

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**

Why concentrate on proteins ?

1) Concentration of target

Proteins are major 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 | $8 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ |
| RNA | $1 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ |
| Hyaluronan | $7 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ |
| Linoleic acid | $9 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ |
| Collagen | $4 \times 10^{11} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ |
| Albumin | $8 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ |

Antioxidants:

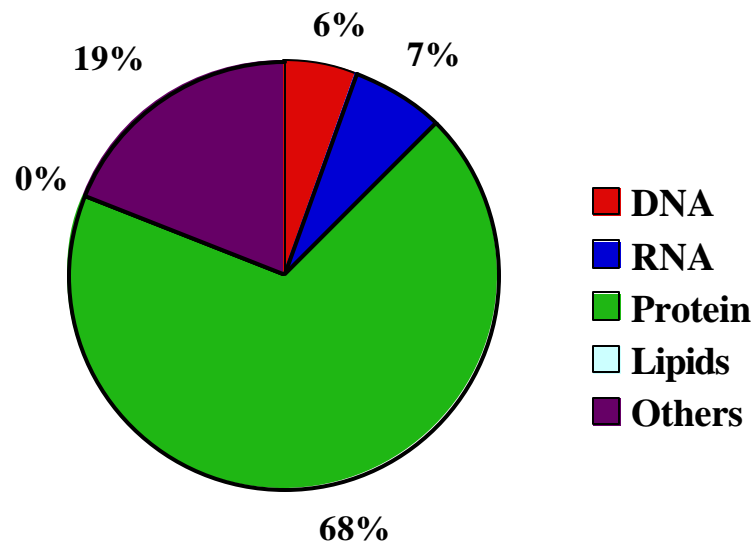
| | |
|------------------|--|
| Ascorbate | $1 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, |
| GSH | $1.4 \times 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$, |
| Trolox C | $6.9 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ |

Why concentrate on proteins ?

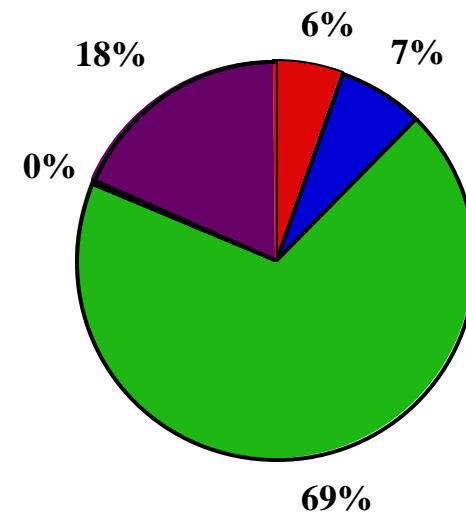
Kinetic and abundance data can be used to predict sites of damage.

For leukocytes:

Singlet oxygen



HO[•]

