Virtual Free Radical School

Protein Oxidation: A primer on characterization, detection, and consequences

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What is protein oxidation?

Covalent modification of a protein induced by reactive oxygen intermediates or by-products of oxidative stress.

Agents that lead to protein oxidation

- Chemical Reagents
  (H_2O_2, Fe^{2+}, Cu^{1+}, glutathione, HOCl, HOBr, $^1O_2$, $ONOO^-$)
- Activated phagocytes (oxidative burst activity)
- γ-irradiation in the presence of O_2
- UV light, ozone
- Lipid peroxides (HNE, MDA, acrolein)
- Mitochondria (electron transport chain leakage)
- Oxidoreductase enzymes
  (xanthine oxidase, myeloperoxidase, P-450 enzymes)
- Drugs and their metabolites

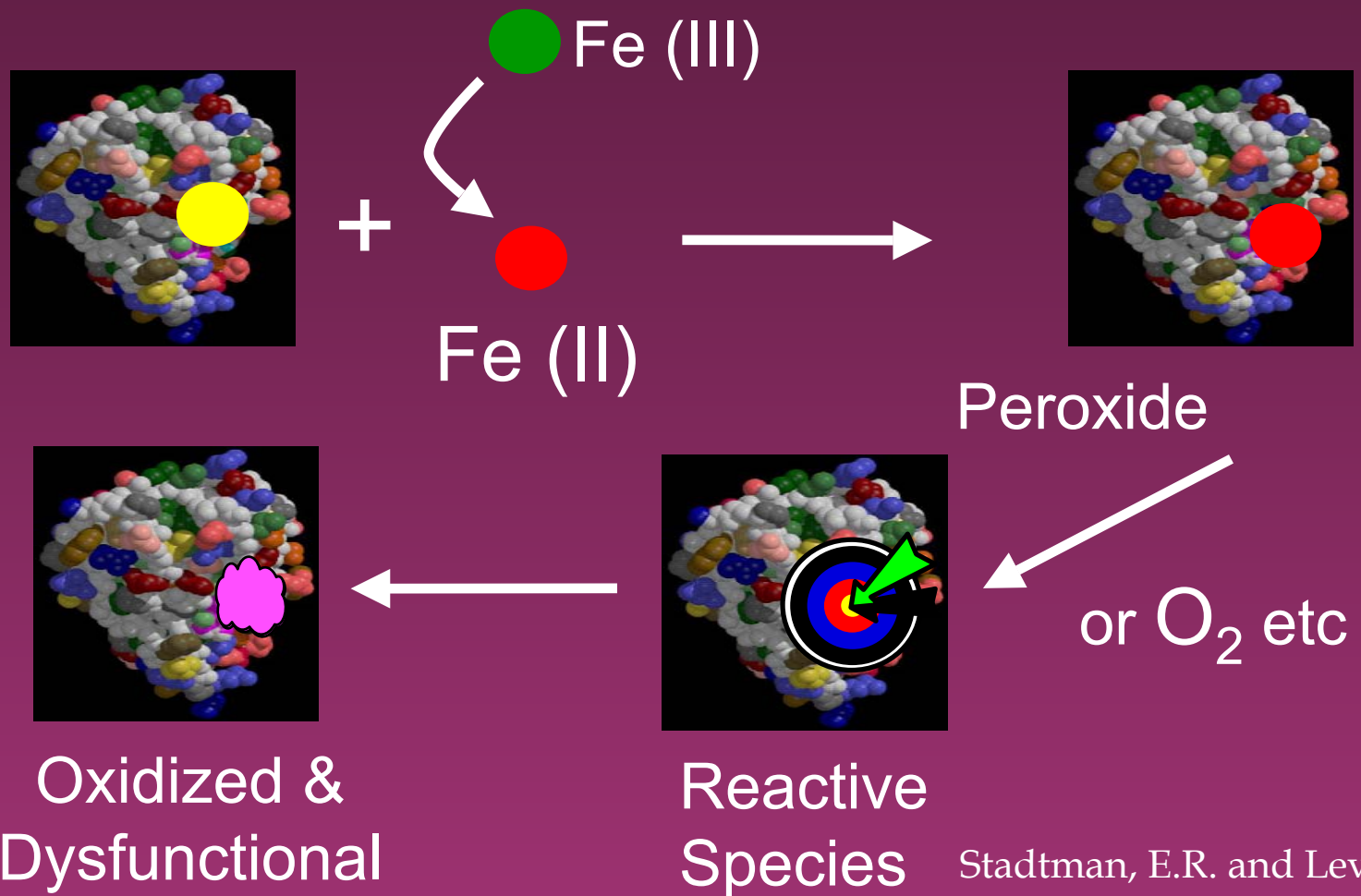
General types of protein oxidative modification

