Photo Oxidation of Proteins in Biologically Relevant Buffers

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Solvent Accessibility - an example: Inhibited Trypsin

By determining the solvent accessible surface it is theoretically possible to determine the reactive residues that are covered during ligand binding. By determining the difference map, key residues should be evident.
Solvent Accessibility - an example: Trypsin

- The four residues in green - three tyrosines and a phenylalanine have a significantly different side chain solvent accessibility by computational analysis.
- Sorry no data on this yet!
Introduction to Protein Interactions

- The ability of an organism to respond to its environment is dependent on a myriad of protein-protein interactions.
- From signal transduction across membranes, to the cyclic AMP binding protein transcriptional regulation in the nucleus, protein-protein interactions form the basis of an integrated information processing network necessary for life.

Insulin Complex - Lodish & Darnell pg 899  
cAMP-CAP - Lodish & Darnell pg 354
Methods to Map Protein-Protein Interactions

- X-Ray crystallography is the best known technique, capable of rendering three-dimensional representations in atomic detail.
- NMR is capable of modeling 3D structures with distance restraint data, however, the size of the technique is mostly limited to proteins <30kDa for physical reasons.
Methods to Map Protein-Protein Interactions

- Mutational analysis of a protein can determine the residues responsible for the stability of the complex. This technique gives a global $\Delta G$ of binding.
- However, a residue that modifies the $\Delta G$ may be necessary for folding, or structure of the protein, and could have no other effect on the interface.

Double Mutant DHFR - a mutation 15 Å from the binding pocket compensates for the first mutation in the binding pocket.
Mapping Protein-Protein Interactions by MS

- All Mass Spectrometry techniques have been based on a modification of the protein MW based on solvent accessibility.
- *DTSSP modification of exposed 1° amines*
- *DCC Activation and modification of exposed carboxylates*
- *Kinetics of backbone amide H/D exchange*
Oxidation Methods

- **Synchrotron Radiation:**
  - High energy X-rays split water generating H\(^+\) and HO\(^-\).

- **Electrospray Oxidation:**
  - High voltage across the capillary generates HO\(^-\).

- **Fenton Reagent:**
  - Fe\(^{2+}\): EDTA with ascorbic acid generates HO\(^-\). However, the Iron EDTA complex must be near a reactive residue for the reaction to occur. This causes uncertainties in the data.

- **Photochemical Oxidation:**
  - UV light excites the O–O bond, causing it to split heterolytically - generating two HO\(^-\). While the radical needs to be generated close to the reactive residue the peroxide is much smaller than Iron:EDTA and its properties are very similar to water.

- **Sharp et al.** 15% H\(_2\)O\(_2\), 50mM NaH\(_2\)PO\(_4\), 150mM NaCl, 5 minutes using a Stratagene cross-linker.

\[
\text{HO} \quad \text{UV} \quad 2 \text{HO}^- 
\]
Alternate Conditions for Photochemical Oxidation

- Use a medium pressure mercury arc lamp - such as are used in nucleotide mutation studies (MLGross).
- Air cool the samples to remove UV blocking glass from the light path.
- Insert the samples through the lid after the lamp has warmed up for 10 minutes - it has reached maximum UV output (~33 mwatts <300nm and 48.4 mwatts <400nm)
Alternate Conditions for Photochemical Oxidation

- **Solution Conditions:**
  - 10uM Apomyoglobin (iron in the heme group causes the Fenton Reaction)
  - 2mM TrisHCl, pH = 7.0
  - 5mM H$_2$O$_2$ This is 1000x less peroxide than used by Sharp et al.
  - Total Volume = 100uL → 1nmole of protein. Only 1uL is needed per injection by electrospray Our total volume is 20x less than used by Sharp et al.

- **Post-Oxidation Purification:**
  - To remove the H$_2$O$_2$ from the solution after oxidation using a desalting column

![Graphs showing UV no H$_2$O$_2$ or H$_2$O$_2$ but no UV and 5mM H$_2$O$_2$](image-url)
Desalting Protocol

- G-25 sephadex beads hydrated in 1mM NH4HCO3 pH = 7.6
- I have desalted >250mM NaCl in 3 steps with sufficient protein recovery for analysis.

