Protein oxidation: concepts, mechanisms and new insights

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Outline of talk

- **Mechanisms of oxidation of proteins by radical oxidants.**
- **Mechanisms of oxidation of proteins by non-radical oxidants**
- **Secondary reactions of reactive protein oxidation intermediates.**
- **Products of protein oxidation.**
- **Protein oxidation products as potential markers of disease.**

Reactions of oxidants with biological targets

Oxidants can damage virtually all biological molecules: DNA, RNA, cholesterol, lipids, carbohydrates, proteins and antioxidants.

Extent of damage to particular targets depends on a number of factors including:

- **concentration of target,**
- **rate constant for reaction of oxidant with target**
- **location of target versus oxidant**
- **occurrence of secondary damaging events**
- **occurrence of transfer reactions**
- **repair and scavenging reactions**

Biochem. J., 1997, 324, 1-18; Biochim. Biophys. Acta, 2001, 1504, 196-219

Why concentrate on proteins ?

1) Concentration of target

Proteins are <u>major</u> components of most biological systems:

Organ level (liver, per kg wet weight):
146 g protein, 2.6 g DNA, 49 g total lipid, 3.9 g cholesterol.

Cellular level (per 10^{12} leukocytes):
100 g protein, 6.9 g DNA, 8.2 g RNA, 15.6 g total lipid, 2 g cholesterol.

Plasma (per dm^3):
73 g protein, 0.4 g free amino acids, 0.5 g total lipid, 1 g carbohydrates, 1.5 - 2.5 g cholesterol.

Low-density lipoproteins (molecules per particle):
1 protein (4535 amino acids), 1600 cholesterol esters, 700 phospholipids, 600 free cholesterol, 26 free fatty acids, 9 tocopherol.

Why concentrate on proteins ?

2) Rate constants for reaction

Rate constants for reaction of $HO^{?}$ with macromolecules:

DNA	**8×10^{8} dm^3 mol^{-1} s^{-1}**
RNA	**1×10^{9} dm^3 mol^{-1} s^{-1}**
Hyaluronan	**7×10^{8} dm^3 mol^{-1} s^{-1}**
Linoleic acid	**9×10^{9} dm^3 mol^{-1} s^{-1}**
Collagen	**4×10^{11} dm^3 mol^{-1} s^{-1}**
Albumin	**8×10^{10} dm^3 mol^{-1} s^{-1}**

Antioxidants:

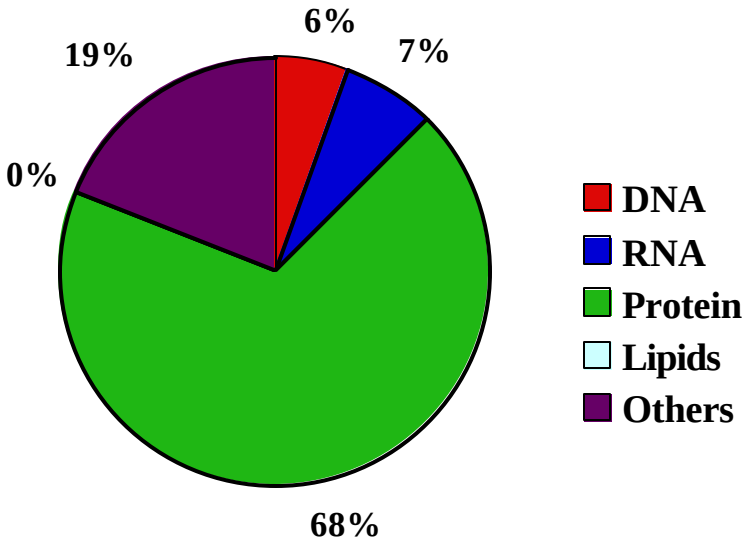
Ascorbate	**1×10^{10} dm^3 mol^{-1} s^{-1},**
GSH	**1.4×10^{10} dm^3 mol^{-1} s^{-1},**
Trolox C	**6.9×10^{9} dm^3 mol^{-1} s^{-1}**

Why concentrate on proteins ?

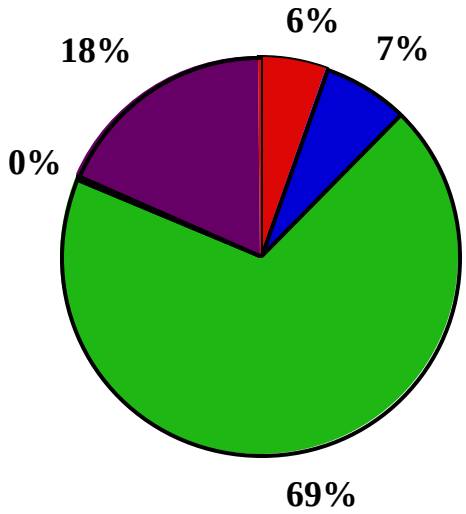
Kinetic and abundance data can be used to predict sites of damage.