- Sulfur oxidation (Cys disulfides, S-thiolation; Met sulfoxide)
- Protein carbonyls (side chain aldehydes, ketones)
- Tyrosine crosslinks, chlorination, nitrosation, hydroxylation
- Tryptophanyl modifications
- Hydro(pero)xy derivatives of aliphatic amino acids
- Chloramines, deamination
- Amino acid interconversions (*e.g.*, His to Asn; Pro to OH-Pro)
- Lipid peroxidation adducts (MDA, HNE, acrolein)
- Amino acid oxidation adducts (*e.g.*, *p*-hydroxyphenylacetaldehyde)
- Glycoxidation adducts (*e.g.*, carboxymethyllysine)
- Cross-links, aggregation, peptide bond cleavage

Amino acids most susceptible to oxidation and their main reaction products

Amino Acid	Physiological oxidation products
Cysteine	Disulfides, mixed disulfides (*e.g.*, glutathiolation), HNE-Cys
Methionine	Methionine sulfoxide
Tyrosine	Dityrosine, nitrotyrosine, chlorotyrosines, dopa
Tryptophan	Hydroxy- and nitro-tryptophans, kynurenines
Phenylalanine	Hydroxyphenylalanines
Valine, Leucine	Hydro(pero)xides
Histidine	2-Oxohistidine, asparagine, aspartate, HNE-His
Glutamyl	Oxalic acid, pyruvic acid
Proline	Hydroxyproline, pyrrolidone, glutamic semialdehyde
Threonine	2-Amino-3-ketobutyric acid
Arginine	Glutamic semialdehyde, chloramines
Lysine	a-Aminoadipic semialdehyde, chloramines, MDA-Lys, HNE-Lys, acrolein-Lys, carboxymethyllysine, pHA-Lys

Reaction scheme showing how metal-catalyzed protein oxidation is a site-specific process



Stadtman, E.R. and Levine, R.L. (2000) *Ann. N.Y. Acad. Sci.* **899**, 191-208

Biochemical consequences of protein oxidative modification

- Loss or gain of enzyme activity
- Loss of protein function (*e.g.*, fibrinogen/fibrin clotting)
- Loss of protease inhibitor activity
  (*e.g.*, α -1-antitrypsin, α 2-macroglobulin)
- Protein aggregation (*e.g.*, IgG, LDL, a-synuclein,
  amyloid protein, prion protein)
- Enhanced susceptibility to proteolysis (*e.g.*, IRP-2, HIF-1 α,
  glutamine synthetase)
- Diminished susceptibility to proteolysis
- Abnormal cellular uptake (*e.g.*, LDL)
- Modified gene transcription (*e.g.*, SoxR, IkB)
- Increased immunogenicity (*e.g.*, ovalbumin; HNE- or acrolein-LDL)

Diseases and conditions in which protein oxidation has been implicated and specific target proteins, if known

- Atherosclerosis (LDL)
- Rheumatoid arthritis (IgG, α-1-proteinase inhibitor)
- Ischemia reperfusion injury
- Emphysema (α -1-proteinase inhibitor, elastase)
- Neurodegenerative diseases
  - Alzheimer's (β-actin, creatine kinase)
  - Parkinson's
  - Sporadic amyotrophic lateral sclerosis
- Muscular dystrophy
- Neonates on ventilators; bronchopulmonary dysplasia
- Adult respiratory distress syndrome
- Aging (glutamine synthetase, carbonic anhydrase III, aconitase)
- Progeria
- Acute pancreatitis
- Cataractogenesis (alpha-crystallins)
- Chronic ethanol ingestion
- Cancer

How can we inhibit protein oxidation?

