Detection, identification, and quantification of oxidative protein modifications

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Exposure of biological molecules to oxidants is inevitable and therefore commonplace. Oxidative stress in cells arises from both external agents and endogenous processes that generate reactive species, either purposely (*e.g.* during pathogen killing or enzymatic reactions) or accidentally (*e.g.* exposure to radiation, pollutants, drugs, or chemicals). As proteins are highly abundant and react rapidly with many oxidants, they are highly susceptible to, and major targets of, oxidative damage. This can result in changes to protein structure, function, and turnover and to loss or (occasional) gain of activity. Accumulation of oxidatively-modified proteins, due to either increased generation or decreased removal, has been associated with both aging and multiple diseases. Different oxidants generate a broad, and sometimes characteristic, spectrum of post-translational modifications. The kinetics (rates) of damage formation also vary dramatically. There is a pressing need for reliable and robust methods that can detect, identify, and quantify the products formed on amino acids, peptides, and proteins, especially in complex systems. This review summarizes several advances in our understanding of this complex chemistry and highlights methods that are available to detect oxidative modifications—at the amino acid, peptide, or protein level—and their nature, quantity, and position within a peptide sequence. Although considerable progress has been made in the development and application of new techniques, it is clear that further development is required to fully assess the relative importance of protein oxidation and to determine whether an oxidation is a cause, or merely a consequence, of injurious processes.

Biological systems are exposed to a wide variety of oxidizing species—both free radicals and two-electron oxidants. These species are often termed "reactive oxygen species," although this is a misleading term, as the reactivity of these species varies enormously (see below). Oxidants are generated both deliberately (*e.g.* to kill invading pathogens or as intermediates in enzymatic reactions) or unintentionally (*e.g.* via electron leakage from electron transport chains, metabolism of drugs, exposure to chemicals, pollutants, and radiation). These processes have been reviewed in Refs. 1, 2.

The formation of these oxidants and their reactions are limited by cellular and organismal defense systems, which include enzymes that remove oxidants or oxidant precursors (*e.g.* superoxide dismutases, peroxiredoxins, thioredoxin/thioredoxin reductase, GSH peroxidase isoforms, and catalases), and water- and lipid-soluble oxidant scavengers, including thiols (*e.g.* GSH and thioredoxin), ascorbic acid (vitamin C), urate, tocopherol isoforms (vitamin E), quinols (*e.g.* reduced coenzyme Q_{10}), carotenoids, and polyphenols. Although these systems are efficient, and in many cases show redundancy (*i.e.* multiple processes remove the same species), they are not 100% effective, and a large body of data indicates that biological targets suffer resulting damage. These protective systems are therefore complemented by systems that either repair damage or remove the modified molecules (*e.g.* methionine sulfoxide reductases, disulfide reductases/isomerases, glutaredoxins, sulfiredoxins, proteasomes, lysosomes, proteases, phospholipases, and DNA repair enzymes) (1, 2).

Despite this battery of preventative and repair systems, many studies have reported increased damage, and accumulation of this, in human, animal, and microbial and plant systems exposed to stress conditions (reviewed in Refs. 1, 2). A higher level of damage may arise from increased oxidant generation, a decrease or failure of defense systems, or (most commonly) a mixture of both processes, as many defense systems are themselves subject to damage or show reduced activity due to co-factor depletion. This concept of an altered balance between formation and removal gave rise to the term "oxidative stress" (2), although it is now apparent that this is an oversimplification of a complex picture, as limited stress ("eustress") can be beneficial in priming and protecting a system against greater damage ("distress") (2). Increasing age is often associated with a decrease in enzyme levels or activity, and in some cases decreased levels of co-factors and essential trace elements, such that increased levels of oxidants and modified products are formed (1, 2). These changes can be accelerated by disease or environmental factors, despite the presence of feedback loops (*e.g.* antioxidant-response elements, including the Nrf-2 pathway; DNA damage–response element; OxyR; SoxRS) that upregulate the synthesis of protective species (1, 2). In this study, we review the basic chemistry and biochemistry of protein modification by oxidants, with a focus on methods available for the detection, identification, and quantification of these changes.

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Proteins as targets of oxidative damage

Proteins are major components of most biological systems and constitute ~70% of the dry mass of cells and tissues. The rate of reaction of an oxidant with a biological target depends on the concentration of the target, multiplied by the rate constant for its reaction with the oxidant. Both of these factors result in proteins being major targets for damage as proteins are both present at high concentrations (up to 1–3 mM in plasma and 5–10 mM in cells) and have high rate constants for reaction with oxidants. Thus, oxidant damage in most biological systems is likely to be skewed toward protein modification (3, 4). This is clearly an oversimplification of a complex situation, as other factors are known to play an important role, including localization of the generating system relative to the target and particularly membrane barriers, micro-environments, binding, or association of the oxidant system to a target, the occurrence of secondary reactions, and intra- and intermolecular transfer reactions (3, 4). However, it is likely that proteins are major sites of damage in many situations, although it should also be noted that the *extent of damage* and its biological *importance* may be very different (3, 4). Thus, low levels of modification of a critical target may have much greater consequences than a high level of damage to noncritical materials.

Radicals (*e.g.* $HO^{\bullet}$, $CO_3^{\bullet-}$, $NO_2^{\bullet}$, $ROO^{\bullet}$, $RO^{\bullet}$, $R^{\bullet}$, and many others), two-electron oxidants (*e.g.* peroxides, $^1O_2$, O_3, ONOOH, HOCl, and related species), and metal–oxo complexes can all modify proteins, although the reactivity and selectivity of these oxidants are highly variable (3, 4). Reactions of secondary products (*e.g.* aldehydes, quinones, and dehydroalanine) are a further source of modifications (5, 6). Together, these generate a wide variety of post-translational modifications that alter amino acid and protein composition and structure, charge, hydrophobicity/hydrophilicity, folding, and function (3, 4, 7, 8).

Nature and reactivity of oxidant species

In the following section, a brief overview is presented on the formation and reactivity of a number of key oxidant species relevant to mammalian systems. Although each of these species is described as single entities, it should be noted that nearly all of these species undergo further interconversion reactions as illustrated in Fig. 1 (although this is situation-dependent) that result in complex mixtures in most reaction systems (3, 4).

Superoxide radical-anions ($O_2^{\bullet-}$)

Superoxide $O_2^{\bullet-}$ radicals are generated continuously by most organisms as a result of the use of O_2 as a terminal electron acceptor in some electron transport chains, such as those of mitochondria, the endoplasmic reticulum cytochrome P450 system, and the plasma membrane NADH/NADPH oxidase systems (1, 2). Incomplete coupling of electron transfer results in single electron leakage to O_2 (estimated at 1–3%) (1). $O_2^{\bullet-}$ is poorly reactive, with iron–sulfur complexes a significant target (9). $O_2^{\bullet-}$ undergoes rapid spontaneous, and superoxide dismutase-catalyzed, disproportionation to give hydrogen peroxide (H_2O_2) and O_2, with the former being a precursor of further oxidizing species, as well as being a reactive species in its own right (10). $O_2^{\bullet-}$ is generated, at very high fluxes over short time periods, by membrane-associated NADPH oxidases (NOXs and DUOXs), at the expense of NADPH via an "oxidative burst" (11). $O_2^{\bullet-}$ is also formed by heme proteins (cytochromes, oxyhemoglobin, and oxymyoglobin) (12), uncoupled nitric-oxide synthase, and xanthine oxidase, among others (1).

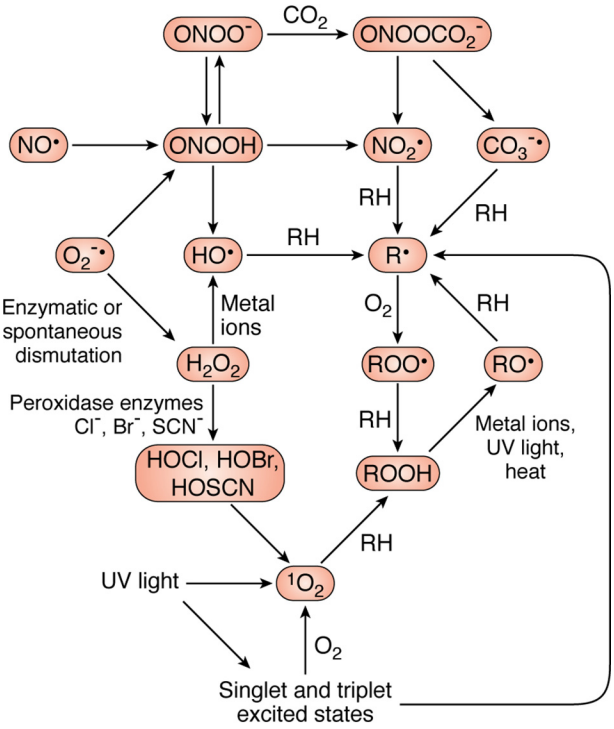

Figure 1. Overview of interconversion processes of common biological oxidants. The extent of these reactions depends on the circumstances and reaction conditions and is therefore only intended as guide to the complexity of examining oxidant reactions. Adapted from Ref. 4.

Hydrogen peroxide (H_2O_2)

In addition to formation from $O_2^{\bullet-}$ by dismutation, H_2O_2 is also generated directly by a number of enzymes (*e.g.* NADPH oxidase-4, monoamine oxidases, hexose oxidases, amino acid oxidases, other oxidoreductases, protein-disulfide isomerases, and Ero1p during protein synthesis and folding (2, 13)). Direct oxidation of biological targets by H_2O_2 is both limited in extent and is usually slow. Thus, direct reaction is limited to Cys, selenocysteine, and Met residues with these typically occurring with very low rate constants (see also below), with the exception of reaction with some specialized enzymes (*e.g.* peroxiredoxins and GSH peroxidases), which have catalytic centers that facilitate rapid O–O bond cleavage. Such environments can elevate the rate constant for reaction by a million-fold (2, 13). H_2O_2 is a substrate for a large family of peroxidase enzymes, with these reactions used both synthetically (*e.g.* in the formation of thyroid hormones by thyroid peroxidase and in the generation of collagen matrices by peroxidasins) and to kill invading pathogens (2, 13).

Hypohalous acids and other reactive halogen species

Reaction of H_2O_2 with heme peroxidases, such as myeloperoxidase, eosinophil peroxidase, and lactoperoxidase, results in the formation of hypohalous acids (HO*X*, *X* = Cl, Br, I, or SCN) (14). These vary markedly in reactivity and oxidizing capacity,

with hypochlorous acid (HOCl, familiar to many as the active component in household bleach) being the most reactive and powerful oxidant (15, 16). HOCl (which exists in equilibrium with Cl_2 at low pH values) is a key component of the innate immune response against pathogens, with this generated at high concentrations in neutrophil phagolysosomes (17). Release of myeloperoxidase to the extracellular space (instead of into phagolysosomes) and subsequent reaction with H_2O_2 can, however, result in host tissue damage, with the level and activity of myeloperoxidase associated with tissue damage in acute and chronic inflammatory conditions (17). Comparison of HOCl and H_2O_2 illustrates a key point about oxidants and their reactivity: H_2O_2 is a strong oxidant (reduction potential 1.32 V) and a more powerful oxidant than HOCl (reduction potential 1.28 V), but this reacts very slowly when compared with HOCl (*cf.* the rate constant, *k*, for reaction of HOCl with the amino acid methionine is $\sim 10^8$-fold higher than for H_2O_2 (4, 15)), so evolution has favored the use of HOCl over H_2O_2, for kinetic rather than thermodynamic reasons. The low reactivity of H_2O_2 is likely to be one of the major reasons why this species is used biologically as a messenger molecule: slow and selective reactivity allows for specificity in transmission of messages as only a limited number of select targets are likely to be activated. Highly-reactive species such as HOCl would not allow such specificity of transfer of information.