For leukocytes:

Singlet oxygen



HO$^{?}$



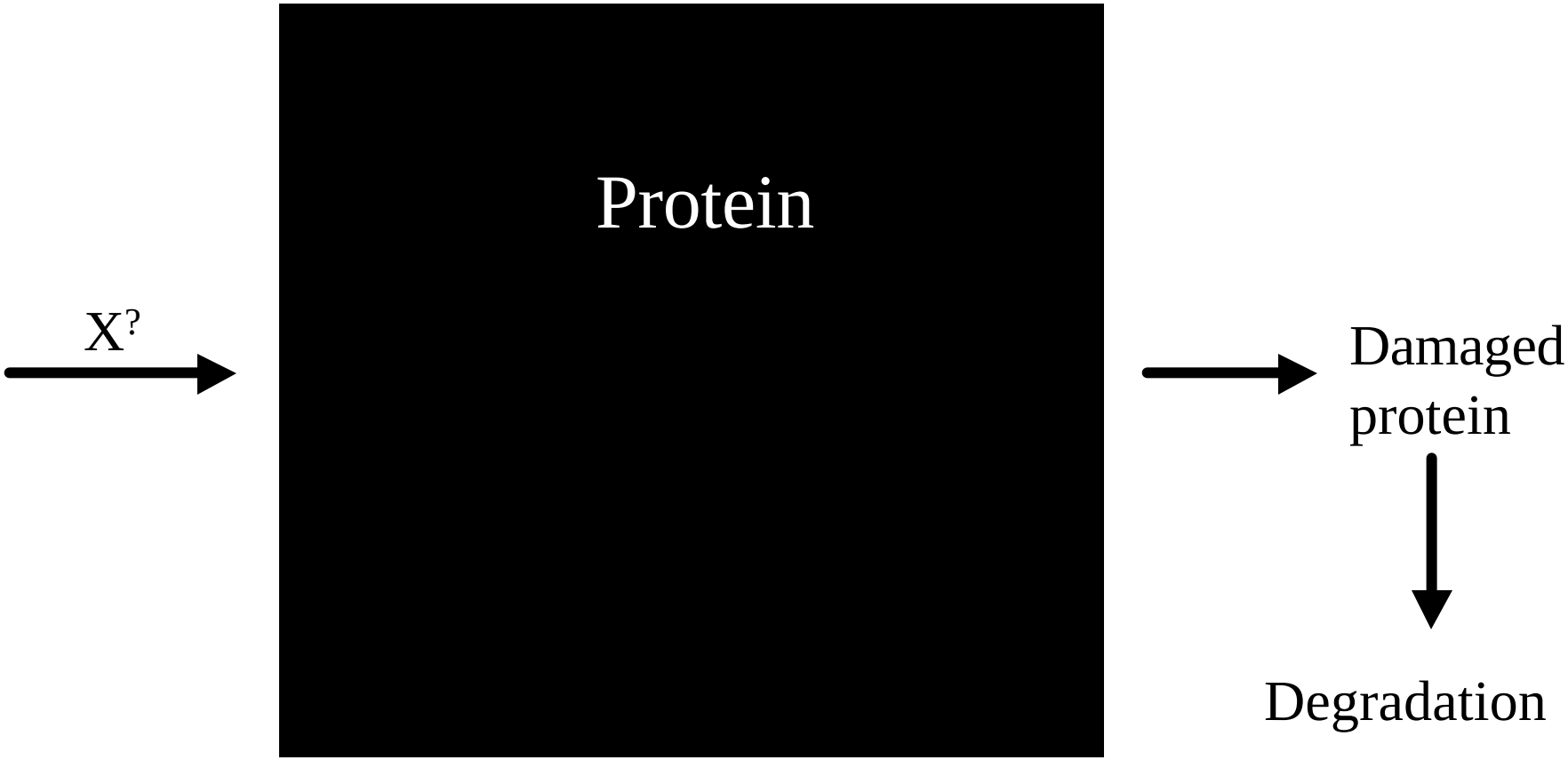
Such data needs to be treated with great caution !

Protein versus DNA versus lipid oxidation

- **Medline / Pubmed searches**
  - **DNA oxidation - 8894**
  - **Lipid peroxidation - 26041**
  - **Protein oxidation - 5463**

- **Oxidation of proteins studied to a much lesser extent than other targets**

Protein oxidation in the 1960's



One radical = one damaged amino acid residue

Why is protein oxidation the poor cousin ?

Not due to a late start - first reports around 1900

- **First volume of J. Biol. Chem.**

Dakin, H.D. (1906) "The oxidation of amido-acids with the production of substances of biological importance" J. Biol. Chem., 1, 171-176.

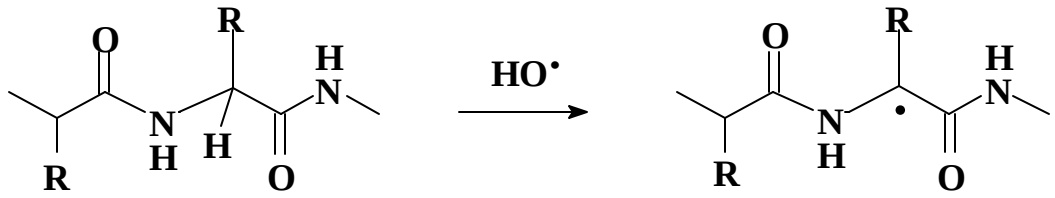
Follow-up papers in 1908, occupied approximately half of the total page-count of J. Biol. Chem. for the entire year.

Why is protein oxidation the poor cousin ?

