in your model?

Many experiments need to be done, this is only the beginning. One would like to show that the sulfenylation occurs on the intact protein and that it occurs at a reasonable rate given the concentrations of the protein and of H_2O_2 inside the cell. One would then like to figure out a biological assay in cells where a protein(s) can be identified that become Ubiquitinylated and have lys48 linkages and show that this specific DUB is present and alters the fate of this (these) protein (proteins). Much effort needs to be expended to convince oneself that this DUB that alters the polyUb is important in the cell before any further experiments regarding regulation of this DUB are undertaken.

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 (see the overview slide on ROS and signaling Lecture 2). 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.

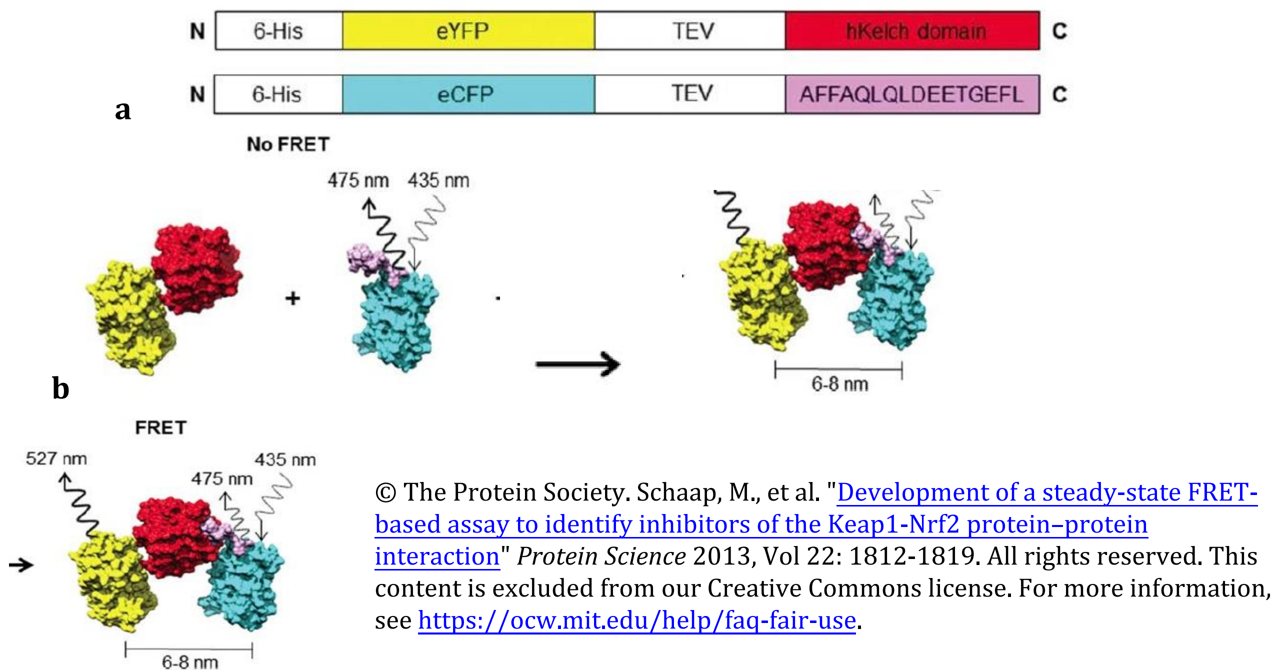

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Figure 3 **a**. Schematic representation of the domains of YFP-Kelch (top) and CFP-Nrf2 (bottom). **b**. Molecular model of the YFP-Kelch and the CFP-Nrf2 FRET pair constructed from the human Keap1 Kelch domain (red) with YFP (yellow) and the 16mer-Nrf2 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.