Nitric oxide ($NO^{\bullet}$)

$NO^{\bullet}$ is a key vascular regulator and messenger molecule, with this generated from arginine by nitric-oxide synthase enzymes (NOSs)[2] (18). The concentration generated by constitutive NOS isoforms is low (pico- to nanomolar), consistent with a regulatory function, but higher levels (up to micromolar) are formed by the inducible NOS isoform of macrophages, at sites of inflammation (19). $NO^{\bullet}$ reacts slowly with most biological targets, consistent with its role as a signaling molecule (18, 20, 21), with fast reactions occurring primarily with transition metal ions (*e.g.* the iron center of heme proteins, including that of soluble guanylate cyclase, the major effector of $NO^{\bullet}$ signaling) and with other radicals (18, 22). Reaction with other radicals can be protective (*e.g.* by terminating lipid peroxidation chain reactions) but also damaging when the product is a powerful oxidant, as is seen in the formation of peroxynitrous acid (19).

Peroxynitrous acid (ONOOH)

Diffusion-controlled (*i.e.* k 10^9–10^{10} M^{-1} s^{-1}) reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$ gives $ONOO^-$(peroxynitrite). This species exists in equilibrium with the corresponding conjugate acid peroxynitrous acid (ONOOH), with the pK_a (6.8) favoring the anion at most biological pH values (19). However, the acid (ONOOH) form is typically the more reactive species with protein targets. Reaction with CO_2 and some boronic acid probes are exceptions, with the rate constant for these targets being higher with $ONOO^-$ (19). The reactivity of ONOOH is therefore modulated by CO_2 (which is in equilibrium with HCO_3^-) as a result of the formation of $ONOOCO_2^-$, which decomposes to give $CO_3^{\bullet-}$ and $NO_2^{\bullet}$ that can either diffuse out of the solvent cage or recombine (reviewed in Ref. 19). ONOOH is a potent oxidizing/nitrating species (19) that can give rise to both two-electron and one-electron oxidation products. The former arises from direct reactions of ONOOH, and the latter is formed from limited homolysis to give $HO^{\bullet}$ and $NO_2^{\bullet}$, and subsequent radical chemistry (19).

Hydroxyl ($HO^{\bullet}$) and other oxygen-centered radicals

Metal ion (primarily Fe and Cu)-catalyzed decomposition of H_2O_2 generates the highly-reactive and powerful oxidant $HO^{\bullet}$ via Fenton and pseudo-Fenton reactions (23); this species is also formed directly from water by high-energy radiation (24). Metal ion–catalyzed decomposition of organic and lipid hydroperoxides gives alkoxyl radicals ($RO^{\bullet}$) that are less powerful oxidants than $HO^{\bullet}$, but more reactive than peroxyl radicals ($ROO^{\bullet}$) that are typically generated from rapid (typically diffusion-controlled) addition of O_2 to carbon-centered radicals ($R^{\bullet}$) (4). The latter arise from hydrogen atom abstraction from biological targets by reactive radicals (24). $R^{\bullet}$ and $ROO^{\bullet}$ are key intermediates in lipid peroxidation chain reactions, *i.e.* typically initiated by hydrogen abstraction from methylene groups of polyunsaturated fatty acids. $ROO^{\bullet}$ are the key chain carriers in lipid peroxidation (25), but $ROO^{\bullet}$ also appear to play a role in (short) chain reactions on proteins (26).

UV light, singlet oxygen ($^1O_2$), and other photochemically-generated species

UV light with very short wavelengths (UVC) is strongly absorbed by atmospheric molecules, such as ozone, and hence does not give rise to significant effects at the earth's surface. In contrast, longer wavelength UV light, particularly UVB (λ 280–320 nm) and UVA (λ 320–400 nm) wavelengths, can oxidize molecules via light absorption by suitable chromophores and generation of either excited state species (*i.e.* species with an electron in a higher orbital, type 2 photoreactions) or radicals (type 1 photoreactions), as a result of photoejection of an electron (27–29). The excited state species (usually triplet species due to the short lifetime of singlet states) can either induce direct oxidation by, for example, electron or hydrogen atom abstraction or by energy transfer to molecular oxygen O_2 to give singlet oxygen ($^1O_2$) (27–29). In addition to its formation by type 2 photoreactions, $^1O_2$ is also formed by some enzyme-catalyzed reactions, via termination reactions of $ROO^{\bullet}$, and some metal ion-catalyzed processes; $^1O_2$ is therefore an important intermediate in both light-induced and "dark" reactions (28, 29).

Negative and positive aspects of oxidative damage

Oxidant species can generate damage to all components of biological systems, including lipids, proteins, and DNA (1, 2, 4), and these modifications have been linked to a wide range of pathologies, including some cancers, neurological disorders (Alzheimer's and Parkinson's diseases and amyotrophic lateral

[2] The abbreviations used are: NOS, nitric-oxide synthase; OPA, *o*-phthaldialdehyde; DMPO, ,5-dimethyl-1-pyrroline *N*-oxide; TNB, as 5-thio-2-nitrobenzoic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid; AGE, advanced glycation end product; DOPA, 3,4-dihydroxyphenylalanine; SEC, size-exclusion chromatography; FOX, ferrous oxidation-xylenol orange; UPLC, ultra-performance liquid chromatography.

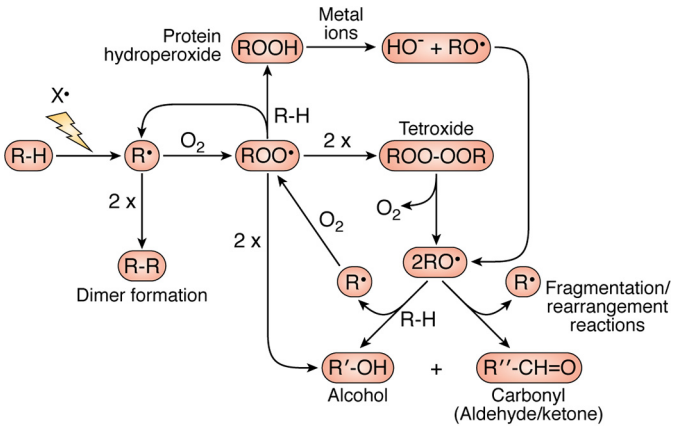

Figure 2. Summary of O_2-dependent reactions of carbon, peroxyl, and alkoxyl radical reactions on proteins and the occurrence of short-chain reactions. In this scheme, R, R′, and R″ are used to designate carbon-centered species with different chemical structures.

sclerosis), sepsis, hypertension, cardiovascular diseases, including atherosclerosis, ischemia-reperfusion injury to multiple organs, renal and ocular damage, cataracts, chronic obstructive pulmonary disease, cystic fibrosis, asthma, rheumatoid and osteoarthritis, motor neuron disease, irritable bowel syndrome, pancreatitis, hepatitis, sunburn and photodamage, and many more (1, 2, 30). Although it is likely that some of these are only associations (*i.e.* oxidation is not causative but merely a consequence of other injurious processes), oxidative damage appears, at least in some cases, to be a contributing factor (*i.e.* causative). Methodologies that can detect and quantify oxidative damage are therefore of considerable importance, not least as potential biomarkers to assess therapeutic strategies. Oxidation can also be a valuable tool in the treatment of some diseases, with radiation and photodynamic therapy of tumors (31–33), the use of redox-cycling drugs (*e.g.* doxorubicin and nitroaromatics (34)), and photochemical tissue bonding in wound closure after surgery being good examples (35). The following section outlines current knowledge of the modifications arising from oxidant interactions with proteins, and subsequently how these can be detected and quantified.

Protein modifications induced by reactive oxidants

The majority of radical reactions with proteins occur via three major pathways— hydrogen atom abstraction from C–H, S–H, N–H, or O–H bonds, electron abstraction from electron-rich sites, and addition to electron-rich centers (aromatic rings and sulfur species) (36). The first of these reactions results in the formation of carbon-centered species (R•), thiyl radical (RS•), nitrogen-centered species (primarily indolyl radicals from Trp), and oxygen-centered radicals (primarily phenoxyl radicals from Tyr). Most R• radicals, including those generated from oxidation of aliphatic side chains (Leu, Ile, Val, and Pro, etc.) react rapidly with O_2 to give ROO• at diffusion-controlled rates ($k \sim 10^9\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$) (4). Although these reactions are fast, the O_2 concentration is low in most biological samples (5–100 μM), which may limit ROO• formation and result in dimers (R–R) when the R• concentration is relatively high (Fig. 2) (37). In contrast, RS•, Tyr phenoxyl radicals, and Trp indolyl radicals react with O_2 with much lower rate constants ($k < 10^7\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ for RS•, $\ll 10^5\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ for TrpN•, and $< 10^3\ \mathrm{M}^{-1}\ \mathrm{s}^{-1}$ for TyrO• (38–40)), resulting in higher yields of cross-linked products, including disulfides (RSSR), Trp–Trp, and Tyr–Tyr (41). Crossed dimers (*e.g.* Tyr–Trp) are also known (41, 42). The chemical structures of some of the most abundant and commonly examined products are given in Fig. 3.

For ROO• generated on aliphatic side chains, hydrogen-atom abstraction is a major reaction with other *X*–H bonds, with this resulting in hydroperoxide (ROOH) formation (Fig. 2). These are major products with multiple different attacking species (4, 43). Dimerization reactions of ROO• have also been characterized, but these appear to be of modest importance, particularly when the radical flux is low (4). When this does occur, and the ROO• is a tertiary species (*e.g.* on Val and Leu), a tetroxide is generated (ROOOOR) that can decompose to give two alkoxyl radicals (RO•) and O_2 (Fig. 2) (44, 45). The RO• can subsequently undergo β-scission fragmentation reactions, to give a carbonyl compound and a further radical (Fig. 2) (46, 47). These processes may be partly responsible for the occurrence of (short) chain reactions on proteins and the generation of protein-bound (or released) carbonyls (46, 47). Alternatively, RO• may undergo hydrogen atom abstraction radicals to generate alcohols (Fig. 2). With primary or secondary ROO•, dimerization reactions yield 1 mol of alcohol, carbonyl, and O_2 (Fig. 2) (44). The major products from radical-mediated oxidation of aliphatic side chains are therefore hydroperoxides, alcohols, carbonyls, and fragmentation products.

Highly-reactive radicals such as HO• react with little selectivity (48), except when these are generated in a site-specific manner, such as by bound metal ions, with this resulting in damage localized to the vicinity of the binding site (49). Other less-reactive radicals can show marked selectivity. As most biologically-important radicals are electrophilic, they react most rapidly, and to the greatest extent, with electron-rich sites (4) resulting in damage to a subset of amino acid side chains; these data are summarized in Table 1. These include the sulfur-containing amino acids Cys, Met, and cystine, and the aromatic residues, Trp, Tyr, Phe, and His. For both Cys and His, the reactions are pH-dependent, with the rate of oxidation occurring more rapidly at higher pH values (4). In the case of Cys, a wide range of products can be formed, including disulfides and oxyacids (50–52). With Met, the major product is usually the sulfoxide (53), and to a much lesser extent the sulfone and carbon-centered radical products (53). For the aromatic amino acids, hydroxylated and dimeric species predominate, although with both Trp and His multiple ring opened products are generated (36, 54). A (nonexhaustive) list of products arising from such reactions is given in Table 2.