- **Complexity of targets ?**
- **Complexity of products ?**
- **Complexity of mechanisms ?**
- **Lack of sensitive, stable, readily detectable markers of damage ?**

Sites of oxidant damage on proteins

- **Backbone - primarily hydrogen atom abstraction at alpha carbon**



- can result in backbone fragmentation

- **Side-chains - 20 different types (excluding unusual amino acids and any post-translational modifications).**
  - **hydrogen abstraction - primarily with aliphatic**
  - **addition - primarily with aromatic**

- usually results in the formation of altered side-chains

Chem Rev, 1987, 87, 381-398; Free Rad Biol Med, 1990, 9, 315-325; J Biol Chem, 1987, 262, 9895-9920

Selectivity of damage by different oxidants

- **The most reactive radicals tend to be the least selective**

  e.g $HO^{?}$ - difference in rate constants is relatively small

  Most reactive: Trp, Tyr, His, Met, Cys, Phe, Arg: *k ca.* 10^{10} dm^3 mol^{-1} s^{-1}

  Least reactive: Ala, Asp, Asn: *k ca.* 10^7 - 10^8 dm^3 mol^{-1} s^{-1}

  Result - most side-chains are oxidised

  Reaction with backbone sites *k ca.* 10^9 dm^3 mol^{-1} s^{-1}

  Significant backbone fragmentation as well as side-chain oxidation

- **Less reactive radicals tend to be more selective**

  e.g. $CCl_3OO^{?}$ - difference in value of rate constants between most reactive side-chain (Trp *k ca.* 9 x 10^7 dm^3 mol^{-1} s^{-1}) and least (aliphatic side-chains - no measurable reaction) very large.

- **Many radicals are electron-deficient (electrophilic) and hence react most rapidly with electron-rich side-chains (Trp, Tyr, His, Met, Cys, Phe). Few nucleophilic oxidants (e^-, $Ph^{?}$, $CO_2^{?-}$).**

Sources of kinetic and associated data

- **Compilations of kinetic data:**

$HO^{?}$ and $H^{?}$	**J. Phys. Chem. Ref. Data, 1988, 17, 513-886**
$HOO^{?}$ / $O_2^{?-}$	**J. Phys. Chem. Ref. Data, 1988, 17, 1027-1284**
Inorganic radicals (*e.g.* $NO_2^{?}$, $CO_2^{?-}$, $CO_3^{?-}$, $Br_2^{?-}$, $N_3^{?}$)	**J. Phys. Chem. Ref. Data, , 1990, 19, 1027-1284**
$ROO^{?}$	**J. Phys. Chem. Ref. Data, 1990, 19, 413-513**
$^1O_2$	**J. Phys. Chem. Ref. Data, 1995, 24, 663-1021**
HOCl	**Chem. Res. Toxicol, 2001, 14, 1453-1464** **Chem. Res. Toxicol, 2003, 16, 439-449**

Website: NDRL/NIST Solution Kinetics Database **-** 14,000 rate constants

http://www.rcdc.nd.edu/RCDC/RadChemHomePage.html

- **Reduction potentials for one-electron reactions involving radicals** **J. Phys. Chem. Ref. Data, 1989, 18, 1637-1755.**

http://www.rcdc.nd.edu/RCDC/RadChemHomePage.html

Selectivity of damage between different sites by a single oxidant

- **Kinetic data does not usually yield information on selectivity of damage at different sites, unless specific absorptions are monitored - usually only possible for aromatic and sulfur-containing residues.**
- **Number of factors influence which sites are most favored**

  - Stability of incipient radical

  (tertiary > secondary > primary; delocalisation on to other atoms)

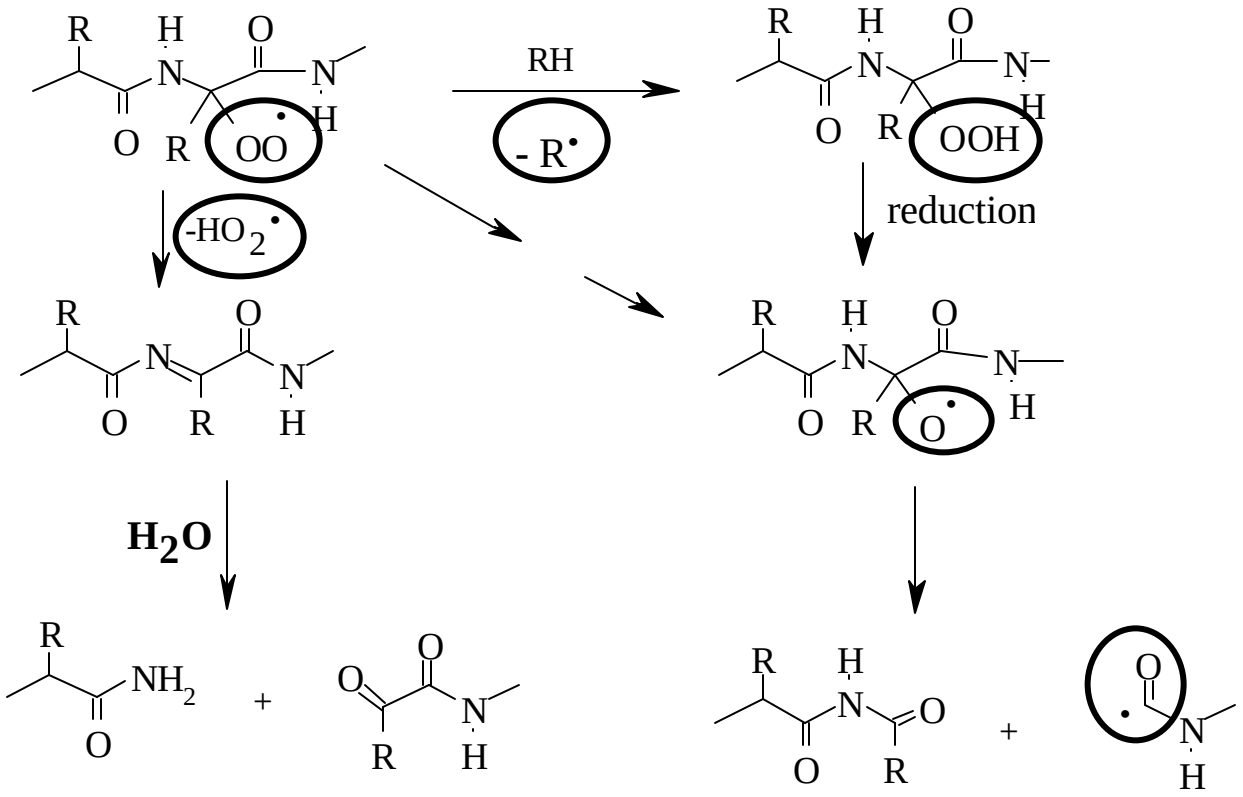
  - Statistics - number of available C-H bonds / sites of addition