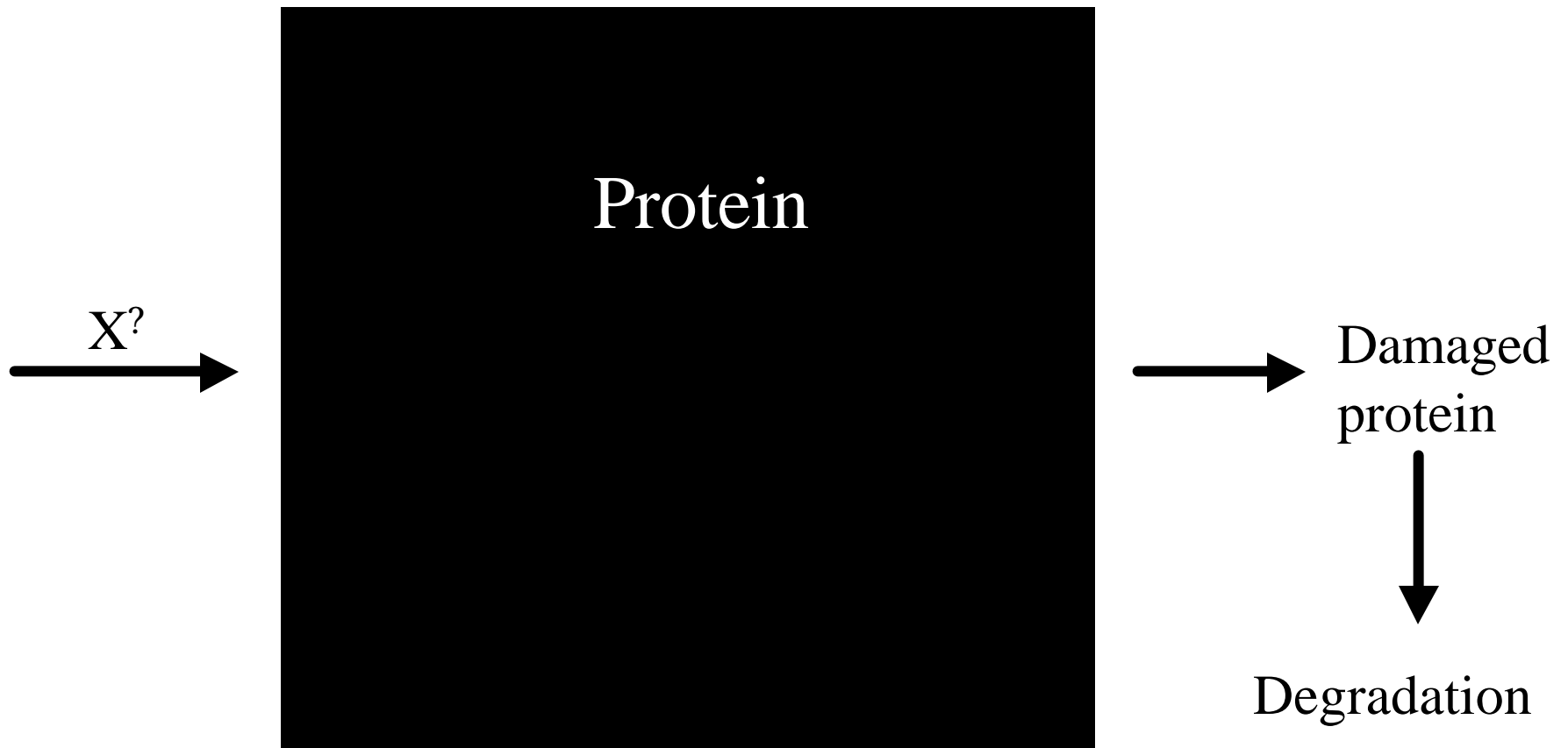


Such data needs to be treated with great caution !

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.

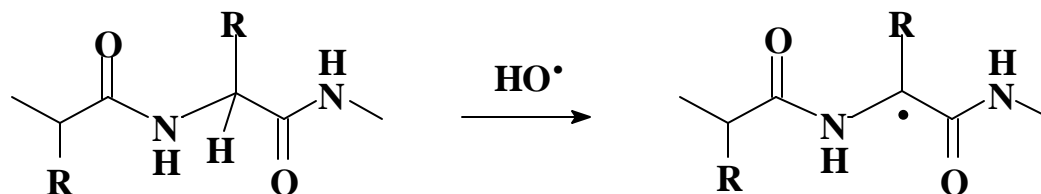
Follow-up papers in 1908, occupied approximately half of the total page-count of J. Biol. Chem. for the entire year.

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

Selectivity of damage by different oxidants

- The most reactive radicals tend to be the least selective
e.g. HO^\bullet - difference in rate constants is relatively small
Most reactive: Trp, Tyr, His, Met, Cys, Phe, Arg: $k \text{ ca. } 10^{10} \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$
Least reactive: Ala, Asp, Asn: $k \text{ ca. } 10^7 - 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$
Result - most side-chains are oxidised
Reaction with backbone sites $k \text{ ca. } 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$
Significant backbone fragmentation as well as side-chain oxidation
- Less reactive radicals tend to be more selective
e.g. $\text{CCl}_3\text{OO}^\bullet$ - difference in value of rate constants between most reactive side-chain (Trp $k \text{ ca. } 9 \times 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$) 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^\bullet , $\text{CO}_2^{\bullet-}$).

Sources of kinetic and associated data

- **Compilations of kinetic data:**

| | |
|---|--|
| HO [?] and H [?] | J. Phys. Chem. Ref. Data, 1988, <u>17</u> , 513-886 |
| HOO [?] / O ₂ ^{?-} | J. Phys. Chem. Ref. Data, 1988, <u>17</u> , 1027-1284 |
| Inorganic radicals (<i>e.g.</i> NO ₂ [?] , CO ₂ ^{?-} , CO ₃ ^{?-} , Br ₂ ^{?-} , N ₃ [?]) | J. Phys. Chem. Ref. Data, , 1990, <u>19</u> , 1027-1284 |
| ROO [?] | J. Phys. Chem. Ref. Data, 1990, <u>19</u> , 413-513 |
| ¹ O ₂ | J. Phys. Chem. Ref. Data, 1995, <u>24</u> , 663-1021 |
| HOCl | Chem. Res. Toxicol, 2001, <u>14</u> , 1453-1464 Chem. Res. Toxicol, 2003, <u>16</u> , 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>