Radical-mediated damage can also be detected on the peptide backbone (36, 55, 56), with this appearing to occur primarily via hydrogen-atom abstraction from the α-carbon (the side-chain attachment site) (56, 57). Subsequent reactions of the initial R• formed in this process result in fragmentation of the peptide backbone, with this occurring via two different pathways involving ROO• and RO• (Fig. 4) (36, 55, 56). These pathways have been reviewed recently (4). With species such as HO•, this can result in a large range of different cleavage sites along a

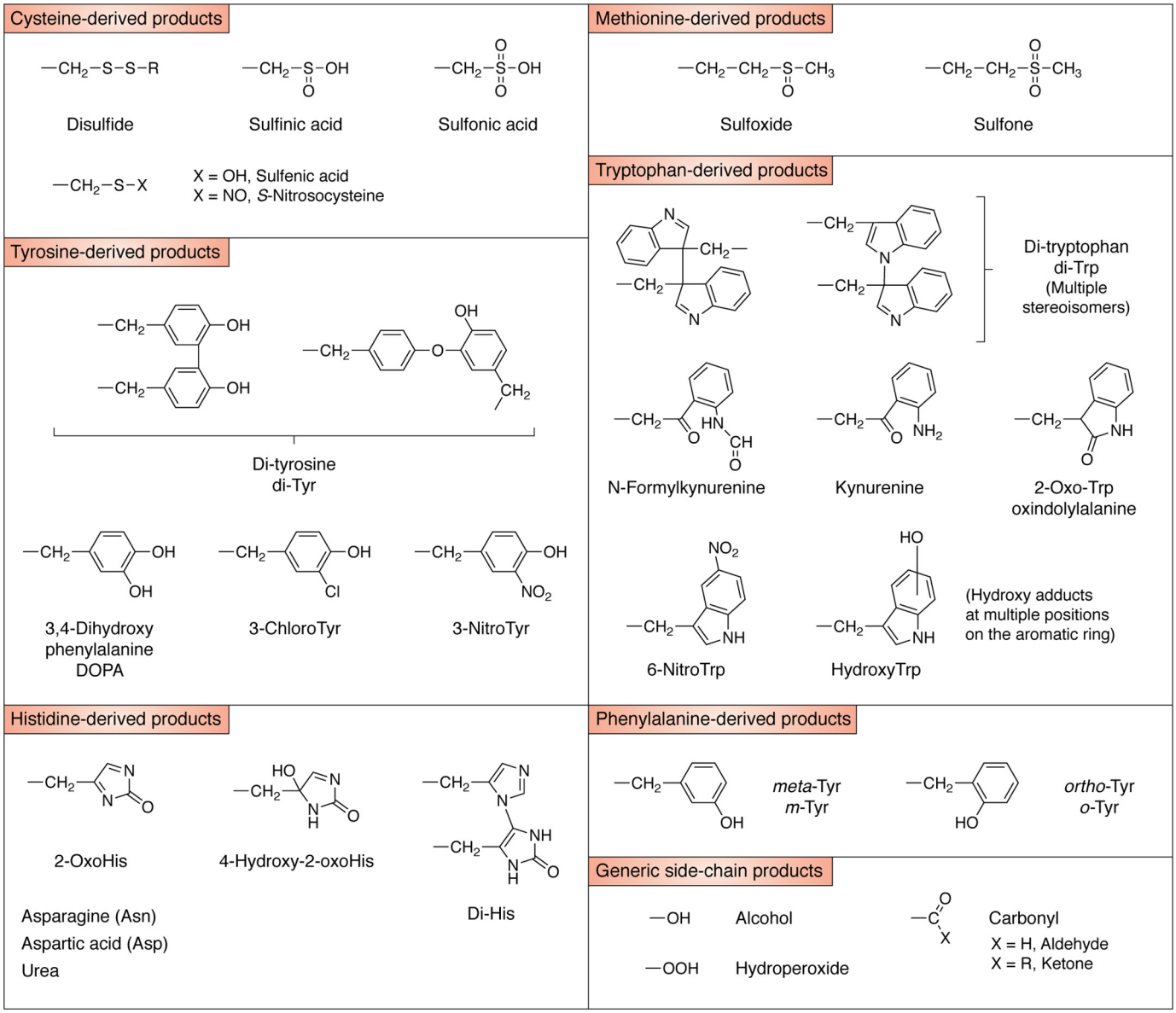

Figure 3. Chemical structures of some of the most abundant and/or commonly examined side-chain oxidation products.

Table 1

Overview of selectivity of different oxidizing species for protein side chains

These data are approximations, as the reactivity of both the oxidant and the side chains are environment- and pH-dependent: reactions at Cys, His, and selenocysteine (Sec) are particularly pH-dependent, with greater reactivity observed with the ionized species. The pK_a values of these residues are also environment-dependent and can vary significantly. These data are only an approximate indication of major sites of damage.

	Major targets
Radical oxidants	
Hydroxyl radical: $HO^{\bullet}$	All, including peptide backbone
Alkoxyl radical: $RO^{\bullet}$	All, but slower reactions than with $HO^{\bullet}$
Peroxyl radical: $ROO^{\bullet}$	Cys, Met, Trp, Tyr
Superoxide radical anion: $O_2^{\bullet-}$	Fe-S clusters
Carbonate radical anion: $CO_3^{\bullet-}$	Cys, Met, Trp, Tyr, His
Nitrogen dioxide radical: $NO_3^{\bullet}$	Cys, Tyr, Trp (and Tyr/Trp radicals)
Two-electron (non-radical) oxidants	
Peroxynitrous acid: ONOOH	Cys, cystine, Met, Trp, selenomethionine (Sec)
UVB light (λ 280–320 nm)	Trp, Tyr, cystine
UVA light (λ 320–400 nm)	No direct amino acid absorption
Singlet oxygen: $^1O_2$	Cys, Met, Trp, Tyr, His, Sec
Hypochlorous acid: HOCl	Cys, Met, cystine, His, α-amino, Lys, Trp, Tyr (slow), Sec, selenomethionine, Arg (slow)
Hypobromous acid: HOBr	Cys, Met, His, cystine, α-amino, Lys, Trp, Tyr, Sec
Hypothiocyanous acid: HOSCN	Cys, Sec
Hydrogen peroxide (H_2O_2) and other peroxides	Cys, Sec, Met (slow), selenomethionine
Quinones and aldehydes	Cys, Sec, Lys, Arg

Table 2

Selected major products (both stable and unstable) generated from oxidation of protein side chains

Products generated from the free amino acids and species formed from glycation/glycoxidation reactions are not included. This list is likely to be incomplete as the material balance (material lost *versus* products identified) is poor in most cases, indicating the probable presence of additional species. For details on the origins of these species and references, see the text. The chemical structures of some of the most abundant, or commonly examined species are given in Fig. 3.

Amino acid side chain	Three-letter code	Single-letter code	Products
Alanine	Ala	A	Serine
			Dehydroalanine
			Lanthionine (dehydroalanine–Cys cross-link)
			Lysinoalanine (dehydroalanine–Lys cross-link)
			Hydroperoxide
			Carbonyl (serine aldehyde, 2-oxo species)
Arginine	Arg	R	Hydroperoxides
			Alcohols
			Carbonyls (glutamic semi-aldehyde and others)
			His–Arg cross-links
Asparagine	Asn	N	Hydroperoxides
Aspartic acid	Asp	D	Hydroperoxides
			Carbonyl
			Decarboxylated species (serine aldehyde)
Cysteine	Cys	C	Cystine (disulfide)
			Sulfenic acid: RSOH
			Sulfinic acid: RSO_2H
			Sulfonic acid: RSO_3H
			Sulfenamide (sulfenyl amide): RS-NHR′
			Sulfinamide (sulfinyl amide): RSO-NHR′
			Sulfonamide (sulfonyl amide): RSO_2-NHR′
			Nitrosocysteine: RSNO
			Nitrocysteine: $RSNO_2$
			Sulfenylchloride: RSCl
			Persulfides: $R(S)_nH$
			Multiple adducts to α-,β-unsaturated aldehydes, aldehydes, and quinones
			Lanthionine (dehydroalanine-Cys cross-link)
			His–Cys cross-link
			Thioethers (addition products)
Glutamic acid	Glu	E	Hydroperoxides
			Alcohols
			Carbonyls (3-oxo species)
			Decarboxylated species (aspartate semi-aldehyde)
Glutamine	Gln	Q	Hydroperoxides
			Carbonyls (3-oxo species)
Glycine	Gly	G	Hydroperoxide
Histidine	His	H	Hydroperoxides and endoperoxides
			Hydroxyhistidine
			2-Oxohistidine
			Nitrohistidine
			Aspartyl urea (ring-opened product)
			Formyl asparagine (ring-opened product)
			Asparagine (ring-opened product)
			Aspartic acid (ring-opened product)
			Di-histidine cross-link (His–His)
			His–Cys cross-link
			His–Lys cross-link
			His–Arg cross-link
Isoleucine	Ile	I	Hydroperoxides
			Alcohols (hydroxyisoleucines)
			Carbonyls (3- and 4-oxo species)
Leucine	Leu	L	Hydroperoxides
			Alcohols (hydroxyleucines)
			Carbonyls (4-oxo species)
Lysine	Lys	K	Hydroperoxides
			Alcohols (hydroxylysines)
			Carbonyls (α-aminoadipic semi-aldehyde and others)
			Chloramines (RNHCl)
			Bromamines (RNHBr)
			Nitriles
			Lysinoalanine (dehydroalanine–Lys cross-link)
Methionine	Met	M	Methionine sulfoxide: RSOR′
			Methionine sulfone: RSO_2R'
			Dehydromethionine
			Carbonyls (aspartate 4-semialdehyde arising from loss of–SMe function)
			Homocysteic acid: RSO_3H (cleavage of S–CH_3 bond)
Phenylalanine	Phe	F	2-Hydroxyphenylalanine (ortho-Tyr)
			3-Hydroxyphenylalanine (meta-Tyr)
			Tyr (4-hydroxyphenylalanine)
			2- or 4-nitrophenylalanine
Proline	Pro	P	Hydroperoxides
			Alcohols (hydroxyprolines)
			Carbonyls (2-pyrrolidinone and ring-opened species such as glutamic semi-aldehyde)
Selenocysteine	Sec	U	Mixed seleno-thiol cross-linked species (RSe-SR′)
			Selenenic acid: RSeOH
			Seleninic acid: $RSeO_2H$
			Selenonic acid: $RSeO_3H$
			Dehydroalanine
Selenomethionine			Selenomethionine selenoxide
Serine	Ser	S	Carbonyls (serine aldehyde, 2-oxo species)

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Table 2 —*continued*

Amino acid side chain	Three-letter code	Single-letter code	Products
Threonine	Thr	T	Carbonyls (2-amino-3-ketobutyric acid and others)
Tryptophan	Trp	W	Hydroperoxides and endoperoxides
			N-Formylkynurenine
			Hydroxy *N*-formylkynurenine
			Di-hydroxy *N*-formylkynurenine
			Kynurenine
			Kynurenic acid
			3-Hydroxykynurenine (and downstream products, including xanthurenic acid, 3-hydroxyanthranilic acid, quinolinic acid, and picolinic acid)
			Hydroxytryptophan (multiple isomers)
			5- and 6-nitrotryptophan
			Chlorotryptophan
			Hydropyrroloindole
			2-Oxindole species
			Di-oxindole species
			Hydroxytryptophandione
			Di-tryptophan (multiple isomers with both C–C and C–N linkages)
			Trp–Tyr cross-link species
Tyrosine	Tyr	Y	Hydroperoxides and endoperoxides
			DOPA
			DOPA quinone
			Trihydroxyphenylalanine (TOPA)
			3-Nitrotyrosine
			3,5-Dinitrotyrosine
			3-Chlorotyrosine
			3,5-Dichlorotyrosine
			3-Bromotyrosine
			3,5-Dibromotyrosine
			Di-tyrosine: Tyr–Tyr cross-link (both C–C and C–O linkages
			Trp–Tyr cross-link species
Valine	Val	V	Hydroperoxides
			Alcohols (3- and 4-hydroxyvalines)
			Carbonyls (3-oxo species)

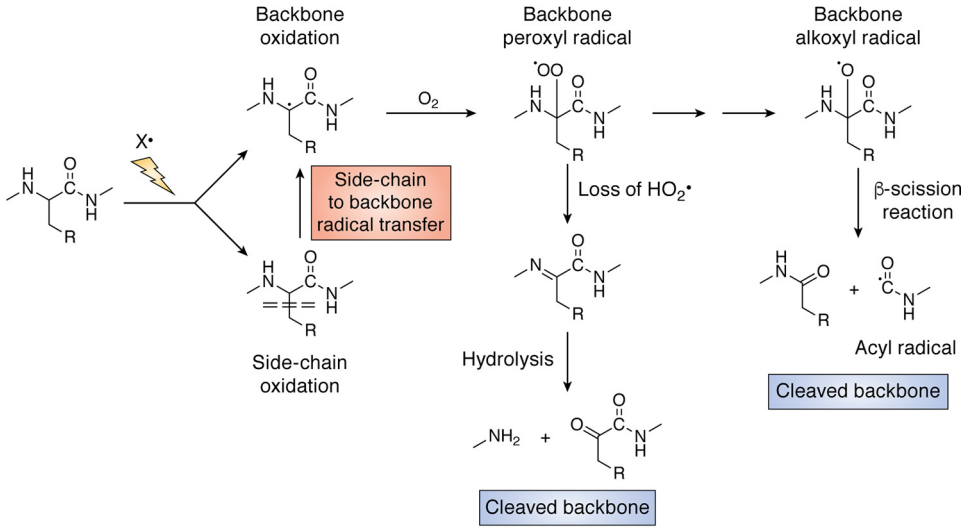


Figure 4. Overview of radical reactions resulting in cleavage of the protein backbone. This can arise from both direct reactions at backbone sites (principally at the α-carbon) and also indirectly via initial oxidation at side-chain sites with subsequent radical transfer to the backbone, either intra- or intermolecularly. For further details see main text and Refs. 3, 4, 51, 52.

protein backbone (often detected as a "smear" on protein gels), although some selectivity in fragmentation has been reported (58, 59), particularly at metal ion–binding sites (60). This may also be due to particular stabilizing factors, such as the capacity to form a planar intermediate that maximizes stabilization of the intermediate α-carbon species (61) or a high level of solvent exposure on the surface of a protein (*e.g.* at turns between helices).