- Antioxidants
  - scavengers (probucol, spin traps, methionine)
  - antioxidant enzymes (catalase, SOD, peroxiredoxins)
  - antioxidant enzyme mimics (ebselen, Tempol, TBAPS)
  - augmentation of cellular antioxidant systems
    - N-acetylcysteine ($\rightarrow \uparrow$intracellular GSH)
- Chelators (DTPA, Desferal®)
- Depletion of O_2

Advantages and disadvantages of using proteins as markers of oxidative stress

- There is no single universal marker for protein oxidation.
  - With so many different potential reaction products, may need to do several different assays if source of oxidants unknown
  - If source of oxidation is known, the range narrows (*e.g.*, metal-catalyzed oxidation does not cause chlorination or nitrosation, and HOCl does not cause lipid peroxidation adducts)

Advantages and disadvantages of using proteins as markers of oxidative stress

- Products are relatively stable
- Types of modification reveal nature of oxidizing species
  - chlorotyrosine from HOCl
  - nitrotyrosine from $^{\bullet}NO + O_2^{\bullet -}$ or HOCl
  - glutamic and aminoadipic semialdehydes from metal-catalyzed oxidation
- Have unique physiological consequences due to the specificity of protein functions
- Sensitive assays are available (detecting <1 pmol of oxidized product)

Advantages and disadvantages of using proteins as markers of oxidative stress

- Different forms of oxidative modification have different functional consequences
  - Met is highly susceptible but oxidation often does not affect protein function
  - Carbonyls are often associated with dysfunction but may require more stringent oxidative conditions

Advantages and disadvantages of using proteins as markers of oxidative stress

- Proteins, lipids, and DNA are modified by different oxidants to different degrees

  e.g., HOCl generated by myeloperoxidase hits

  protein >> lipids >> DNA

  e.g., H_2O_2 treatment of cells hits

  DNA ≥ lipids >> proteins

Methods for detection of oxidative protein modifications[#]

Modification	Methods of Detection
Disulfides	SDS-gel electrophoresis ± ß-ME
	DTNB
	Reaction with BIAM*[&] or MPB* → SDS-PAGE
Glutathiolation	RP-HPLC/mass spectrometry
	S^{35}-Cys/Chx → SDS-PAGE
	Biotinylated glutathione ethyl ester[$]
Methionine sulfoxide	CnBr cleavage/amino acid analysis
Carbonyls	DNPH**-coupled assays:
	Spectroscopy
	HPLC
	Western blotting
	ELISA
	Immunohistochemistry
	Reduction with NaB^3H_3

[#] See Table 4 in Shacter, E. (2000) *Drug Metab. Rev.* **32**, 307-326. [&] Kim *et al*. (2000) *Anal. Biochem.* **283**, 214-221; [$] Sullivan *et al*. (2000) *Biochemistry* **39**, 11121-11128.

* Biotinylated iodoacetamide or maleimido-propionyl biocytin; **, Dinitrophenylhydrazine

Methods for detection of oxidative protein modifications, con[#]

Modification	Methods of Detection
2-oxo-His	Amino acid analysis
Dityrosine	Fluorescence Proteolysis or hydrolysis → HPLC
Chlorotyrosine	Hydrolysis/nitroso-naphthol/HPLC HBr hydrolysis → GC/MS
Nitrotyrosine	Immunoassay Hydrolysis → HPLC HPLC/electrochemical detection
Tryptophanyl modifications	Fluorescence Amino acid analysis (alkaline hydrolysis) Proteolysis/MS
Hydroperoxides	KI/I_3^-/spectroscopy Na□BH_4/hydrolysis/OPA-HPLC

[#] See Table 4 in Shacter, E. (2000) *Drug Metab. Rev.* **32**, 307-326.