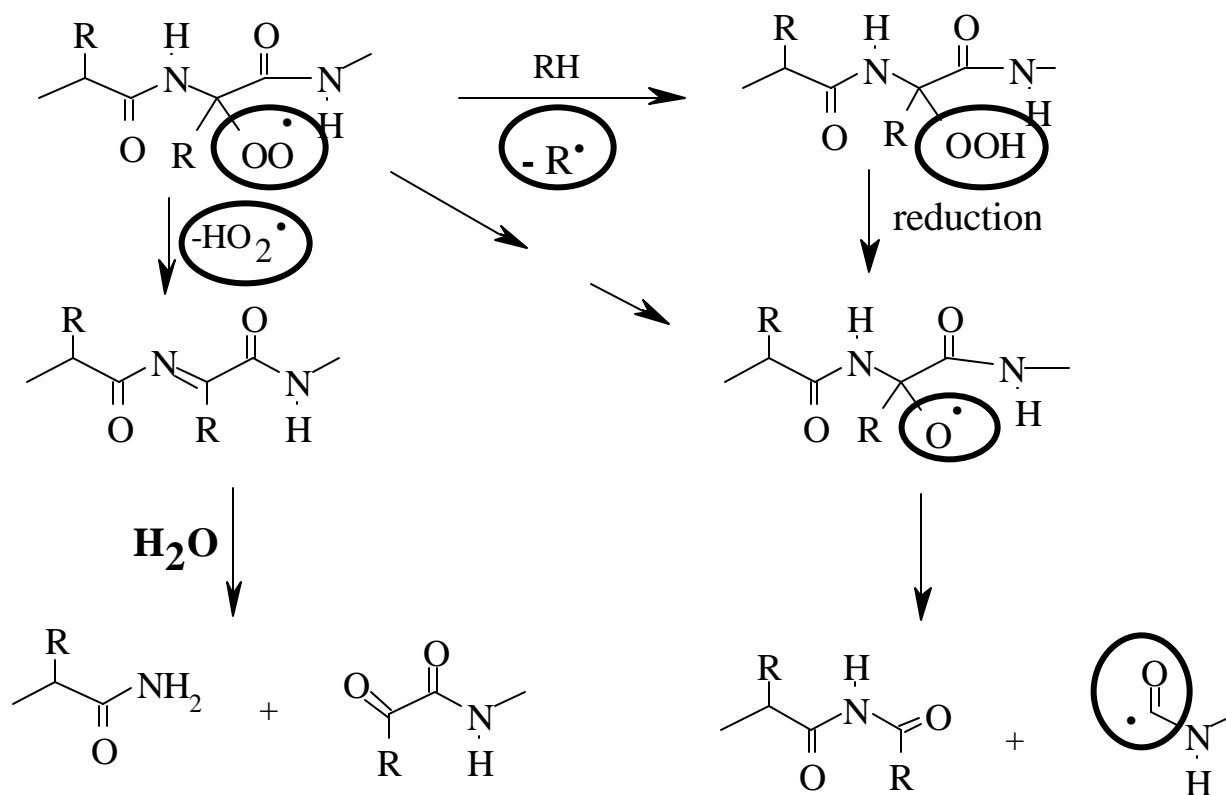
Selectivity of damage between different sites by a single oxidant

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

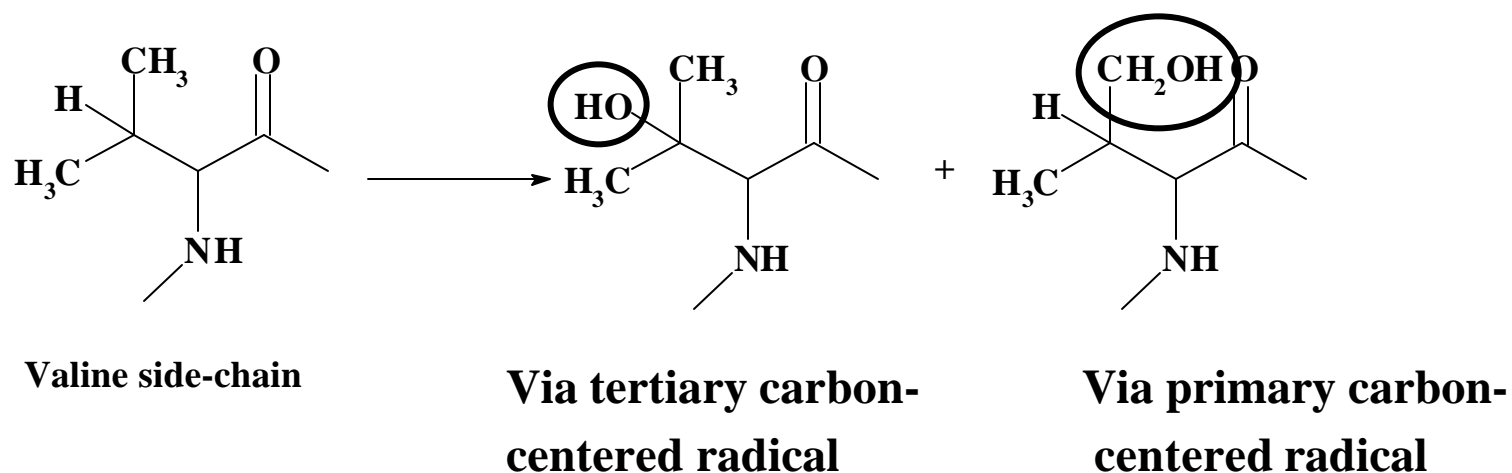


Mechanisms of side-chain oxidation by radicals: aliphatic residues

Hydrogen atom abstraction gives common intermediates

but

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



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

Mechanisms of side-chain oxidation by radicals: aliphatic residues

Initial carbon-centred radicals undergo rapid reaction with O₂ to give peroxy radicals. Dimers formed in absence of O₂.