Two-electron oxidants typically show markedly greater selectivity than most radicals, due to the higher-energy barriers for many of these reactions (Table 1) (4). For species such as ONOOH, direct two-electron processes can occur (19), including reaction with Cys, cystine, and Met residues to give oxygenated species (Table 1). Oxidation of metal-ion centers can also occur via two-electron pathways. However, these reactions occur in competition with homolysis of ONOOH to give radicals (and hence one-electron oxidation products), and reaction of the anion $ONOO^-$ with CO_2 to give the corresponding carbonate adduct (although the identity of this species is disputed (62, 63)). The adduct has been reported to have a short life-time (a few nanoseconds (62)) and to decompose to give $NO_2^{\bullet}$ and $CO_3^{\bullet-}$, and thereby generates radical-mediated products. As $NO_2^{\bullet}$ is formed from both ONOOH and $ONOOCO_2^-$, nitrated products are commonly detected, with these being mainly generated from Tyr and Trp residues via dimerization reactions of $NO_2^{\bullet}$ with the $TyrO^{\bullet}$ and $TrpN^{\bullet}$ species formed by $HO^{\bullet}$ or

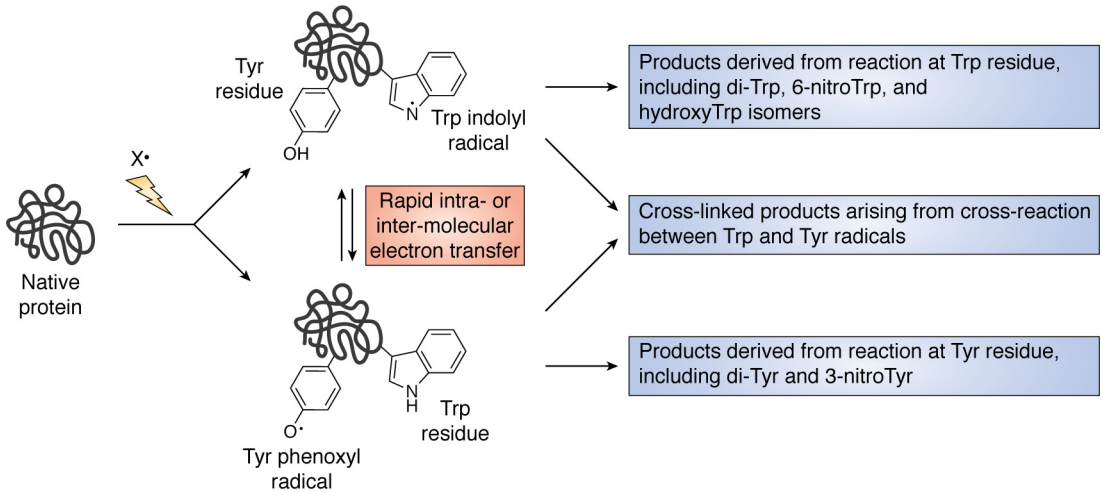


Figure 5. Initial oxidation at electron-rich sites (*e.g.* Tyr and Trp residues but also Met, His, and Cys) can result in rapid electron transfer both within, and between, protein molecules. This can result in subsequent reactions and products being formed at sites that were not the initial site of oxidation and at locations remote from the initial site.

$CO_3^{\bullet-}$ (19). More limited modifications are detected from radical chemistry at Phe and His (64, 65), but oxidation of Cys and Met likely occurs via both one- and two-electron reactions (65).

With hypohalous acids (HOCl, etc.), reaction occurs most rapidly with the sulfur amino acids (Cys > Met > cystine) to give a mixture of species (Tables 1 and 2) (66). Reaction also occurs, albeit less rapidly, with nitrogen nucleophiles (*i.e.* His, the α-amino group, and Lys) to give short-lived chloramines (RNHCl) (15, 66, 67) that retain the oxidizing capacity of HOCl but react much less rapidly and with greater selectivity (67, 68). Reaction also occurs with Trp and Tyr, although with lower rate constants (15). With Trp, oxygenated (and possibly chlorinated) species are formed (66), and with Tyr, the major product is 3-chloro-Tyr (69), a well-established biomarker of this oxidant, even though this species is formed slowly and in low yield (Table 2) (15).

$^1O_2$ reacts primarily with sulfur (Cys, Met, and cystine) and aromatic residues (Trp, Tyr, and His) (27, 29), with reaction at the former species to give the dimer (cystine) and oxygenated products (Cys oxyacids, Met sulfoxide, and oxygenated disulfides) (27, 29). With Trp, Tyr, and His, the initial products are endoperoxides formed by cycloaddition reactions, with these subsequently undergoing ring opening to give hydroperoxides, oxygenated products, and further cyclized materials (Table 2) (27, 29). As with the radical chemistry of Trp and His, these reactions can result in ring opening reactions and a similar (complex) mixture of species. The products from these amino acids therefore do not allow the initial oxidant to be easily identified.

From the above discussion it is clear that different oxidants have very different chemical behaviors and reaction kinetics, and these differences can be magnified or decreased by a range of other factors that influence oxidant selectivity. This is discussed further in the following section.

Factors affecting oxidant selectivity

The extent of damage by a particular oxidant can be modulated by multiple factors, including the accessibility of the oxidant to the target residue (*e.g.* Trp residues are often buried within protein structures and have limited solvent accessibility), and also electrostatic interactions with residues on the protein surface (*e.g.* the presence of charge on oxidants such as $O_2^{\bullet-}$ and $CO_3^{\bullet-}$). Neutral species may induce greater damage than charged species, and also at more remote locations, due to the greater propensity of such species to traverse membranes and hence diffuse away from their site of generation. The neutral species may also be better electrophiles and provide better leaving groups. This is exemplified by the greater reactivity of HOCl over $^-$OCl and ONOOH over $ONOO^-$ (15, 19). Ionization of side-chain residues on a protein increases their electron density, increasing their capacity to act as a nucleophile and be more readily oxidized; thus, the thiolate anion, RS^-, is more reactive than the parent RSH (and similarly for the related selenium-containing amino acid, selenocysteine, Sec), and the Tyr phenolate anion is more readily oxidized than the neutral phenol (70, 71).

Readily oxidized residues (*e.g.* Cys, Trp, and Tyr) can undergo long-range electron transfer reactions and thereby function as radical "sinks" both within and between proteins (72–75). Such transfers can occur over long distances (*e.g.* in ribonucleotide reductase, DNA photolyase, and photosystem II), such that initial oxidation at one site can be rapidly transferred to another remote residue, with the electron transfer occurring through bonds or space (Fig. 5) (76, 77). Consequently, the initial site of oxidation may not be the final site of modification. As the one-electron reduction potentials of Trp and Tyr are similar, radical formation at one residue can result in equilibration between residues, assuming suitable electron transfer pathways are available (73). One consequence of this is that radical termination reactions (*e.g.* dimerization of two $TyrO^\bullet$, two $TrpN^\bullet$, or cross-reaction of these species with $NO_2^\bullet$ to give nitrated products) may occur via the most *accessible*, or *reactive*, $TyrO^\bullet$ or $TrpN^\bullet$ rather than at the site of initial radical generation. Thus, cross-link formation involving Tyr and Trp radicals, and formation of products such as 3-nitro-Tyr, may be determined by the

accessibility and reactivity of a particular residue, rather than the *extent* of initial reaction at that site (Fig. 5).

In the light of these data, the next section summarizes commonly used methods to detect protein alterations, starting with modifications at the intact protein level (*i.e.* changes that markedly affect protein mass and structure: "gross changes") and then progressing to techniques that identify and quantify changes at an amino acid level, and at specific sites within a protein sequence.

Detection and quantification of protein oxidation

Gross modification of parent proteins

Oxidation of proteins can generate both fragmentation and aggregation of proteins. The latter can involve both covalent cross-linking as well as noncovalent interactions. Separation methods based on mass or charge (*e.g.* one- or two-dimensional electrophoresis and column chromatography) with subsequent detection methods (*e.g.* silver staining or immunoblotting) can provide limited information about such changes. This works best with purified proteins or limited mixtures, but it has severe limitations with complex samples and also when comparing healthy *versus* diseased samples, as the protein pools may be very different in such cases, even when two-dimensional gels are used to enhance resolution. Immunoblotting with specific antibodies can provide high sensitivity and specificity detection, but this approach is severely limited with regard to both the quantification and identification of modifications. Artifactual proteolysis or aggregation is also a serious concern. Both oxidant-mediated fragmentation and aggregation can be investigated using these approaches, but as fragmentation is often nonspecific or poorly-specific, discrete bands or spots (from 2D gels) are rare, with "band smears" being the usual outcome.

Aggregation or cross-linking is more readily analyzed, as dimers (for example) generated by any pairing of residues are likely to provide bands/spots of similar mass. Care clearly needs to be taken as multiple proteins are typically present in each band or fraction. Reduced antibody recognition of a specific native epitope can be used as a method of assessing modification to that site, in either immunoblotting studies or ELISA. These approaches are limited by the availability of specific antibodies but have been used successfully in a large number of studies ranging from isolated proteins to tissues, and they have the advantage of very-high sensitivity. Increased information can be obtained if antibodies against both parent protein epitopes, and specific products (see below), are available (78–85). An example of this approach are the studies that have examined HOCl-mediated damage to the extracellular matrix underlying endothelial cells. Binding of three specific antibodies (anti-fibronectin, anti-laminin, and anti-thrombospondin) was decreased on treatment with HOCl, implicating damage to these proteins (86). However, analysis of such data can be complex, as damage may also enhance antibody binding by exposing cryptic epitopes. Thus, low doses of HOCl appear to increase the affinity of anti-fibronectin antibodies to plasma fibronectin, whereas high concentrations have the opposite effect (87). This has been rationalized in terms of the generation of an extended fibronectin conformation at low HOCl doses, and aggregation with high concentrations.

An absence of positive data from ELISA or immunoblotting studies does not preclude the presence of damage, as epitopes may become inaccessible on protein oligomerization or as a result of other structural changes. Quantification is also challenging as this is very dependent on the sensitivity of the antibody: strong signals may be detected for low-abundance material, whereas abundant species may give a weak (or no) signal with a poor antibody. Separation of modified species by HPLC, for example, has been employed successfully with oxidants that are highly selective and that induce damage at a limited set of residues. An example is the separation of modified apolipoproteins AI and AII (88, 89) after mild oxidation of high-density lipoproteins or plasma, with loss of the parent isoforms, and the formation of newly-oxidized species, as detected by HPLC (88). Subsequent MS analysis of the fractions identified the modifications as loss of parent Met and generation of the corresponding sulfoxide.