  - Accessibility (buried versus exposed; steric and charge interactions)

Backbone fragmentation induced by radicals

Common intermediate implicated in majority of mechanisms

- **Formation of ? -carbon radical via direct, or indirect reaction - detection by EPR spin trapping (*e.g.* Chem. Res. Toxicol., 2000, 13, 1087-1095).**
- **Subsequent formation of peroxyl radical in presence of O_2.**
- **Little backbone fragmentation in absence of O_2.**

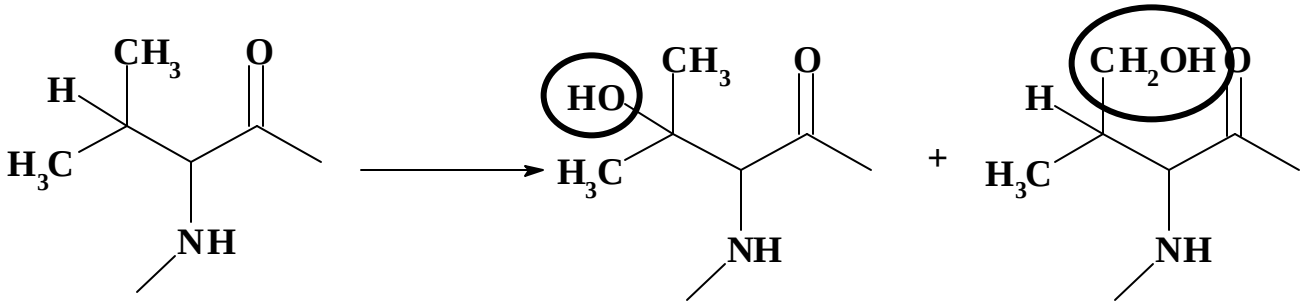


Mechanisms of side-chain oxidation by radicals: aliphatic residues

Hydrogen atom abstraction gives common intermediates

<u>but</u>

ratio of intermediates formed at different C-H positions varies with attacking radical.



Valine side-chain

Via tertiary carbon-centered radical

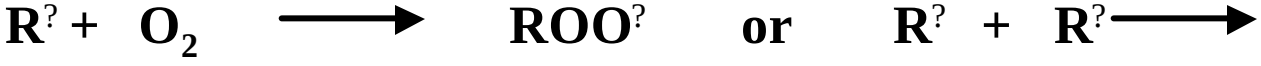
Via primary carbon-centered radical

Direct, rapid-flow, EPR spectroscopy studies can give information on selectivity of initial radical attack for amino acids and peptides.

J. Chem. Soc., Perkin Trans. 2, 1998, 2617-2622; Biochim. Biophys. Acta, 2001, <u>1504</u>, 196-219

Mechanisms of side-chain oxidation by radicals: aliphatic residues

Initial carbon-centred radicals undergo rapid reaction with O_2 to give peroxyl radicals. Dimers formed in absence of O_2.

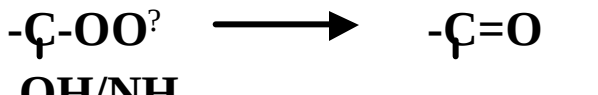


Fate of peroxyl radicals

- **Reaction with another peroxyl radical to give ROO-OOX (X = R, H).**

- **Hydrogen atom abstraction - gives hydroperoxide:**

Elimination reactions - special case for Ser, Thr and few other residues



Major products from aliphatic side-chains: peroxides, alcohols and carbonyls.

Biochem J, 1995, 305, 643-649; Arch Biochem Biophys, 1996, 336, 163-172; Biochem. J. 1997, 324, 1-18

Specific aliphatic side-chain oxidation products

Glutamic acid	**hydroperoxides**
Leucine	**hydroperoxides** **alcohols** **? -ketoisocaproic acid** **isovaleric acid** **isovaleraldehyde** **isovaleraldehyde oxime** **carbonyl compounds**
Glycine	**Aminomalonic acid**
Valine	**hydroperoxides** **alcohols** **carbonyl compounds**
Lysine	**hydroperoxides** **alcohols** **carbonyl compounds**
Proline	**hydroperoxides** **alcohols** **5-hydroxy-2-aminovaleric acid** **carbonyl compounds**
Arginine	**hydroperoxides** **5-hydroxy-2-aminovaleric acid**
Isoleucine	**hydroperoxides,** **alcohols,** **carbonyl compounds**
Methionine	**Methionine sulphoxide**
Cysteine	**Cystine,** **Oxy acids**

Free Radic. Biol. Med., 1999, 27, 1151-1163

Peroxides are major initial products of $HO^{?}$ attack on amino acids, peptides and proteins in the presence of O_2

Substrate	% yield of hydroperoxide groups formed from initial HO .
N-Ac-Lys-NH_2	26
Gly-Lys-Gly	34
Poly-lysine	64
Melittin	16
Protamine	33
Insulin	12
RNase A	53
BSA	36

Formation of peroxides on proteins

Does it matter what the attacking radical is ? - NO

High energy radiation (?- or X-rays, UV, visible light with sensitizer)
Metal ions / ascorbate
Metal ions / peroxide systems ($HO^{?}$, $RO^{?}$)
Thermo-labile azo compounds + O_2 ($ROO^{?}$)
Peroxynitrite
Activated white cells
Hemoprotein / peroxide systems