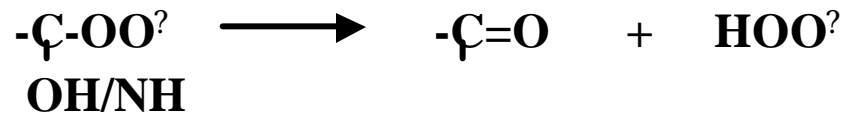
Fate of peroxy radicals

– Reaction with another peroxy 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.

Specific aliphatic side-chain oxidation products

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

Peroxides are major initial products of HO[·] attack on amino acids, peptides and proteins in the presence of O₂

| Substrate | % yield of hydroperoxide groups formed from initial HO[·] |
|--------------------------------|---|
| N-Ac-Lys-NH₂ | 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 + O₂ (ROO[?])

Peroxynitrite

Activated white cells

Hemoprotein / peroxide systems

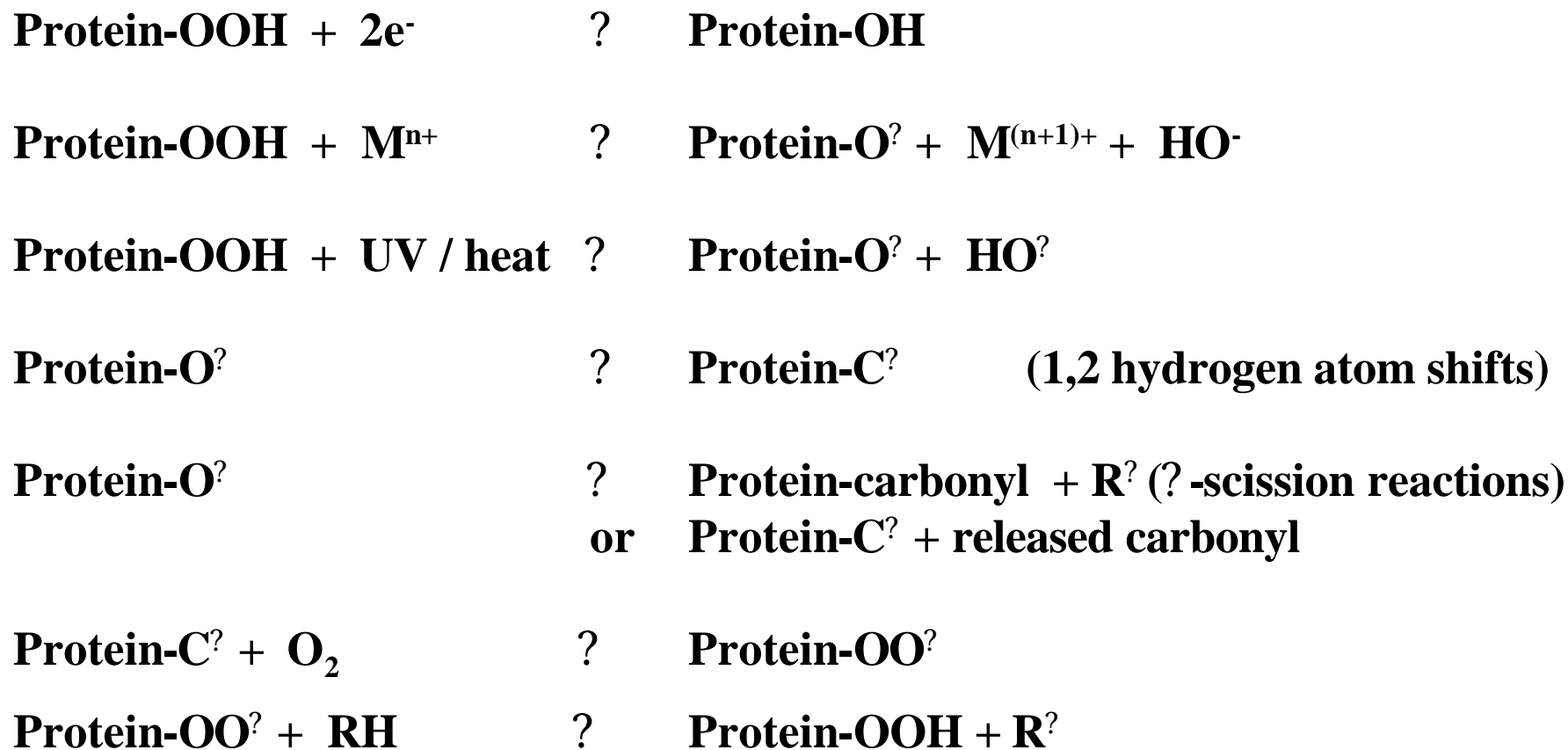
Does it matter what the amino acid / peptide / protein is ? - NO