Other biophysical techniques (*e.g.* CD, light scattering, small-angle neutron scattering, small-angle X-ray scattering, turbidity methods, X-ray crystallography, and NMR spectroscopy) can also yield information on protein structure, particularly the generation of fragments or aggregates, as these methods are sensitive to changes in protein mass, the size of particles, modified secondary structure, and altered charge and solubility. X-ray crystallographic studies have provided evidence for increased electron density between residues in aggregated proteins supporting the presence and identification of particular cross-links and their nature (exact site of linkage and intra- *versus* inter-molecular; see, for example, data for oxidized peroxiredoxin 5, thioredoxin 2, and γS-crystallin (90–92)). However, with the exception of X-ray crystallography and NMR, these methods do not provide definitive information as to the sites and modifications, and these techniques are (currently) limited to homogeneous samples (often single proteins) with high modification levels.

Total amino acid analysis

This methodology can provide important quantitative data on the consumption of parent species, arising from all potential modification reactions, and for some species the yields of products can also be assessed (*e.g.* methionine sulfoxide, see below). Such data are important with regard to obtaining a material balance, something that has been difficult to achieve even with the simplest systems. An overview of this approach is provided in Fig. 6. Differences between loss and total product formation can provide vital information with regard to the generation of alternative (known or unknown) species (see also below). Typically, proteins are isolated (*e.g.* by precipitation from homogenates/lysates using TCA or organic solvents), cleaned up (*e.g.* delipidation), and subsequently subjected to hydrolysis to give the free amino acids and products (Fig. 6) (54). Processing is preferentially carried out in the presence of enzyme inhibitors and antioxidants to decrease artifacts (54). Proteolysis can be achieved using acid conditions (with this resulting in loss of Cys, cystine, and some Trp species, although this depends on the acid), alkaline conditions (which preserves Trp species, but

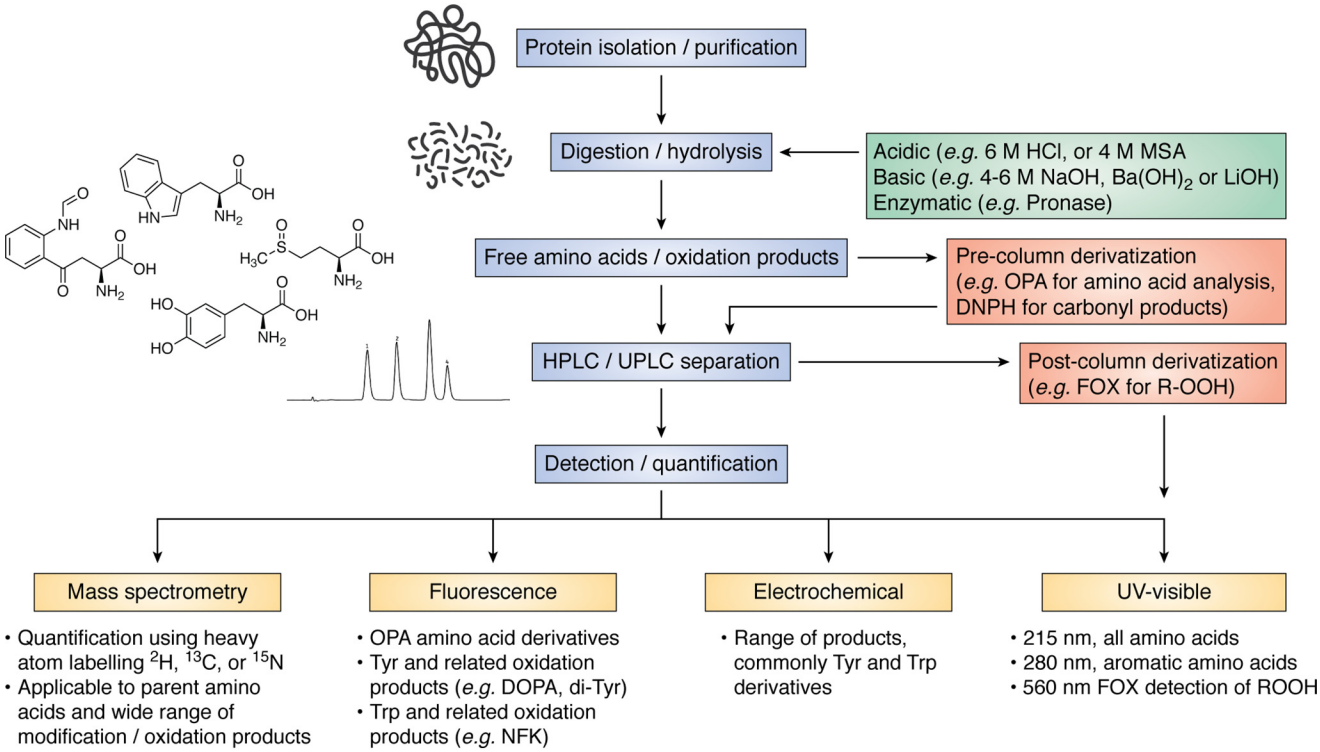


Figure 6. Workflow to assess protein amino acid composition and their associated modifications. Proteins isolated and purified before digestion or hydrolysis to their constituent amino acids. Free amino acids and/or related oxidation products are then separated by LC. For some applications, pre-column or post-column derivatization of the amino acids and related products is required before separation to enable detection and quantification using one or more detection methods, which typically include MS, fluorescence, UV or visible absorption, or electrochemical (EC) detection. Abbreviations used are as follows: MSA, methane sulfonic acid; NFK, *N*-formylkynurenine.

results in loss of other species such as the product DOPA), or nonspecific proteases, such as Pronase (54, 80, 93). The free amino acids (and any products) are then separated (*e.g.* by HPLC/UPLC) and quantified by mass spectrometry (MS), fluorescence (either directly, for some aromatic species, or by pre-column fluorescent tagging of free amino groups using reagents such as *o*-phthaldialdehyde, OPA), UV absorption, or electrochemical methods (Fig. 6) (54, 80, 93). The combination of acid hydrolysis (using methane sulfonic acid) and OPA tagging allows data to be obtained for all common amino acids with the exception of Cys/cystine (which are acid-sensitive), Asn and Gln (which are converted to Asp and Glu, respectively), and Pro (which does not react with OPA, being an imine) (54). Enzymatic hydrolysis results in lower levels of artifactual oxidation due to the mild conditions (typically overnight incubation at 37 °C, pH 7.4) and hence preservation of acid/base-sensitive materials, but quantification can be problematic due to self-digestion of the protease resulting in release of additional amino acids although this is often limited (80).

Quantitative data can be obtained by use of standard curves generated using amino acid mixtures, with heavy atom labeling (usually ^{2}H, ^{13}C, or ^{15}N) in the case of MS analysis (54). Lys quantification can be problematic due to the second side-chain amino group, which results in multiple peaks if labeling (such as tagging with OPA or other amine-reactive tags) is incomplete. An internal standard (*e.g.* homo-Arg) allows sample recovery and derivatization efficiency to be assessed (54). The use of sacrificial oxidation targets (*e.g.* tryptamine), anoxic conditions, antioxidants, and other inhibitors are important to prevent significant losses, and data are typically normalized to nonmodified amino acids to compensate for any losses during processing (54). Comparison with expected amino acid compositions is recommended for studies using pure proteins, to ensure that materials are not lost (or the extent compensated for) during processing. Some modifications (*e.g.* Trp products during acid hydrolysis) are also known to be lost during processing (see below), which may result in an underestimation of the level of damage. MS analysis can also be readily used for studies on free amino acids, peptides, and for proteins. For the last of these, analysis is usually undertaken on loss of specific peptides after digestion using trypsin (or other enzymes) or at the intact protein level. Absolute quantification can, however, be tricky to achieve (see below).

The total amino acid analysis approach is limited in that it only provides *overall* levels and not data on the *sites* of modifications within a sequence, nor the data on what proteins they might be present on, when analyzing complex mixtures. However, these data are an important complement to other approaches, such as MS peptide mass mapping, where only a limited number of species are typically analyzed.

Loss of some amino acids can be quantified by alternative methods. Direct fluorescence (typically λ_{ex} 280–285 nm and λ_{em} 340–345 nm) has been widely used to examine Trp residues (94), but this can be problematic, as Trp fluorescence is environment-sensitive (hence its use in examining protein unfolding (95)) and is difficult to use in the presence of other species that absorb or exhibit fluorescence at these wavelengths. This may include proteins with high concentrations of

Tyr residues, heme proteins, species arising from Trp degradation, and some glycoxidation products. Lys and Arg can be quantified by reagents that give strongly fluorescent derivatives, such as fluorescamine (96) and 9,10-phenanthrenequinone (97) respectively. These methods are rapid, sensitive, and give limited artifacts during sample preparation, but neither reagent is entirely specific, and hence needs to be used with care.

A number of methods have been developed to allow quantification of the loss of the key redox-sensitive amino acid Cys on proteins. Detection and quantification of Cys products are covered below. Methods include spectrophotometric (*e.g.* using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), Ellman's reagent (98, 99), or 4,4′-dithiodipyridine (100)), fluorometric (*e.g.* using ThioGlo 1, (10-(2,5-dihydro-2,5-dioxo-1*H*-pyrrol-1-yl)-9-methoxy-3-oxo-3*H*-naphthol[2,1-b]pyran-2-carboxylic acid methyl ester), a naphthopyranone maleimide derivative, and related species (101), biotinylated reagents with various detection methods, MS (102), and click chemistry (103, 104). These approaches can be used both directly on complex samples to give an overall readout or after protein separation using 1D or 2D gels (105) and HPLC/UPLC (101, 102). With fluorescent or biotinylated thiol derivatization reagents, care should be taken to ensure specificity of the probe (54). The use of MS with isotope-coded tags is a powerful method for determining the sites and extent of Cys oxidation in complex biological mixtures (reviewed in Ref. 106).

The oxidation of the major low-molecular-mass cellular thiol, glutathione (GSH), is widely employed as an oxidative stress marker (*e.g.* Ref. 107), particularly when expressed relative to its major oxidation product, the disulfide GSSG, or the total of these species (*e.g.* Ref. 108). As GSSG formation can be reversed by GSH reductase (109, 110), this reaction can be exploited to determine levels of GSH and the total of GSSG and GSH spectrophotometrically. GSSG levels can then be determined by difference in the values. The GSH concentration can be determined as described above, and also via the consumption of NADPH, the co-factor for GSH reductase (109, 110). GSSG levels can also be determined using genetically-encoded probes, such as roGFP fused to glutaredoxin; these probes are not responsive to thioredoxin-mediated changes probably for steric reasons. These probes allow real-time imaging of redox changes at specific and defined locations within living cells, although they also have some significant caveats; in particular, these are not direct oxidant probes, for they only report on the redox status of the specific environment being examined (111, 112). Kits are available for such measurements. Direct measurements of both GSH and GSSG can be achieved after separation (*e.g.* by HPLC/UPLC) with various detection methods, including electrochemistry (*e.g.* Ref. 113) and fluorescent tagging (*e.g.* with dansyl chloride or monobromobimane (114)). The levels and ratio of Cys/cystine and other thiols present in plasma have also been used as oxidative stress indicators in plasma (115). The absolute amounts, and ratio, of protein-bound Cys and low-molecular-mass thiols can be individually assessed after separation of the high- and low-molecular-weight fractions (*e.g.* by use of spin filters or protein precipitation).

Reduced and oxidized thiols on proteins separated on 1D or 2D gels can be assessed by a number of methods (reviewed in Ref. 116), including the use of fluorescent-tagging (*e.g.* 5-iodoacetamidofluorescein, a fluorescent derivative of iodoacetamide) or biotin-tagging (*e.g.* Ref. 117). Reversible thiol modifications can be examined by use of derivatization reagents after oxidant treatment, reduction of the reversible modification, and use of a second orthogonal tag (also see below and Ref. 117).