Does it matter what the amino acid / peptide / protein is ? - NO

Formed on most amino acids, and all peptides and proteins tested
Detected on both isolated proteins and proteins in cells

Yield and exact structure of peroxides formed is dependent on target, the attacking radical, O_2 concentration, presence of reductants / antioxidants / metal ions (*etc*…)

Redox Rep, 1997, 3, 99-110; Biochem J, 1997, 324, 1-18; Biochim Biophys Acta, 2001, 1504, 196-219

Fate of protein peroxides

Protein-OOH + $2e^-$? Protein-OH

Protein-OOH + M^{n+} ? Protein-$O^{?}$ + $M^{(n+1)+}$ + HO^-

Protein-OOH + UV / heat ? Protein-$O^{?}$ + $HO^{?}$

Protein-$O^{?}$? Protein-$C^{?}$ (1,2 hydrogen atom shifts)

Protein-$O^{?}$? Protein-carbonyl + $R^{?}$ (? -scission reactions)
or Protein-$C^{?}$ + released carbonyl

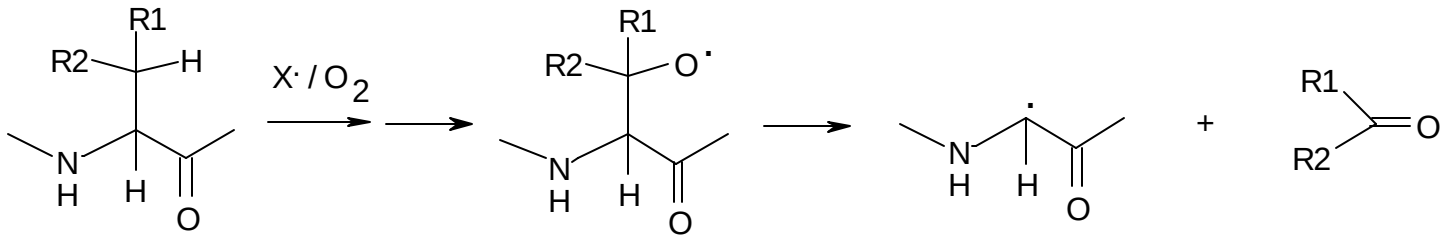
Protein-$C^{?}$ + O_2 ? Protein-$OO^{?}$

Protein-$OO^{?}$ + RH ? Protein-OOH + $R^{?}$

Biochem. J., 1995, 305, 643-649; Arch. Biochem. Biophys., 1996, 336, 163-172;
Chem. Res. Toxicol, 2000, 13, 1087-1095; Free Radic. Biol. Med., 2002, 32, 1171-1184.

Transfer of damage within proteins

- **Evidence for radical transfer from side-chains sites to backbone via mediation of alkoxyl radicals**
  - **Favorable process due**
    - **to stability of ? -carbon radical**
    - **relief of steric crowding**
    - **stability of carbonyl product**



Detected by EPR spin trapping

Quantified by HPLC

- **Results in loss of side-chain group as reactive aldehyde / ketone and formation of backbone radical -> backbone cleavage**
- **Similar reactions with thiyl radical from cysteine ?**

Chem Res Toxicol, 2000, 13, 1087; Free Radic Biol Med, 2002, 32, 1171, J Am Chem Soc, 2003, 125, 2042

Occurrence of chain reactions

Number of reactions of protein radicals that give rise to other radicals:

1) **Decomposition of dimers formed between two peroxyl radicals**

   ROO-OOR $\longrightarrow$ 2RO$^{?}$ + O_2

2) **Hydrogen atom abstraction by an initial peroxyl radical**

   ROO$^{?}$ + XH $\longrightarrow$ ROOH + X$^{?}$

3) **Fragmentation reactions of ? -hydroxperoxy radicals on Ser and Thr**

4) **Decomposition of hydroperoxides to alkoxyl radicals**

<u>All</u> of these reactions give rise to further radicals and hence either

- chain reactions on proteins, or

- damage to other biomolecules

Oxidation of Cys, cystine and Met residues

- **Important targets: rapid reaction with range of oxidants (both radical and non-radical) and easily oxidised.**

- **Met converted to sulfoxide: -S- → -S(=O)- ⇢ -S(O_2)-**
- **Cys oxidised via two major pathways:**

RSH → RSSR (cystine) via thiyl radical

RSH → RS-X → → RSO_2H + RSO_3H (X=OH, Cl, etc)

↓

RSSR

- **Cystine can be oxidised to RSS(=O)R**
- **Both Met sulfoxide and cystine can be <u>repaired</u>**

- **Only major examples of repair of oxidised amino acid residues on proteins**

Free Radic Biol Med, 1995, <u>18</u>, 93; PNAS, 2001, <u>98</u>, 12920, Free Radic Biol Med, 1995, <u>31</u>, 1432; Int J Radiat Biol, 1989, <u>55</u>, 539; von Sonntag - The Chemical Basis of Radiation Biology

Mechanisms of side-chain oxidation by radicals: aromatic residues

Major reaction is addition, though electron abstraction can also occur.

Electron abstraction reactions usually yield hydroxylated products.