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, O₂ concentration, presence of reductants / antioxidants / metal ions (*etc...*)

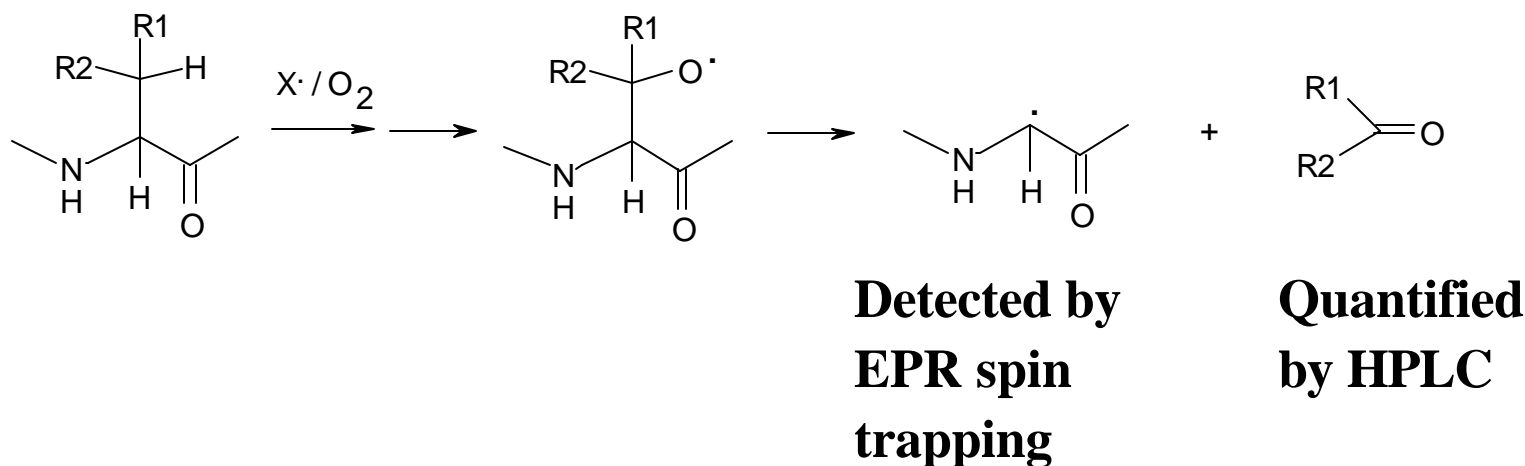
Fate of protein peroxides



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 alkoxy radicals**
 - Favorable process due
 - to stability of α -carbon radical
 - relief of steric crowding
 - stability of carbonyl product



- **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 ?**

Occurrence of chain reactions

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

- 1) Decomposition of dimers formed between two peroxy radicals



- 2) Hydrogen atom abstraction by an initial peroxy radical



- 3) Fragmentation reactions of α -hydroxyperoxy radicals on Ser and Thr

- 4) Decomposition of hydroperoxides to alkoxy radicals

All of these reactions give rise to further radicals and hence either

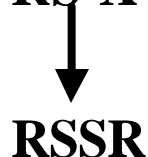
- chain reactions on proteins, or
- damage to other biomolecules

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: $-S- \longrightarrow -S(=O)- \dashrightarrow -S(O_2)-$

- Cys oxidised via two major pathways:



- Cystine can be oxidised to $RSS(=O)R$
- Both Met sulfoxide and cystine can be repaired
- Only major examples of repair of oxidised amino acid residues on proteins

Free Radic Biol Med, 1995, 18, 93; PNAS, 2001, 98, 12920, Free Radic Biol Med, 1995, 31, 1432; Int J Radiat Biol, 1989, 55, 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.