Direct quantification of disulfides (*e.g.* cystine) is complex but can be achieved by mild protein digestion methods (*e.g.* using chemicals such as cyanogen bromide or enzymatic approaches), which cleave the polypeptide backbone between the half-cystinyl residues, under conditions that minimize thiol-disulfide exchange and disulfide reduction. Diagonal electrophoresis was used in early studies (reviewed in Ref. 118), but this is now often examined using MS partial digestion and LC separation (*e.g.* Ref. 119). Other MS methods have also been developed for cystine (115).

Detection of protein oxidation intermediates

Modification of proteins and peptides by oxidants can result in the generation of a number of intermediate species that have modest lifetimes and stabilities. As the assay of these materials requires specialized methods, these are discussed separately from the analysis of long-lived ("stable") products, which are discussed later in this review. An overview of these methods is provided in Fig. 7.

Radicals

Radicals, both initiating species and those formed on peptides and proteins, can be detected by a number of approaches including direct spectroscopic methods such as UV-visible, resonance Raman, conductivity, and electron paramagnetic resonance (EPR). Because of the short half-lives of most radicals, techniques with rapid response times are required, and of the above methods, only EPR is specific for radicals (120). Measurements using other techniques can be readily confounded by more abundant nonradical species, and hence they are only typically used with very clean systems coupled to rapid radical generation methods (*e.g.* pulse radiolysis, flash photolysis, and stopped flow) (120). These other methods, although limited in applicability, can provide valuable kinetic (rate constant) data.

The use of EPR spectroscopy to detect and identify amino acid, peptide, and protein radicals in both isolated and complex systems has been reviewed elsewhere (121–123). Although this is a very powerful analytical technique for identification of radicals (the "gold standard"), quantification is very challenging due to the short lifetime of these species. This can be (partially) overcome by use of ancillary techniques such as rapid-flow methods, *in situ* photolysis or radiolysis, freeze-quenching, and spin trapping (*e.g.* using nitrone compounds, such as 5,5-dimethyl-1-pyrroline *N*-oxide, DMPO), although each of these has advantages and disadvantages (reviewed in Refs. 121–123). Spin trapping is the most widely used as it allows studies on fluids, cells, tissues, and intact animals (*e.g.* mice and rats; see *e.g.* Refs. 120, 122, 123). In this technique, a compound (the spin trap, typically a nitroso or nitrone) is added, with the aim of generating more stable, detectable adducts (Fig. 7). Analysis of the resulting spectra can then yield information on the species present (121–123). The technique is artifact-prone and needs

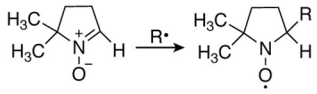
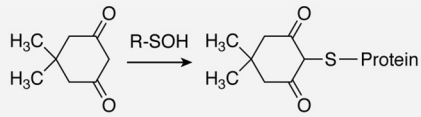
Species	Reagent	Chemistry	Detection method
Radical (R•)	Nitroso spin trap (*e.g.* 2-Methyl-2-nitrosopropane, MNP)	$H_3C-C(CH_3)_2-N=O \xrightarrow{R^\bullet} H_3C-C(CH_3)_2-N(R)-O^\bullet$	Electron paramagnetic resonance (EPR)
	Nitrone spin trap (*e.g.* 5,5-Dimethyl-1-pyrroline *N*-oxide, DMPO)		EPR Immunological methods (*e.g.* Western blotting, ELISA, microscopy, etc.)
Hydroperoxides (R-OOH)	Iodide (I^-)	$ROOH + 2\,H^+ + 2\,I^- \longrightarrow ROH + H_2O + I_2$ $I^- + I_2 \rightleftharpoons I_3^-$ (Acidic conditions)	Absorbance at 358 nm
	Fe^{2+} and xylenol orange (FOX)	$ROOH + Fe^{2+}\text{-XO} \longrightarrow ROH + Fe^{3+}\text{-XO}$	Absorbance at 560 nm
	Coumarin boronic acid	Coumarin boronic acid ⟶ 7-Hydroxycoumarin	Fluorescence λ_{ex} 320 nm, λ_{em} 430 nm
Chloramines (R-NHCl)	None		UV absorbance ~ 250 nm for RNHCl ~ 290 nm for RNHBr
Bromamines (R-NHBr)	5-Thio-2-nitrobenzoic acid (TNB)	$2\,TNB + RNHCl \longrightarrow DTNB + RNH_2 + H^+ + Cl^-$	Absorbance at 412 nm
	3,3′,5,5′-Tetramethylbenzidine (TMB) and iodide (I^-)	$RNHCl + I^- + H_2O \longrightarrow RNH_2 + HOI$ (or ICl) HOI (or ICl) $+ TMB \longrightarrow TMB_{ox} + I^- + H_2O$	Absorbance at 650 nm
Sulfenic acids (R-SOH)	Dimedone		Mass spectrometry Immunological methods for biotinylated dimedone probes

Figure 7. Approaches and experimental methods to detect reactive intermediates on proteins.

to be carried out with care. The resulting data can be definitive with regard to the species present, but also have a number of inherent caveats. In particular, the sensitivity of the method allows minor pathways to be detected and potentially misinterpreted, and the data do not provide information as to the absolute radical concentrations. Differences in the rates of trapping (or adduct decay) may make a minor species appear important when compared with a major pathway that yields very transient species. The long lifetimes of some protein–radical adducts have allowed this approach to be combined with other analytical methods, including MS, to provide detailed information (reviewed in Ref. 121).

A related method, immunospin trapping, has been developed to detect protein radicals (reviewed in Refs. 124, 125), with this method utilizing the decay of a nitroxide spin adduct to a nitrone species to generate an antigen that is recognized by an antibody. This then allows the sensitive and specific detection of the former radical species, on isolated proteins, in cells, and in animals (primarily rodents). Immunospin trapping system can also be combined with LC/MS/MS and with the anti-DMPO antibody used to screen separate fractions (*e.g.* from HPLC or size-exclusion columns) for the presence of adducted DMPO. This allows residues that previously contained a radical to be readily detected, as the mass addition arising from the presence of DMPO can be readily detected (124, 125).

Hydroperoxides—Hydroperoxides are major products of both radical- and $^1O_2$-mediated damage to proteins in the presence of O_2. These species are formed on many side chains in high yield by a wide range of insults, including oxygen-derived radicals, $^1O_2$, activated white cells, ONOOH, and metal ion-catalyzed systems (Table 2) (reviewed in Ref. 4). Decomposition of these species, which have lifetimes of minutes to many hours, by metal ions or UV light can give further radicals ($RO^\bullet$, $R^\bullet$, and $ROO^\bullet$) that propagate damage, including to lipids, other proteins, DNA, and RNA (4, 126, 127). Two-electron reduction by both low-molecular-mass reductants (*e.g.* GSH) and some protein and enzyme systems (128) gives the corresponding (stable) alcohols as products. In contrast, reaction of $ROOH/H_2O_2$ with critical Cys residues present on proteins or enzymes can inhibit enzyme activity and exacerbate damage (51, 52, 129, 130). Alcohols consistent with the formation and subsequent decay of hydroperoxides have been detected in healthy and disease specimens (*e.g.* human lens cataracts (131) and atherosclerotic lesions (132)), consistent with the occurrence of this chemistry *in vivo*. Hydroperoxides can be quantified by multiple methods, including iodometric titration, the ferrous oxidation–xylenol orange (FOX) assay, and also by use of boronic acid probes (Fig. 7) (133–135). In the first of these, reaction of ROOH with iodide ions (I^-) in the presence of acid generates triiodide (I_3^-) that can be quantified by its absorbance at 358 nm. This method is quantitative and has a well-defined 1:1 stoichiometry, but it needs be performed under strict anoxic conditions due to the sensitivity of acidified iodide solutions to O_2; it is therefore technically demanding (133). The FOX (ferrous oxidation–xylenol orange) method assays hydroperoxide-mediated oxidation of a Fe(II)–xylenol orange complex to the Fe(III) form, with the latter

quantified via its absorbance at 560 nm (134, 135). This assay is generic for all hydroperoxides, and also H_2O_2 (so samples are typically pre-treated with catalase to remove this species), and has been adapted to allow quantification of both protein- and lipid-derived hydroperoxides (134). This method has a low sensitivity to O_2, but it is not compatible with some buffers and has a poorly-defined stoichiometry; consequently, data are typically reported as H_2O_2 equivalents obtained by use of a standard curve generated using this species (136). Boronic acid probes that give fluorescent products on reaction with hydroperoxides have also been introduced (137), and these can provide real-time data, although they may be limited to *in vitro* systems, as multiple other oxidants also react with these profluorescent species (138, 139).

Chloramines/bromamines—Reaction of nucleophilic nitrogen centers (*e.g.* imidazole, amines, and amides), with hypohalous acids (HOCl and HOBr) generates *N*-chloro and *N*-bromo species (RNH*X*, where X = Cl, Br (14, 140)). These species can be formed on both the N-terminal amine and the side chains of Lys and His and to lesser extent Arg, Asn, Gln, and backbone amides (14). Quantification is possible by their UV absorption bands (Fig. 7) (λ ~250 and ~290 nm, for RNHCl and RNHBr respectively (141)), but these overlap with many other species in complex systems, so quantification is usually achieved by reaction with an added probe such as 5-thio-2-nitrobenzoic acid (TNB, which is oxidized to the corresponding dimer, DTNB (141)), with quantification achieved via the loss of absorbance from TNB at 412 nm. Oxidation of TNB to DTNB also occurs with many oxidants (*e.g.* HOCl, HOBr, HOSCN, H_2O_2, and other peroxides, ONOOH, $^1O_2$, and many radicals), and hence it is not specific for any particular species. Iodometric titration can also be employed (see section on "Hydroperoxides"), but again this lacks specificity. Iodide ions have also been used to catalyze the oxidation of 3,3′,5,5′-tetramethylbenzidine and dihydrorhodamine, by chloramines, to optically absorbing or fluorescent products (Fig. 7) (142); this method is more specific but also has some drawbacks, including slow reaction (142). *N*-Chloro species have also been identified by LC/MS, but the instability of these species at elevated temperatures limits quantitative assessment (143).

Sulfenic acids and related species—Sulfenic acids (RSOH, with the formation of these species often termed *S*-sulfenylation), sulfenyl chlorides (RSCl), and *S*-nitrosated species (RSNO, *S*-nitrosylated) are major intermediates formed by two-electron oxidants with Cys residues and related species (50–52, 144). *S*-Nitrosylated and *S*-nitrated ($RSNO_2$) species are also formed by one-electron mechanisms involving reaction of $RS^{\bullet}$ with $NO^{\bullet}$ and $NO_2^{\bullet}$, respectively. RSOH and the corresponding sulfenyl amides RS-NHC(O)R′ (145) are key intermediates in the catalytic and regulatory processes of some proteins and enzymes (52), particularly as these are formed in a reversible manner and hence may provide protection against irreversible oxidation of critical Cys residues and provide a facile "on-off" switch for enzyme activity and act as redox switches (146). Most of these species (with the exception of RSNO) react rapidly with other thiols (and other nucleophiles and oxidants) to give disulfides, thiosulfinates, and sulfinic (RSO_2H) or sulfonic acids (RSO_3H) (147). The higher oxyacids are usually irreversible oxidation products, although RSO_2H can be reduced by sulfiredoxins (148). As sulfenic acids are key redox switches, considerable effort has been expended in developing methods to quantify these intermediates (148, 149). Most methods for the detection of RSOH rely on chemical derivatization or trapping methods, with the prototypic species being 5,5-dimethyl-1,3-cyclohexanedione (dimedone), which reacts with RSOH (and other species (150)) to form a stable thioether adduct (50–52), which can be quantified by MS or by use of fluorescent or biotinylated tags (Fig. 7) (144, 151–153). Reduction of sulfenic acids by arsenite has been utilized to develop a "biotin-switch" method for labeling protein RSOH, with the reduced amino acid subsequently labeled with biotin-maleimide. The adducts can then be detected using immunoblotting with streptavidin-horseradish peroxidase or separated using streptavidin-agarose (154).