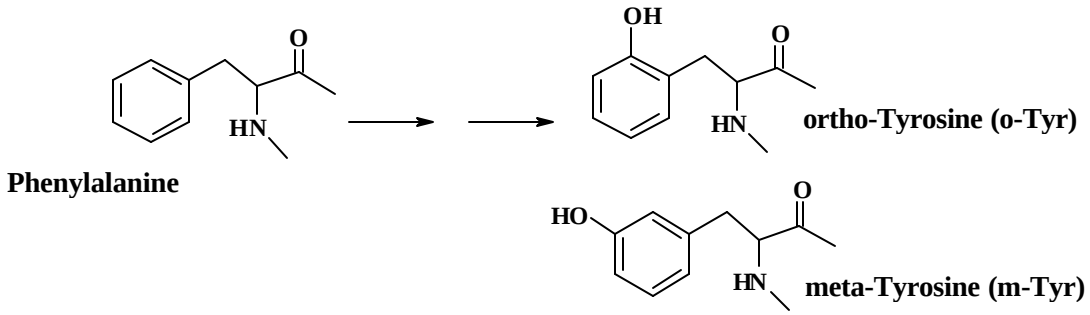
Addition reactions tend to yield a greater diversity of products as this depends on the added species.

Similar products tend to be formed in presence and absence of O_2.

Specific aromatic side-chain oxidation products

Phenylalanine

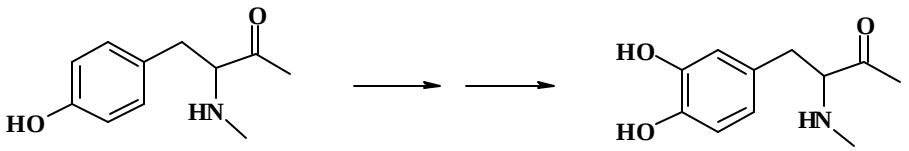
- **o-, m-tyrosine**
- **dimers**



Phenylalanine

Tyrosine

- **DOPA**
- **di-tyrosine**

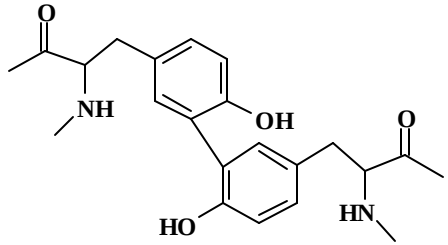


Tyrosine

3,4-dihydroxyphenylalanine (DOPA)

Tryptophan

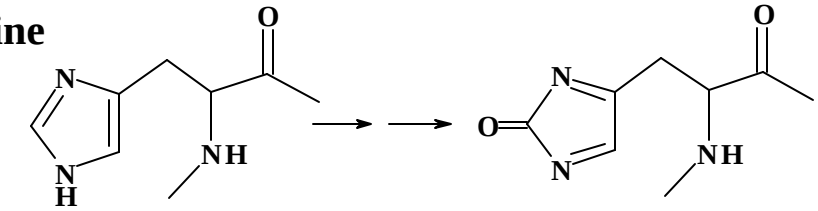
- **N-formylkynurenine,**
- **kynurenine,**
- **5-hydroxytryptophan,**
- **7-hydroxytryptophan**



di-Tyrosine (di-Tyr)

Histidine

- **2-oxo-histidine**



Free Radic. Biol. Med., 1999, 27, 1151-1163

Transfer of damage within proteins

- **Evidence for long range transfer of radical sites within proteins:**
  - **transfer from initial site to a readily oxidised residue (Trp, Tyr, Met, Cys) to give more stable radical - can be equilibria.**
  - **can occur over very large distances, but depends on protein structure**
  - **occurs in competition with reaction of initial radical with O_2**
  - **most common when initial radical is poorly, or unreactive, with O_2**
  - **examples:** **Trp <-> Tyr, Trp <-> Cys, Tyr <-> Cys**

    Oxidised porphyrin ring from Trp, Tyr, Cys

J. Am. Chem. Soc., 1989, 111, 5141-5145; J. Am. Chem. Soc., 1994, 116, 12010-12015; Int. J. Radiat. Biol., 1989, 55, 539-556; J. Biol. Chem., 1997, 272, 2359-2362

Mechanisms of protein oxidation by non-radical oxidants

- **Species such as $^1O_2$, HOCl, HOBr, peroxynitrite, O_3, UV light**

- **Reactions can be very selective and generally on side-chains**

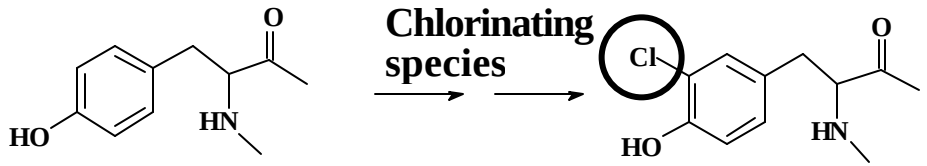
  Little fragmentation, but considerable aggregation

  - **$^1O_2$ - damage to Cys, Met, Trp, Tyr and His.**
  - **HOCl / HOBr - primarily damage to Cys, Met, His, Lys, Trp, ? - amino group.**
  - **Peroxynitrite - damage to Cys, Tyr, Trp.**
  - **UV light - damage to cystine, Trp, Tyr and His.**

- **Some products well-defined - *e.g.* 3-chloroTyr, 3-nitroTyr, methionine sulfoxide, disulphides.**