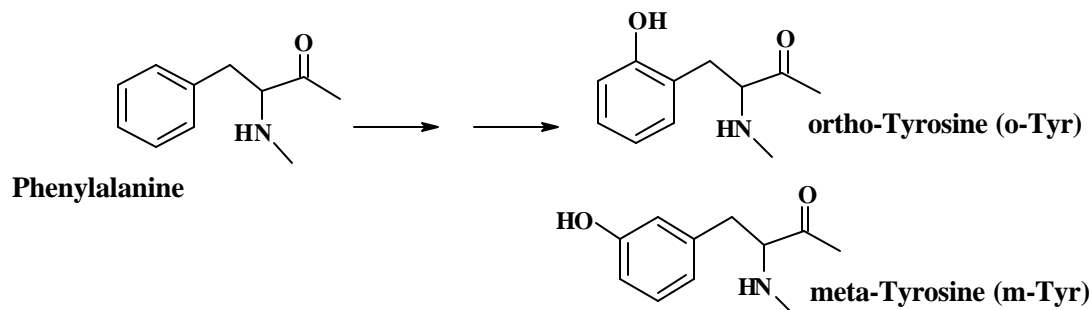
Addition reactions tend to yield a greater diversity of products as this depends on the added species.

Similar products tend to be formed in presence and absence of O₂.

Specific aromatic side-chain oxidation products

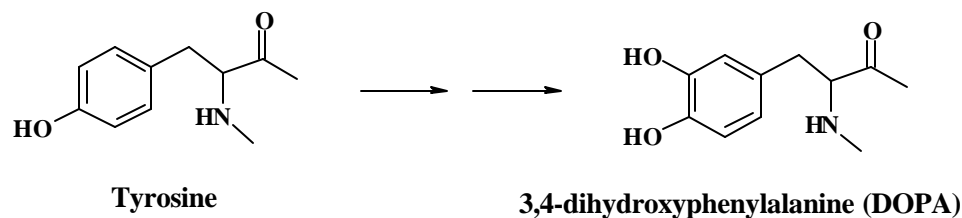
Phenylalanine

o-, m-tyrosine
dimers



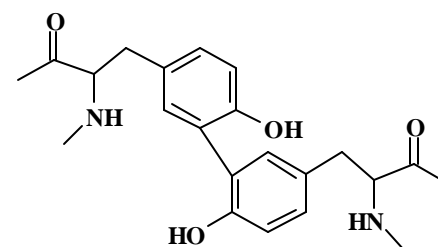
Tyrosine

DOPA
di-tyrosine



Tryptophan

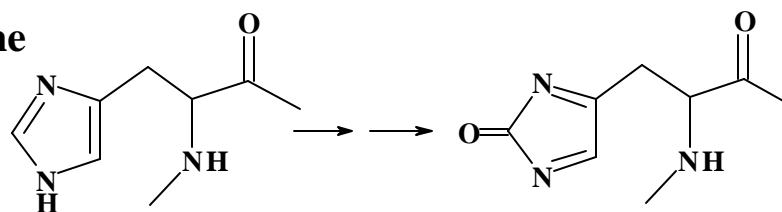
N-formylkynurenine,
kynurenine,
5-hydroxytryptophan,
7-hydroxytryptophan



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 O₂
 - most common when initial radical is poorly, or unreactive, with O₂
 - examples: Trp <-> Tyr, Trp <-> Cys, Tyr <-> Cys

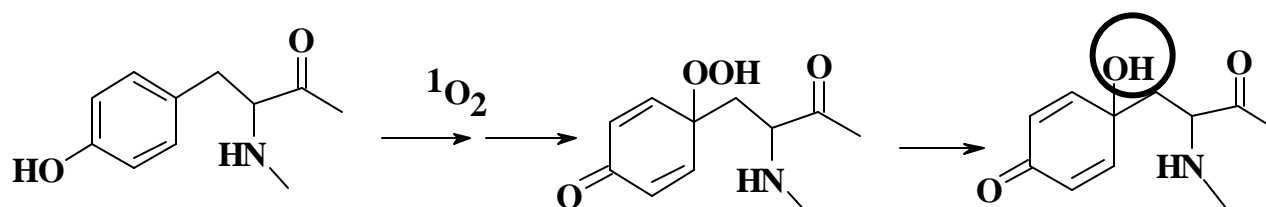
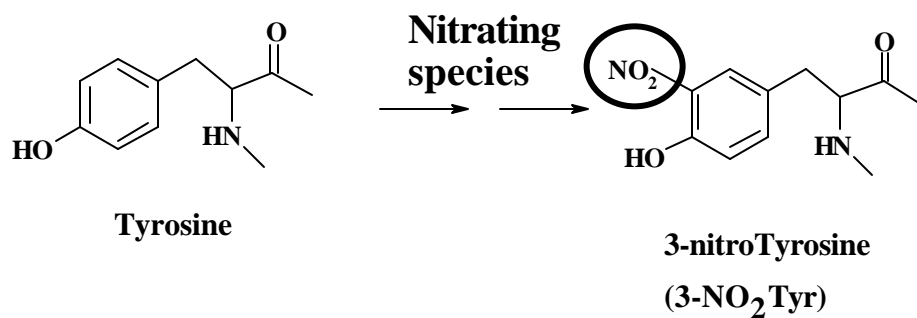
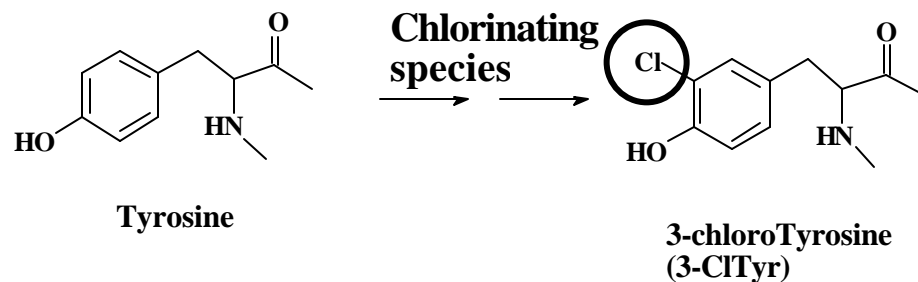
Oxidised porphyrin ring from Trp, Tyr, Cys