Methods for detection of oxidative protein modifications, con[#]

Modification	Methods of Detection
Lipid peroxidation adducts	Immunoassays DNPH $Na\square BH_4$/hydrolysis/OPA-HPLC Hydrolysis → GC/MS
Amino acid oxidation adducts	$NaCNBH_3$ reduction/hydrolysis /H^1-NMR/MS
Glycoxidation adducts	Derivitization → GC/MS
Cross-links, aggregates, fragments	SDS-gel electrophoresis HPLC
Thiyl radicals	ESR

[#] See Table 4 in Shacter, E. (2000) *Drug Metab. Rev.* **32**, 307-326.

A little more about protein carbonyls

- Carbonyl groups are stable (aids detection and storage)
- Present at low levels in most protein preparations
  (~1 nmol/mg protein ~ 0.05 mol/mol ~ 1/3000 amino acids)
- See 2- to 8- fold elevations of protein carbonyls under conditions of oxidative stress *in vivo*
- Induced *in vitro* by almost all types of oxidants (site-specific metal catalyzed oxidation, γ-irradiation, HOCl, ozone, $^1O_2$, lipid peroxide adducts)
- Sensitive assays are available (≤ 1 pmol)

Amino acids that undergo metal-catalyzed oxidation to form carbonyl products

- Proline (γ-glutamylsemialdehyde)
- Arginine (γ-glutamylsemialdehyde)
- Lysine (amino-adipicsemialdehyde)
- Threonine (amino-ketobutyrate)

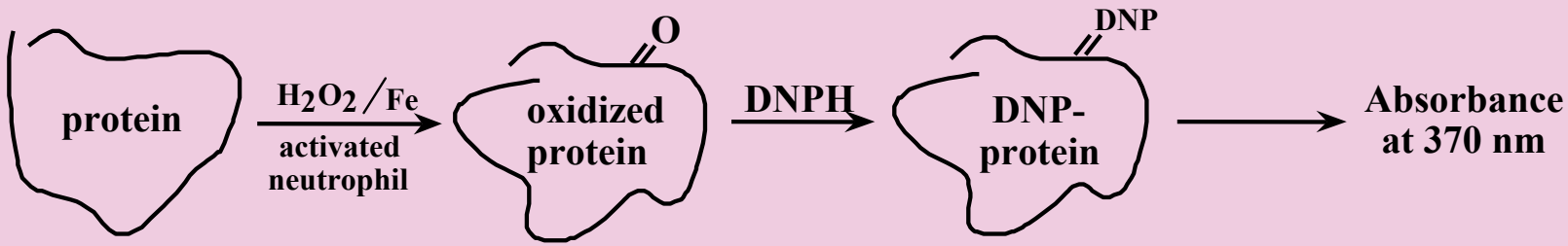
Detection of protein carbonyls

- Measure total protein carbonyls levels after reaction with DNPH* followed by spectroscopy (A370), ELISA, or immunohistochemistry

- Measure carbonyl levels in individual proteins within a mixture of proteins (tissue samples, cell extracts) by reaction with DNPH followed by Western blot immunoassay