- **Some poorly defined -> peroxides generated by $^1O_2$ and O_3.**

Aromatic side-chain oxidation products generated by non-radical oxidants



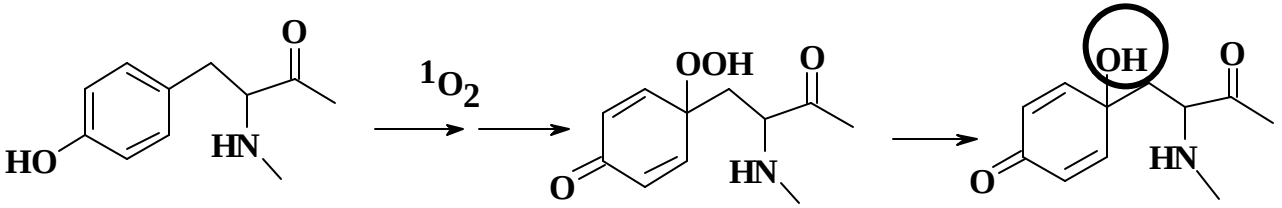
Tyrosine

3-chloroTyrosine (3-ClTyr)

Tyrosine

3-nitroTyrosine

(3-NO_2Tyr)



Free Radic. Biol. Med., 1999, 27, 1151-1163; Photochem. Photobiol, 2002, 76, 35-46

Damage to other targets induced by reactive intermediates of protein oxidation

- **Protein peroxides can induce damage to other proteins by:**
  - **Non-radical reactions (oxidation of Cys and Met residues)**
  - **Radical-mediated reactions**

Biochem. J., 1999, 338, 629-636; Biochem. J., 1999, 344, 125-134; Chem. Res. Toxicol., 2000, 13, 665-672; Biogerentology, 2002, 3, 95-102; Eur. J. Biochem., 2002, 269, 1916-1925; FEBS Lett., 2002, 527, 289-292.

Oxidation of other residues by non-radical reactions

- **Evidence for oxidation of cysteine residues by peroxides**
  - **Protein thiols, peroxides and enzyme activity lost concurrently**
  - **Cysteine oxidised to disulphide and oxy acids**

- **Evidence for oxidation of methionine residues by peroxides**
  - **Evidence for the generation of methionine sulfoxide**
  - **Inactivation of methionine-dependent enzymes ?**

Eur. J. Biochem., 2002, 269, 1916-1925; FEBS Lett., 2002, 527, 289-292; Redox Rep., 2003, 8, 81-86

Damage to other targets induced by reactive intermediates of protein oxidation

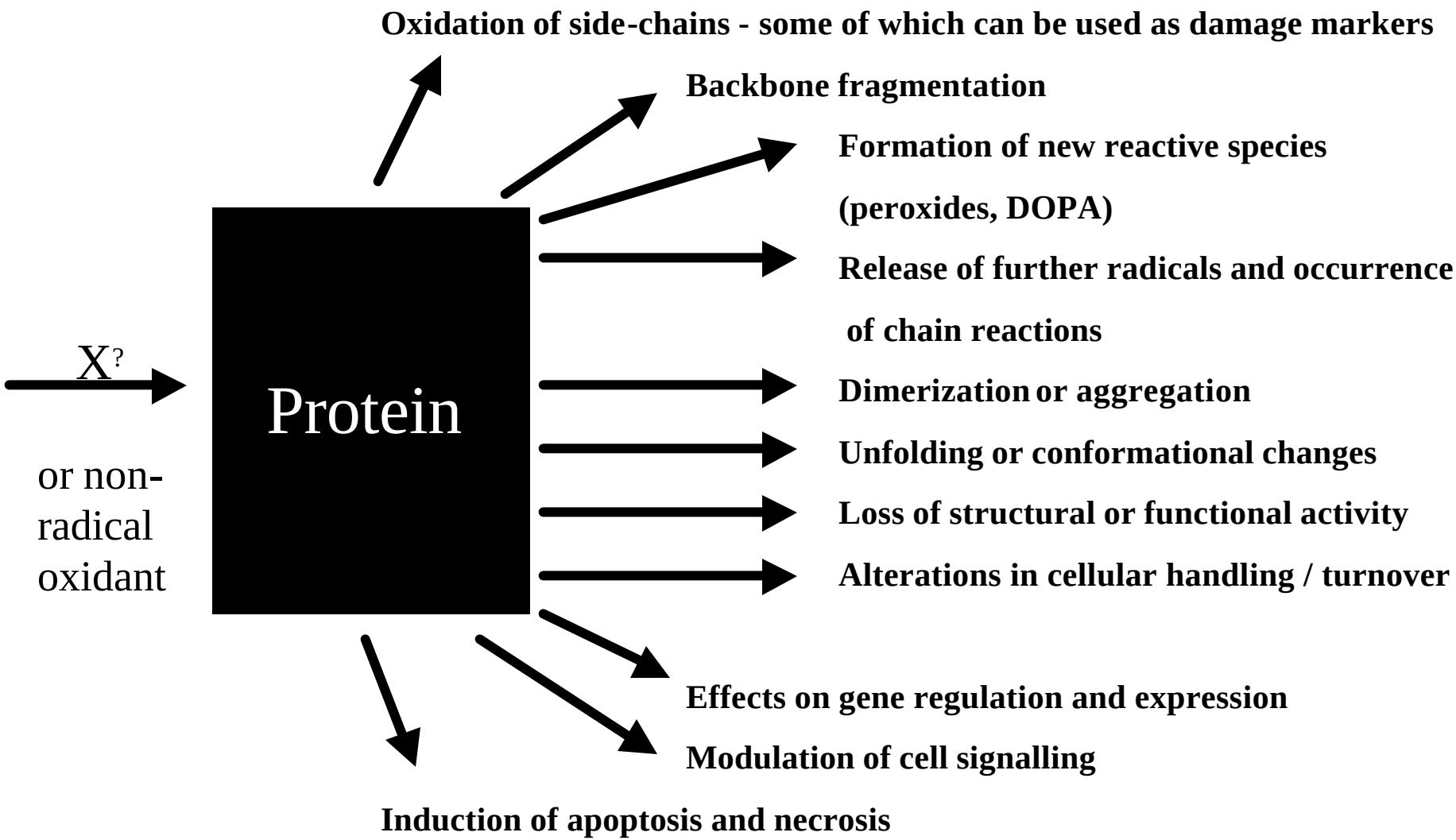
- **Protein peroxides can induce damage to other proteins by:**
  - **Non-radical reactions (oxidation of susceptible thiols)**
  - **Radical-mediated reactions**