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 $^1\text{O}_2$, HOCl, HOBr, peroxyxynitrite, O_3 , UV light
- Reactions can be very selective and generally on side-chains
Little fragmentation, but considerable aggregation
 - $^1\text{O}_2$ - damage to Cys, Met, Trp, Tyr and His.
 - HOCl / HOBr - primarily damage to Cys, Met, His, Lys, Trp, ? - amino group.
 - Peroxyxynitrite - 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 $^1\text{O}_2$ and O_3 .

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



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; Biogerontology, 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**

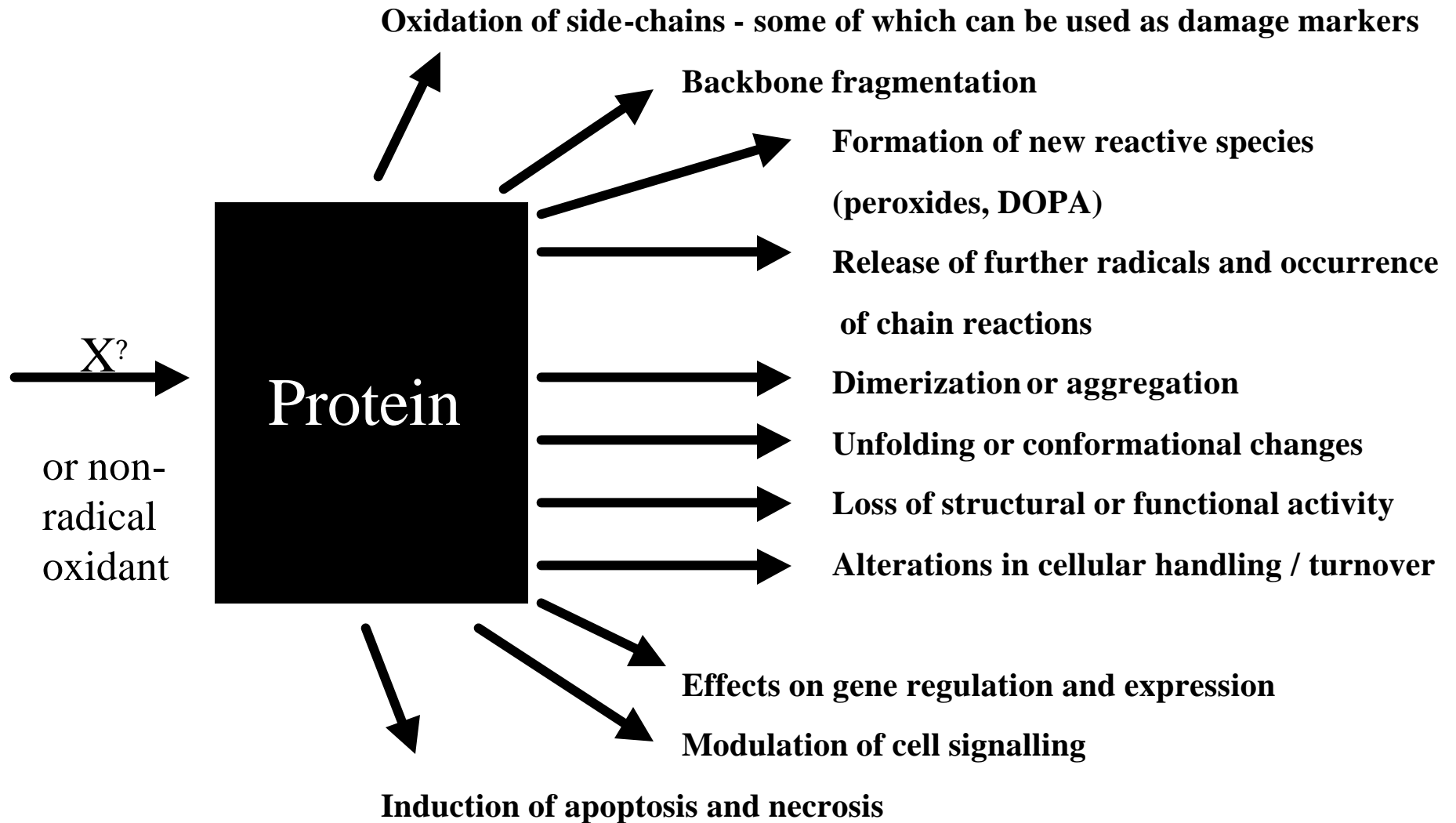
- **Evidence for oxidation of methionine residues by peroxides**
 - **Evidence for the generation of methionine sulfoxide**
 - **Inactivation of methionine-dependent enzymes ?**