*DNPH, dinitrophenylhydrazine

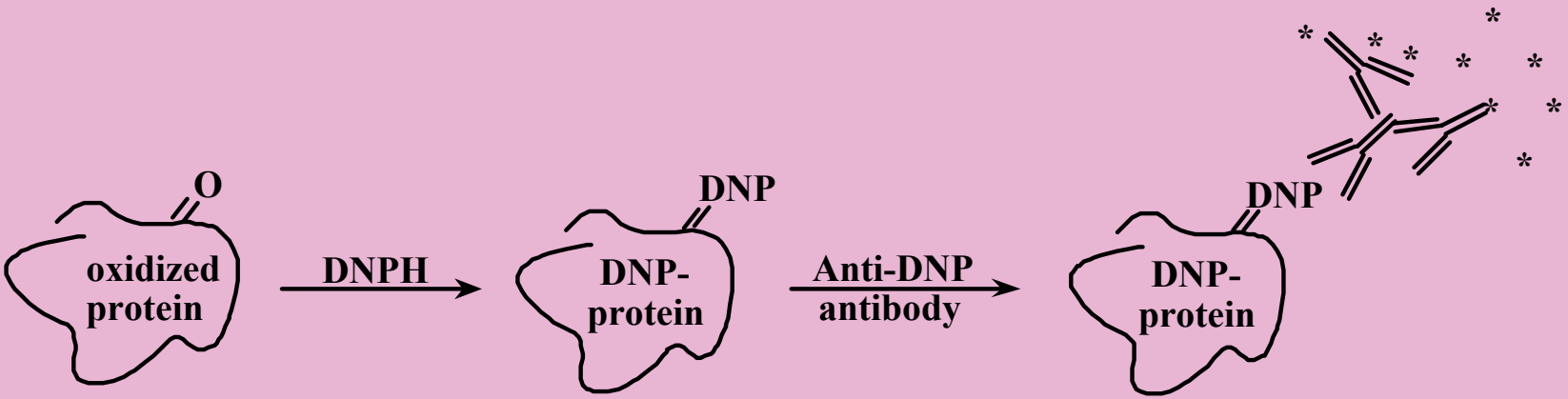
Measurement of total carbonyls (Spectrophotometric DNPH assay)



e.g. arg ---> γ-glutamylsemialdehyde

Dinitrophenylhydrazone-protein

Immunoassays for protein carbonyls



***e.g.*, Western blot, ELISA, immunohistochemistry**

Western blot assay for protein carbonyls

- Detects individual oxidized proteins within a mixture of proteins
- Requires ~ 50 ng of protein
- Sensitivity of ≤1 pmol of protein carbonyl
  - ~50 ng of a 50 kDa protein oxidized @ 0.5 mol/mol
- Reveals differential susceptibility of individual proteins to oxidative modification*

* Shacter *et al*. (1994) *Free Radic. Biol. Med.* **17**, 429-437

Notes

- Carbohydrate groups of glycoproteins do not contribute to carbonyl levels*
- Free aldehyde groups from lipid peroxidation adducts (*e.g.*, MDA) can react with DNPH
  - » Adduct needs to be stable
  - » if reduction with $NaBH_4$ is required to stabilize the adduct, DNPH reactivity will not be seen
- Western blot assay is only semi-quantitative
  - » use titration to estimate carbonyl content**

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*Lee, Y-J. and Shacter, E. (1995) *Arch. Biochem. Biophys.* **321**, 175-181

** Shacter, E. *et al.* (1994) *Free Radic. Biol. Med.* **17**, 429-437

Reagents and equipment*

- 20 mM DNPH in 20% trifluoroacetic acid (TFA)
- 24% SDS in water
- Neutralizing solution (2M Tris/30% glycerol ± 20% β-ME)
- Sample protein(s)
- Oxidized and native protein samples
- SDS-gel electrophoresis and Western blotting apparatus and conventional solutions
- Anti-DNP antibody (Sigma D-8406, IgE)
- Rat anti-mouse IgE, conjugated for immunoassay detection (biotin, HRP)

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* See Shacter (2000) *Meth. Enzymol.* **319**, 428-436 or
Levine, R.L., Williams, J., Stadtman, E.R., and Shacter, E. (1994) *Meth. Enzymol.* **233**, 346-357

Technical Pointers

- Can be used on cell and tissue extracts
- **Dissolve the DNPH in 100% TFA and then dilute with H_2O**
- Total protein bands can be visualized with Amido black stain after washing the blot
- Always run positive and negative controls
  - » internal standards of oxidized and non-oxidized control protein
  - » adjust exposure time if doing chemiluminescence
- Run controls without DNPH or primary antibody
  - » to establish specificity