- **Protein peroxides and DOPA can induce damage to DNA via radical-mediated reactions**
  - **Oxidation of bases (*e.g.* 8-oxodG)**
  - **Induction of strand breaks**
  - **Formation of protein-DNA cross-links**

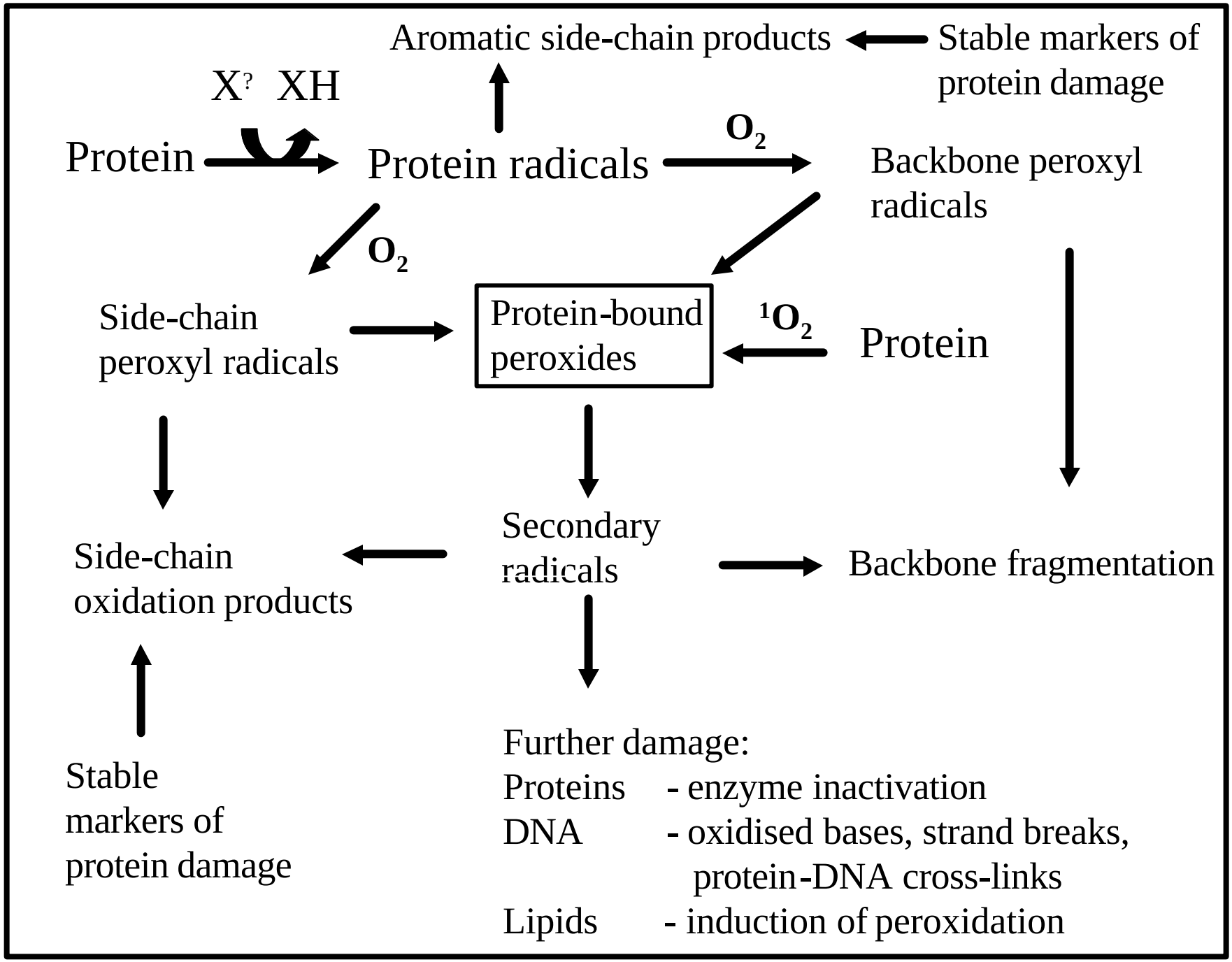
- **Protein peroxides can induce lipid oxidation via radical-mediated reactions**

Biochem. J., 1999, 338, 629-636; Biochem. J., 1999, 344, 125-134; Chem. Res. Toxicol., 2000, 13, 665-672; Biogerentology, 2002, 3, 95-102; Eur. J. Biochem., 2002, 269, 1916-1925; FEBS Lett., 2002, 527, 289-292.

Consequences of oxidation of proteins



Biochim. Biophys. Acta, 2001, 1504, 196-219; J. Photochem. Photobiol. B, 2001, 63, 114-125.


Aromatic side-chain products
Stable markers of protein damage
X? XH
Protein
Protein radicals
O2
Backbone peroxyl radicals
O2
Side-chain peroxyl radicals
Protein-bound peroxides
1O2
Protein
Side-chain oxidation products
Secondary radicals
Backbone fragmentation
Stable markers of protein damage
Further damage:
Proteins - enzyme inactivation
DNA - oxidised bases, strand breaks, protein-DNA cross-links
Lipids - induction of peroxidation

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