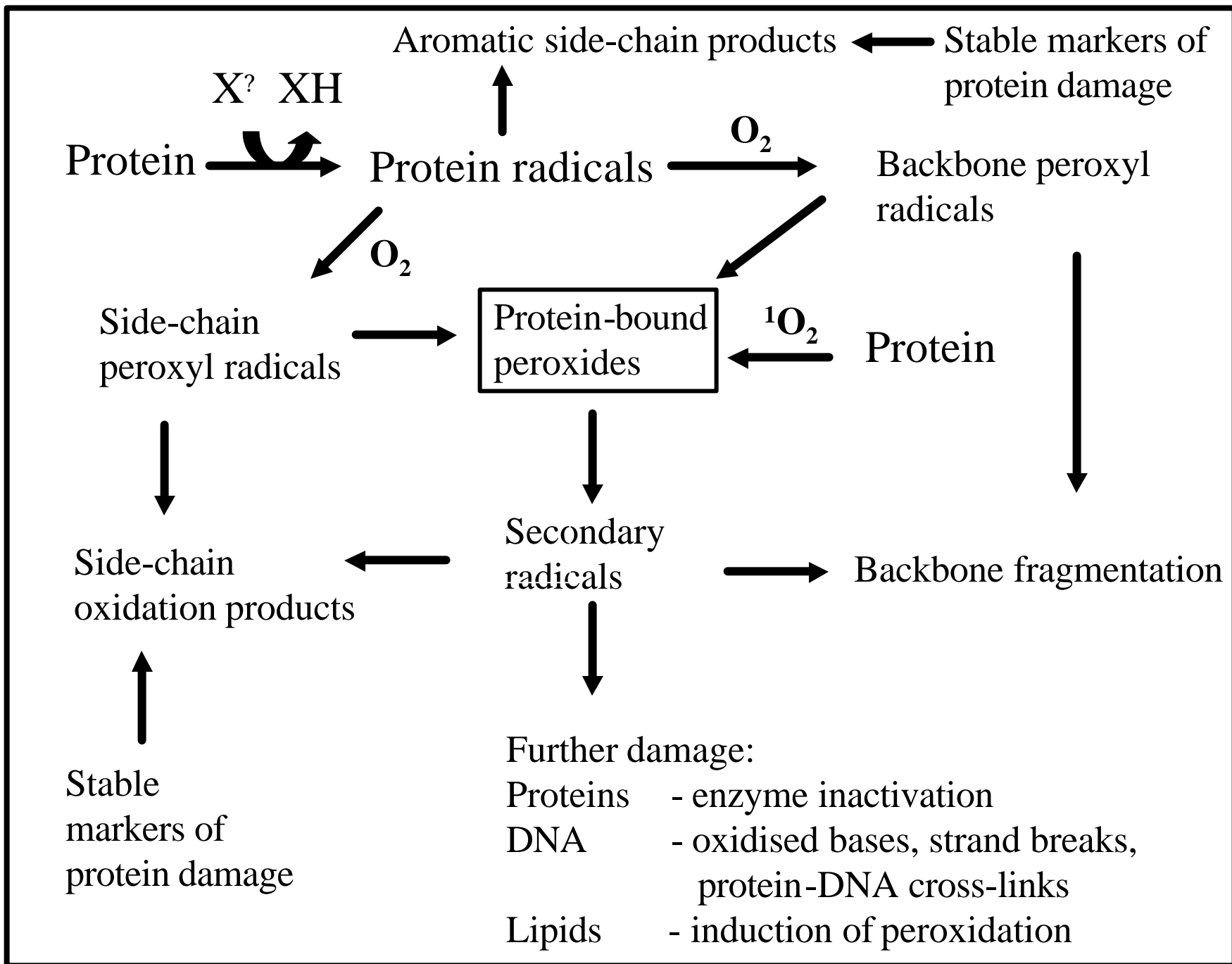
Damage to other targets induced by reactive intermediates of protein oxidation

- **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; Biogerontology, 2002, 3, 95-102; Eur. J. Biochem., 2002, 269, 1916-1925; FEBS Lett., 2002, 527, 289-292.

Consequences of oxidation of proteins





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