Sample - 100uL of 10uM → 100uM = 1nmole → 10nmole

Peroxide stays in column cf. 2500rpm

Protein located in Flowthrough

90uL for Digestion <900pmol

Add Trypsin, O/N incubation at 37°C

10uL for LCMS <90pmol

Check whole MW by MS

Check peptide MW by MS
Apomyoglobin Sequence - a model protein

- GLSDGEWQQV LNVWGKVEAD IAGHGQEVLIRLFTGHPETLEKFDKFKHLLKTEAEMKASEDLKHHGTVVLTALGGILKKG HHEAELKPLAQSHATHKHIP
  IKYLEFISDA IHIHLHSKHPGDFGADAQGAMTKALELFRODIAAKYKELGFOQ

Chart from Sharp et al.
Increased Oxidation - 0mM or 5mM $H_2O_2$, UV

- Apomyoglobin with 5mM $H_2O_2$ but no UV light
- Note the presence of a weak mono oxidation

- Apomyoglobin with 5mM $H_2O_2$ and 5 minutes of UV light
- Note multiple oxidations of the protein
Tryptic Digest Comparison of Apomyoglobin

Oxidized

Control
Oxidation of 1502 - Met 131

8Da x +2 charge = 16Da difference
Oxidation 1815 - Trp 7 & probably Leu 11

8Da x +2 charge = 16Da difference

16Da x +2 charge = 32Da difference

24Da x +2 charge = 48Da difference
Oxidation of 1885 - Phe 106 or Tyr 103

5.3Da \times +3 \text{ charge} = 16\text{Da difference}
Sequencing of an Oxidized Tryptic Peptide

New Peak at +8 m/z, z = 2; So this is +16
Sequencing of [M+H+16]^+ and [M+H]^+ Peptides
Apomyoglobin - Slab View

- Myoglobin - 1WLA
- Heme in Green
- Tryptophan, Tyrosine, Phenylalanine, Methionine in Red

Tryptophan 7 Side Chain Solvent Exposed Area = 14.8 Å²

Tryptophan 14 Side Chain Solvent Exposed Area = 4.1 Å²
Apomyoglobin Oxidation Summary

Potential Oxidation Sites

Chart from Sharp et al.
Lysozyme Conditions

- **100uM Lysozyme in 100mM NaH$_2$PO$_4$ 150mM NaCl**
  - This is a suitable buffer for an activity assay.
- **After 5 minutes of oxidation with varying amounts of peroxide we saw significant oxidation.**

0mM lysozyme

5mM lysozyme

50mM lysozyme
Lysozyme Whole Spectrum

Lyso1A 39 (1.395)

Control

Oxidized
50mM H$_2$O$_2$

TOF MS ES+ 1.04e4

TOF MS ES+ 2.32e4
Lysozyme Oxidation

Control
no H$_2$O$_2$

Oxidized with
50mM H$_2$O$_2$
Lysozyme Oxidation: Identified Peaks

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<th>m/z</th>
<th>Z</th>
<th>[M+H]^+</th>
<th>Detected in</th>
<th>Sequence</th>
<th>aa#’s</th>
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<td>50mM 100% Ox.</td>
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- KVFGRCCEAA AMKRHGLDNY RGYSLGNWVCA AAKFESNFNT QATNRN TDGTS
  TDYGILQINS RW WDGRTP GSRNLCNI PIC SALLSDITA SVNCAKK IVS
  DGNGMNAWVA WR NRCK GTDV QA WIR GCRL

- 58% Sequence coverage
Radical Scavengers in Hydroxyl Radical Production

• Maleknia et al. - synchrotron radiation technique:
  – In addition, buffers can act to different degrees as radical scavengers. The standard buffer is sodium cacodylate, which does not interfere with radiolysis chemistry. Urea and guanidine hydrochloride can also be used as denaturants that do not appreciably scavenge radicals. We have found that Tris and EDTA have significant radical scavenging properties. Higher exposure times are required under these experimental conditions to balance the scavenging property of these reagents.

• Heyduk et al. - the Fenton chemistry technique:
  – … MOPS is the most often used buffer for this cleavage reaction in our laboratory. Other buffers will also work unless they effectively scavenge hydroxyl radicals (as, for example Tris and glycerol).
Are “Radical Scavenging” Buffers Incompatible?

BES 50MM  34 (1.219) TOF MS ES+ 2.35e4
BICINE 50MM  31 (1.111) TOF MS ES+ 5.32e4
EEPS 50MM  35 (1.254) TOF MS ES+ 2.71e4
MOPS 50MM  35 (1.254) TOF MS ES+ 5.73e4
PIPES 50MM  37 (1.326) TOF MS ES+ 3.09e4
TAPS 50MM  30 (1.077) TOF MS ES+ 3.81e4
Conclusions

• Photochemical oxidation of proteins can be done under much gentler conditions compared to the previously published method.
• Photochemical oxidation generates, so far, data in complete accord with known solvent accessibility of residues on well characterized proteins.
• Photochemical oxidation can easily handle the presence of “radical scavenging” buffers, an important development not previously reported in other publications.
• A method has been developed to modify proteins using only 5mM hydrogen peroxide in 5 minutes using a medium pressure mercury lamp setup that can be purchased for less than $2500.
• A post-reaction method has been developed enabling MS interfering contaminants to be removed prior to either MS analysis or protease digestion.
References and Thanks

• Dr. Michael Gross, Amber Russell and Jonathan Chang
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