Other DNPH immunoassays for protein carbonyls

ELISA

Buss *et al*. (1997) *Free Radic. Biol. Chem*. **23**, 361-366

2D gel electrophoresis/immunoblotting

Yan *et al*. (1998) *Anal. Biochem*. **263**, 67-71

Immunohistochemistry

Smith *et al*. (1998) *J. Histochem. Cytochem*. **46**, 731-735

A little more about protein sulfur group oxidations

- In general, Cys and Met are the amino acids that are most susceptible to oxidation
- Distinguished from other oxidative protein modifications in that cells have mechanisms to reverse the oxidation

  e.g., methonine sulfoxide reductase

  e.g., glutathione or thioredoxin redox systems
- Hence may serve a regulatory function
- Reversible oxidation/reduction of methionine may protect proteins from more damaging forms of oxidative modification (*e.g.*, carbonyl formation)*

* Stadtman, E. R., Moskovitz, J., Berlett, B. S., and Levine, R. L. (2002) *Mol. Cell. Biochem.* **234-235**, 3-9

A little more about HOCl-induced protein oxidation

- Primary products are chloro- and di-tyrosyl residues, amino acyl aldehyde adducts, and chloramines
- Represent unique products of myeloperoxidase activity, reflecting neutrophil and monocyte activity
- Serve as markers for oxidants generated as part of the inflammatory response
- Are elevated in atherosclerotic plaques
- Can be detected with sensitive and specific assays

See Heinecke, J.W. (2002) *Free Radic. Biol. Med.* **32,** 1090-1101
Winterbourne, C.C. and Kettle, A.J. (2000) *Free Radic. Biol. Med.* **29**, 403-409
Hazell, L.J. *et al.* (1996) *J. Clin. Invest.* **97**, 1535-1544

A little more about lipid peroxidation adducts

- Indirect oxidative protein modification through attachment of lipid peroxidation breakdown products (*e.g.*, hydroxynonenal, malondialdehyde, acrolein) to Lys, Cys, and His residues in proteins
- Generated by a variety of oxidizing systems, predominantly metal-catalyzed oxidation and γ-irradiation
- Elevated in atherosclerosis and neurodegenerative diseases
- Detected with immunoassays specific for each type of protein adduct

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See Uchida, K. (2000) *Free Radic. Biol. Med.* **28**, 1685-1696

Some recent review articles on protein oxidation

1. Stadtman, E. R., and Levine, R. L. (2000) Protein oxidation. *Ann N Y Acad Sci* **899**, 191-208
2. Shacter, E. (2000) Quantification and significance of protein oxidation in biological samples. *Drug Metab. Rev.* **32**, 307-326
3. Davies, M. J., Fu, S., Wang, H., and Dean, R. T. (1999) Stable markers of oxidant damage to proteins and their application in the study of human disease. *Free Radic Biol Med* **27**, 1151-1163
4. Sayre, L. M., Smith, M. A., and Perry, G. (2001) Chemistry and biochemistry of oxidative stress in neurodegenerative disease. *Curr Med Chem* **8**, 721-738
5. Winterbourn, C. C., and Kettle, A. J. (2000) Biomarkers of myeloperoxidase-derived hypochlorous acid. *Free Radic Biol Med* **29**, 403-409
6. Baynes, J. W., and Thorpe, S. R. (2000) Glycoxidation and lipoxidation in atherogenesis. *Free Radic Biol Med* **28**, 1708-1716
7. Greenacre, S. A., and Ischiropoulos, H. (2001) Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res* **34**, 541-581
8. Shringarpure, R., Grune, T., and Davies, K.J. (2001) Protein oxidation and 20S proteasome-dependent proteolysis in mammalian cells. *Cell Mol Life Sci* **58**, 1442-1450