S-Nitrosated, sometimes (incorrectly) named as *S*-nitrosylated, Cys residues are also key signaling species and may act as a reservoir of $NO^{\bullet}$ (reviewed in Refs. 155, 156). Protein *S*-nitrosation has also been implicated in multiple disease states, particularly those involving neurodegeneration and inflammation (reviewed in Refs. 157, 158). The classical "biotin-switch" technique that has been widely used quantifies these species (158–160). In its classical form, nonmodified thiols are first blocked, and following the removal of excess alkylating reagent, ascorbate is added to selectively reduce any *S*-nitrosothiols (but not other species) to free thiols that are then labeled and detected by immunoblotting or fluorescent tagging following separation. Multiple iterations and improvements of this method have been proposed to enhance its specificity and to allow more rigorous quantification of RSNO levels. Many drawbacks have been reported, and considerable care and appropriate controls need to be employed to minimize artifacts (161, 162).

Multiple studies have reported MS methods to detect RSNO species (163, 164). As with other unstable intermediates, considerable care needs to be taken to avoid artifactual changes in the levels and sites of modification, as it is well-established that some RSNO species undergo ready transnitrosation reactions (164–166).

In biological systems, nitrosation is readily reversed, and this appears to be primarily driven by enzymatic reactions with reduction of low-molecular-mass species, such as *S*-nitrosated GSH (GSNO), being catalyzed by the widespread enzyme *S*-nitrosoglutathione reductase (GSNOR) (165–167), whereas removal of protein species is catalyzed by members of the thioredoxin family (168).

Persulfidation (RSSH formation) is a relatively recently identified modification of proteins that can act as both a redox control mechanism and sensor of redox stress (169). These species are formed, at least in part, via downstream reactions of H_2S, a relatively recent addition to the family of a gaseous signal transmitter family, the other members being $NO^{\bullet}$ and CO. Conversion of Cys to Cys-SSH occurs through sulfuration or persulfidation processes and may involve oxidized H_2S species and particularly polysulfides (H_2S_n), as well as other pathways. H_2S is generated from Cys and homocysteine by widely-expressed enzymes, including cystathionine β-synthase, cystathionine γ-lyase, cysteine aminotransferase, and 3-mercaptopyruvate sulfurtransferase. As these species are expressed in the vascular

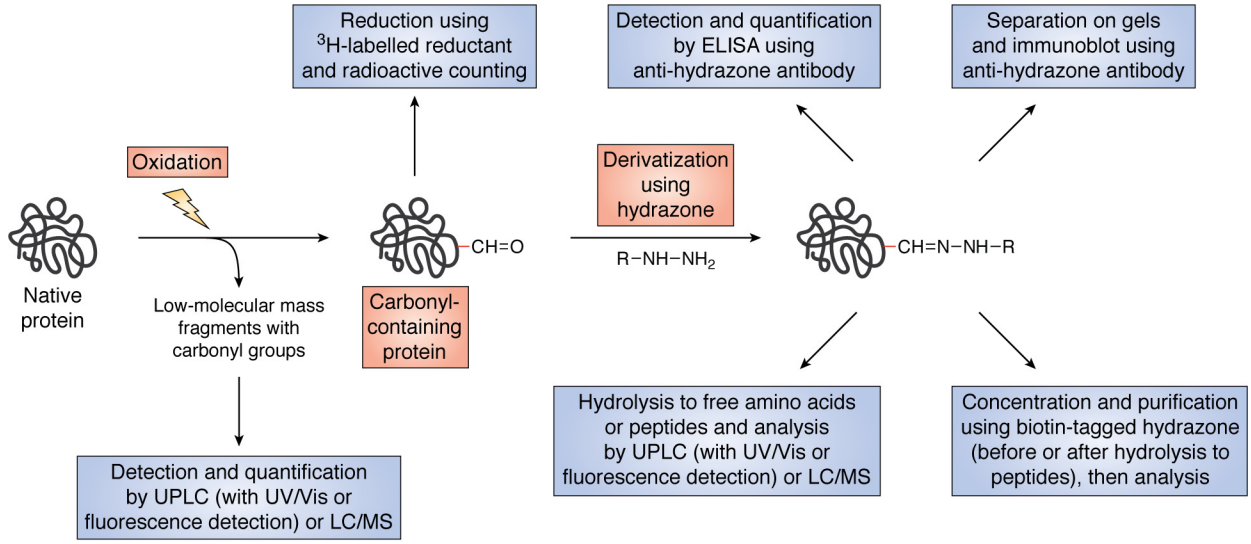


Figure 8. Overview of methods for the detection and analysis of carbonyls (both protein-bound and low-molecular mass) arising from protein oxidation.

wall, H_2S has been proposed as a regulator of vascular tone, neuronal health, the integrity of endothelial cells barriers, smooth muscle cell proliferation and survival, angiogenesis, and as a modulator of inflammation (169–171) Persulfides can be detected and quantified using a tag-switch method (172); this should not be confused with the biotin-switch method (173), by MS methods, and also by use of fluorescent dyes, although the last of these, like most fluorescent dye approaches (138, 174), is likely to be artifact-prone (175–178).

Detection and quantification of products

The above discussion of methods available to detect loss of parent materials and detection of intermediate species illustrates some of the positives and negatives of these approaches. In particular, it is hopefully clear that detecting minor losses of a parent amino acid against a large background of native species is challenging, as is the detection of transient reactive intermediates, which are often present at low concentrations. In contrast, detection of stable products can afford more compelling data and often yields higher-quality quantitative data (as detection of a small increase against a theoretical background of zero can be achieved more readily), although again none of the methods available are without significant pitfalls and caveats. A description of available methods together with their advantages and drawbacks is presented below.

Generic markers of protein oxidation—Carbonyls are generated on proteins by multiple pathways and on a wide variety of residues, although with very variable yields (reviewed in Refs. 8, 36, 179). They can also arise from glycation/glycoxidation reactions, which may confound use of these as a quantitative, and exclusive, marker of oxidation. These species are also not specific to particular oxidants (47). Carbonyls can be formed on most amino acids (Table 2), although some yield higher concentrations than others (reviewed in Ref. 36). Metal ion–catalyzed oxidation systems give relatively high yields on Arg, Pro, and Lys residues, but modification is not exclusive to these sites (180, 181). Recent advances in MS identification methods—and particularly enrichment techniques—has expanded knowledge of the amino acids that give these species their chemical identity and yields (182). Different oxidants give different patterns of carbonyls, and both protein-bound and low-molecular-mass fragments can be formed (46, 47). The low-molecular-mass species arise from fragmentation reactions of $RO^{\bullet}$ (see above and Refs. 46, 47), and these can be significant contributors to the total yield, although they are infrequently quantified. Quantification solely of protein-bound species is therefore likely to underestimate the total extent of damage (47, 183). Carbonyl levels have been shown to increase with age as well as with multiple diseases (reviewed in Refs. 8, 179, 184, 185).

Carbonyls can be quantified via their reaction with 2,4-dinitrophenylhydrazine (and related species) to give the hydrazone, with these assayed by optical absorbance (at 370 nm) or by antibodies against standards (Fig. 7) (179, 186, 187). A number of commercial kits are available that use this technology in either ELISA or immunoblotting approaches after separation on 1- or 2-D gels. The former gives the total yield of protein carbonyls, whereas the latter provides qualitative data on the proteins on which these may be present. Similar separation methods have been employed with fluorescent tags (*e.g.* fluorescein 5-thiosemicarbazide (188)).

Similar chemistry underlies the use of biotin-hydrazine and related species, which react to give the corresponding hydrazones. The latter can be reduced using cyanoborohydride, and a biotin tag can be used for enrichment before MS analysis (Fig. 8) (182, 189). This can be undertaken at both the protein and peptide level (190–192), but in the former case the high abundance of native peptides after proteolysis can result in the carbonyl products being missed, due to ion suppression and the use of only a limited number of the most abundant ions (typically parent peptide species) for further investigation (191, 192). The high affinity of biotin for avidin/streptavidin can result in a poor release of enriched materials in some cases, but this problem now seems to have been resolved by the use of 95 °C water as the eluent (182). Carbonyls can also be reduced with tritiated borohydride with subsequent and radioactive

counting (Fig. 8), but this assumes that carbonyls are the only reducible species, which may not always be correct (187). The methods available for detecting carbonyls have recently been reviewed (188).

Most Cys oxidation products, with the exception of sulfenyl chlorides (which are very transient), are formed by multiple different species and hence are not diagnostic of the initial species. Furthermore, the wide range of species (and particularly cystine) that can be generated from Cys do not typically allow these species to be easily used as markers of oxidation. As outlined above, sulfenic acids can be quantified, but their reactive nature usually prohibits accurate assessment of the absolute extent of Cys oxidation. The higher oxyacids are commonly detected in MS experiments, but they are often not quantified as they can be generated as artifacts during processing and because oxyacids can also be generated from oxidative cleavage of cystine (probably via intermediate thiosulfinate species) (193, 194). Sulfenic and sulfinic acids can also be generated enzymatically and hence are not exclusive markers of reactive, diffusible oxidants. Thus, cysteine dioxygenase enzymes, which are common in plants, have been characterized in mammalian tissues, and these nonheme iron-containing enzymes can oxidize N-terminal Cys residues to the sulfinic acid. Interestingly, these enzymes also contain an internal Cys–Tyr thioether cross-link, which markedly enhances the enzymatic activity (195). These enzymes are key regulators of hypoxia responses in both animals and plants (195). Furthermore, there is abundant evidence for enzymatic Cys oxidation occurring by way of redox relays involving proteins with highly-reactive Cys residues, such as peroxiredoxins and STAT3, with initial oxidation of one protein by oxidants such as H_2O_2 allowing subsequent oxidative transfer to target proteins in a controlled and specific manner. This process appears to be a key pathway in H_2O_2-mediated cell signaling (196–199).

Methionine sulfoxide, which exists as two stereoisomers (*R*- and *S*-), is readily formed by many oxidants, although the rate constants for its formation vary by ~10 orders of magnitude (4). Recent studies have however shown that the sulfoxide can also be generated on proteins, and particularly the key cytoskeletal protein actin, by the enzymatic action of the MICAL-family of proteins (200). This oxidation appears to play a key role in the regulation of actin depolymerization and also potentially membrane trafficking (see Refs. 200 and Fremont *et al.* (201). The detection of methionine sulfoxide may therefore not always be a marker of diffusible reactive oxidant species. Although the sulfoxide can be oxidized further to the sulfone, this is typically a slow and minor process (202). Levels in diseased tissue samples are often elevated, but the species is also readily formed by artifactual oxidation (*cf.* its ready detection in many MS analyses (203, 204)); hence, the true *in vivo* values may be overestimated. Methods have been developed to circumvent this problem (203, 204), but these are often not readily applicable to complex samples as they require complex cleavage and derivatization procedures (204) or complete oxidation of all Met residues using $H_2{}^{18}O_2$ and the use of the +2-Da mass shift arising from ^{18}O incorporation to differentiate artifactual oxidation from "real" oxidation (203). Although an improvement, this method still has problems with peptides containing multiple Met residues, but this may be resolved using a method employing theoretical isotope distributions (205). The use of significant levels of peroxide in this method may also induce other alterations. Biological levels may also be perturbed by the repair of this product by methionine sulfoxide reductases (206, 207). However, the detection of elevated (relative to control) levels can be a useful indicator of enhanced damage, and its ready and rapid oxidation indicate that this is a sensitive marker. A recent study has reported a genetically-encoded ratiometric fluorescent biosensor MetROx that allows quantification of the *R* stereoisomer (207).

Immunological detection of oxidation products—Antibodies have been raised against a number of both generic and specific products formed on peptides and proteins. The antibodies vary significantly with regard to their specificity and selectivity, with some commercial species having a very poor reputation. In other cases, sensitive antibodies appear to have been generated, but either the exact epitope is unknown or there is significant cross-reactivity with other materials. This approach therefore has to be used with extreme care, and (both positive and negative) data need to be validated using alternative methods. Some of the better antibodies show tremendous sensitivity (as good or better than the most sensitive MS machines), but quantification is a significant problem, irrespective of whether these are employed in immunoblotting or ELISA formats (reviewed in Ref. 208). Thus, the absence of a signal does not necessarily imply the absence of a product (*e.g.* this may be due to epitope inaccessibility), and a strong signal does not necessarily result from a high yield of product. Different oxidation sites on a protein, or on different proteins, may react with an antibody to a greater or lesser extent depending on the position of the epitope in the structure and the surrounding environment. This is compounded in the case of polyclonal species and by use of poorly-defined materials as the original antigen; the latter may result in the recognition of multiple species.