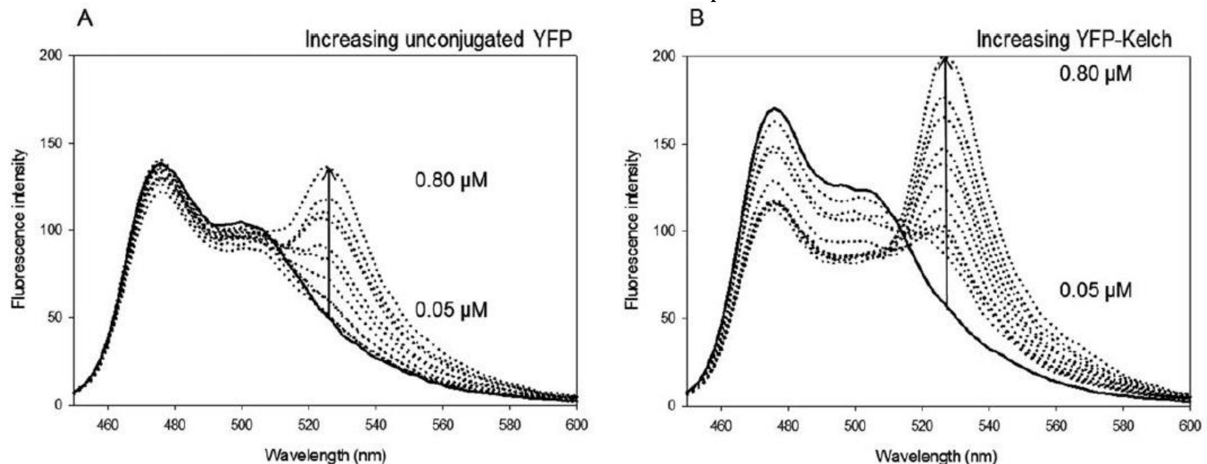

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Figure 4 Fluorescent emission spectra of direct titration of 0.11 μM CFP-Nrf2 with **A**, the unconjugated YFP or with **B**, the YFP-Kelch at increasing concentrations from 0.05 to 0.8 μM. The solid line represents the donor alone before titration.

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.

This data was taken from Protein Science 2013 22 1812-19. The details of the experiments in this problem set and additional experiments are described. Two titrations, a control with YFP alone and one conjugated to Kelch were carried out. Also in the figure are displayed the emission spectra of the acceptor and donor fusion proteins for reference. The TEV protease is a further control as cleavage at this site would result in rapid loss of FRET which does occur (data not shown). The efficiency of FRET is quantitifed by measuring the decrease in donor emission at 475 nm and an increase at 527 nm. An optimum FRET efficiency (FE) was recorded at 0.11 μM CFP-Nrf2 and 0.28 μM YFP-Kelch (FE = 0.23). The FRET signal is stable for 24 h important for assay development. The changes observed are reasonably robust and one would expect to see a loss in fluorescence with a moderately large change. If the signal is stable, then this pair could be useful in an assay.

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.

High salt (effects electrostatic interactions) and DMSO from "inhibitor addition to assist solubility" could effect interactions between this FRET pair. Thus one needs to optimize the FE given the exact conditions of the high though put assay, to maximize the chance of finding an inhibitor of this interaction.

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.

The type of control one needs to carry out is shown in Figure 5. A peptide from Nrf2 which contains the ETGE motif and has a known Ki and is a competitive inhibitor, based on other methods of analysis, serves as the control. What you see is an increase in donor emission at 475 nm and a loss of signal at 527 nm due to disruption of the interaction. One can use this change and different concentrations of the inhibitor to calculate a Ki. This Ki can be compared with one determined by other methods to establish that the assay is working as expected. If this is successful then one would like to carry out experiments in a high throughput fashion were many inhibitors can be assayed simultaneously. The concentrations in the screen are key as you want to maximize your signal, but have it disrupted with concentrations of inhibitor as low as possible.

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.

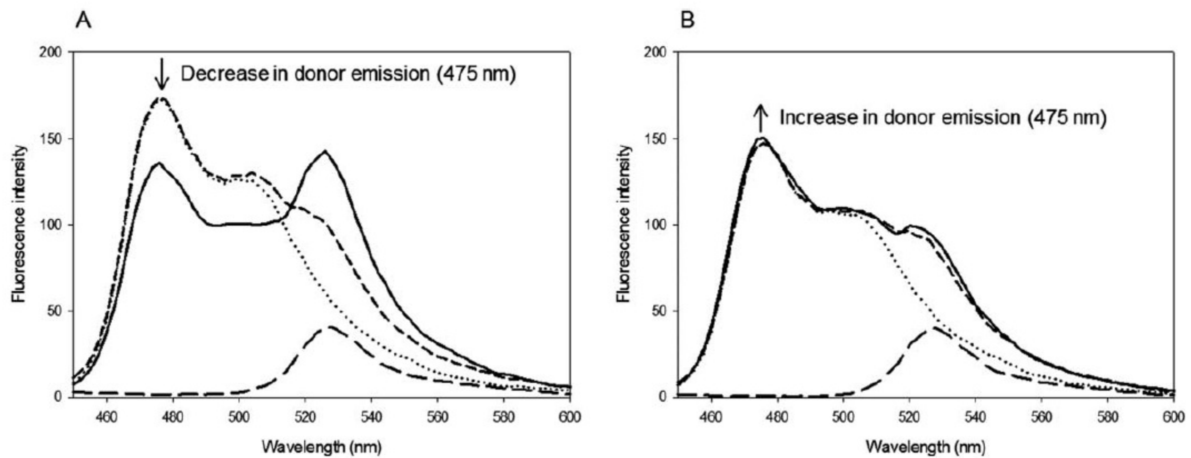

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Figure 5 Fluorescence emission spectra of YFP-Kelch and CFP-Nrf2 in the absence (A) or the presence (B) of a 10 μM unlabeled Nrf2-derived peptide inhibitor. Shown are the emission spectra of the FRET pair (solid line), donor alone (dotted line), acceptor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

Questions:

4. Explain the results shown in Figure 5.

The inhibitor disrupts the 475, 527 nm features see model above and preceding question.

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