Accurate quantification requires authentic epitope standards, which are typically not available for peptides and proteins. Relative quantification (*i.e. versus* the parent materials) may be less problematic, but still prone to error. Nonetheless, immunoblot or ELISA data can be a useful method for determining relative changes and as a screening tool. Antibodies can also be of tremendous use in enriching or semi-purifying low concentration materials. Unfortunately, some peptide and protein oxidation products have proven to be difficult to generate antibodies against; these include some chlorinated and brominated species (*e.g.* 3-chloro-Tyr and 3-bromo-Tyr) and methionine sulfoxide. Good commercial antibodies are available for 3-nitro-Tyr, 6-nitro-Trp, and the cross-linked species Tyr–Tyr. An antibody 2D10G9 (HOP-1) raised against HOCl-modified proteins (209) also recognizes HOBr-induced damage (210), and although the exact epitope that is recognized is unknown, this antibody has nonetheless yielded useful data on HOCl-mediated damage (209, 211–213). Some commercial antibodies raised against advanced glycation end products (AGEs) also recognize multiple species (or even different products) as a result of the use of poor original antigens. Appropriate controls are therefore critical. Similarly, antibodies have also been raised against derivatized products, with a well-estab-

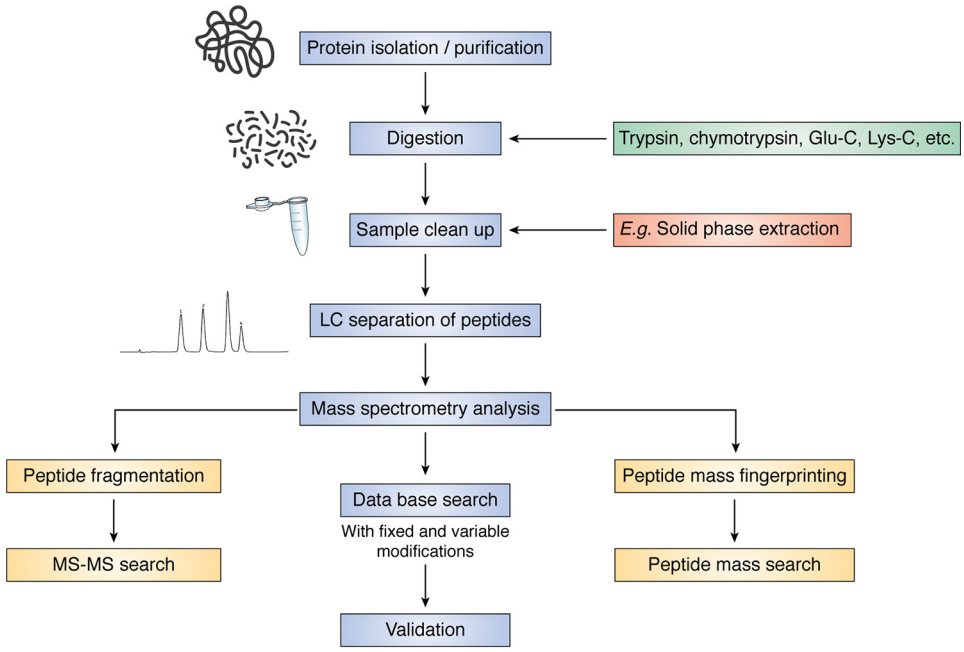


Figure 9. Workflow to assess protein modifications by peptide mass–mapping approaches. Proteins were isolated and purified before digestion to peptides with trypsin or other protease enzymes. Peptides analyzed by MS following cleanup and LC separation. Peptide mass fingerprinting analysis and/or peptide fragmentation analysis by MS-MS with appropriate database searches using fixed or variable modifications followed by validation to limit artifacts.

lished example being those raised against the 2,4-dinitrophenylhydrazones formed from the reaction of carbonyls with 2,4-dinitrophenylhydrazine (see above and Ref. 214). These antibodies, which are widely available in commercial kits, can be used for both immunoblotting (on 1D and 2D gels (215, 216), with derivatization carried out after isoelectric focusing so as to not alter the pI of the proteins (217)) and in ELISA (214). Considerable efforts have been expended to develop standardized methods to allow data to be compared with confidence across different labs (218, 219).

Detection and quantification of specific oxidation products—Aromatic side-chain modification products are widely used to assess protein oxidation, as these are typically easily oxidized and give stable products, some of which can be readily quantified (Fig. 6). Some of these materials are also informative with regard to the generating oxidant, unlike the situation with carbonyls, alcohols, most Cys products, and Met sulfoxide. Thus, 3-chloro-Tyr, 3-bromo-Tyr, and 3-nitro-Tyr/6-nitro-Trp are established biomarkers of chlorinating, brominating, and nitrating oxidants, respectively (69, 220–222). Chlorination appears to be exclusive (in mammalian systems) for myeloperoxidase-derived HOCl (14), and bromination can arise from HOBr generated by both myeloperoxidase and eosinophil peroxidase (220), and nitrating species appear to be predominantly generated via reactions of $NO_2^{\bullet}$ that can arise from ONOOH, $ONOOCO_2^-$, and also peroxidase-catalyzed oxidation of NO_2^- (19). Other Tyr and Trp oxidation products are less informative, but are still valuable markers of damage, with DOPA and Tyr–Tyr generated by multiple species (223). Similarly, oxidation of Phe to give 2-hydroxytyrosine (*o*-Tyr) and 3-hydroxytyrosine (*m*-Tyr), conversion of Trp to hydroxylated and ring-opened species (*e.g.* *N*-formylkynurenine), and His to ring-opened species occur with multiple oxidants, including $HO^{\bullet}$, some $ROO^{\bullet}$ and $RO^{\bullet}$, ONOOH, HOCl, and HOBr, and some metal–ion oxo complexes (36, 54, 93). A number of these species, although moderately long-lived, can undergo additional reactions (*e.g.* conversion of *N*-formylkynurenine to kynurenine and other species, DOPA to the quinone and cyclized products, and His-adducts to Asn and Asp (36, 54, 93)).

Multiple methods have been developed to quantify these products, including HPLC/UPLC with UV (all species), fluorescence (*e.g.* DOPA, *o*- and *m*-Tyr, and Tyr–Tyr), electrochemical (*e.g.* Tyr–Tyr, 3-chloro-, 3-bromo-, and 3-nitro-Tyr), and MS detection, GC/MS, and immunological methods (immunoblotting/ELISA) (Fig. 6) (42, 54, 93, 224). Most GC methods have been superseded by LC approaches due to an increased risk of artifacts during derivatization to make the species volatile (224). DOPA (and other catechol species) can also be detected (but not quantified) by redox staining methods after separation of the proteins by SDS-PAGE or column chromatography (225).

These methods can be employed at an amino acid level (*i.e.* after hydrolysis, see Fig. 6) to give free amino acids and products at the peptide level (*e.g.* by MS peptide mass mapping, Fig. 9) and in some cases at the intact protein level. The first of these has the advantage that this gives the *total yield* of a particular species, but results in the loss of *positional* information. In contrast, peptide mass mapping, gives precise *positional* data but is less quantitative (see below). Intact protein studies have the advantage that they do not require the extensive handling and processing of the other methods and can provide an overall picture of the extent of oxidation (as judged, *e.g.* by the presence of ions corresponding to the addition of single or multiple oxygen atoms (194)), but they do not typically provide *positional* data or quantitative data. Recent advances in column technology allow large numbers of parent and modified amino acids to

be detected in short LC runs, potentially allowing a more complete picture to be obtained as to the extent of modification of multiple species in single runs. However, control for ionization efficiency and suppression effects typically require the use of heavy isotope (^{2}H, ^{13}C, and ^{15}N) standards (226). Although these are readily available for the parent amino acids, the pool of isotopic homologues for modified amino acids is low.

Amino acid, peptide, and intact protein MS methods are therefore complementary and ideally should be employed together with measurement of parent loss (see above), allowing a material balance to be obtained. A number of recent studies have illustrated the importance of such data, as it is clear that in many cases there is a significant missing mass, consistent with the presence of multiple unidentified products (42, 227).

The powerful and increasingly widely used technique of peptide mass mapping of both native proteins and post-translational modifications, including oxidation products, has been discussed extensively elsewhere (228–232), and hence only very brief details will be given here (Fig. 9). The protein is digested enzymatically (*e.g.* with trypsin, but increasingly with other species) to release peptides that are then analyzed by LC/MS with specific peptide ions, typically the most abundant, subject to further fragmentation to give the amino acid sequence from the series of ions (usually b and y) detected (231–233). A range of different fragmentation techniques can be employed to obtain good sequence coverage (231, 232). Complete coverage (both for the parent and also modified species) is highly desirable but often is not achieved due to the generation of either very short or very long peptides (which can be outside of the detectable mass range of a spectrometer, but this is machine-dependent) or other factors (231, 232). A reduction in sequence coverage is often also encountered with modified proteins, particularly if extensive cross-linking or alteration/blocking of cleavage sites occurs (*e.g.* modifications at Lys or Arg, which prevent cleavage by trypsin) (231, 232). This can sometimes be circumvented by use of alternative cleavage enzymes. Data analysis can be automated, with both fixed and variable modifications included in the analysis. The former allows processing artifacts (*e.g.* deamination) to be eliminated, but in some cases oxidation species are also included (*e.g.* Cys and Met sulfoxide), which prevent data being obtained on these species (228, 231, 232). Variable modifications are typically specified (*e.g.* +16 and +32 from addition of one or two oxygen atoms, etc.) at defined amino acid residues. Lists of known products have been compiled in both papers (see Refs. 49, 228) and in databases (*e.g.* NCBI database, www.ncbi.nlm.nih.gov), and multiple search engines are available, but increasing the number of modifications examined increases the computational power required in a significant manner.

Directed searches are not ideal, but often employed, as only a defined subset of modifications are detected, and this therefore provides a potentially misleading picture. Open and unbiased searches (*i.e.* with no pre-defined modifications) are preferable (49), but are very time- and resource-demanding. All automated analyses should be manually validated and checked for chemical reality, which is again demanding on resources. Both loss of the parent peptides and generation of the modified species should ideally be examined, as this can limit artifacts and provide data on the extent of conversion of a particular residue to products. The number of ions chosen for secondary fragmentation, and hence sequencing, can also be a limiting and significant factor, as modified species are typically present at low levels and may be lost in a sea of higher abundance species. However, fragmentation of a larger number of ions severely impacts the run times.

Sample treatment before analysis is often necessary and can limit the information obtained. Because of the high propensity for artifactual oxidation at Cys residues during processing, and also to enhance sequence coverage of proteins containing native disulfide bonds, reduction and alkylation is typically carried out (234). This therefore results in loss of most information about oxidant-mediated changes at both Cys and cystine, although the higher oxyacids (Cys–SO_2H and Cys–SO_3H) are still detected (193). Several recent studies have, however, shown that reduction and alkylation can also decrease the levels of other modifications (*e.g.* 3-chloro-Tyr (235, 236) and others (234, 237)), although the exact reasons for this and its quantitative importance remain to be determined. Sequencing without reduction and alkylation can markedly decrease sequence coverage with disulfide-rich proteins (235, 236), as the peptides will remain covalently linked via the disulfide, and this is a particular problem with disulfide-rich species.

Quantification of modifications from peptide mass mapping studies is important, but it can be complex and subject to a large number of limitations. Data are often presented as relative site occupancies with this defined as the percentage of a particular modification at a specific site, relative to the sum of all the detected peptides (native and modified) containing the site (238). Accurate quantification therefore requires knowledge, and detection, of all modifications that occur at the site, and this is rarely achieved. If the extent of missing modified peptide data is low and the percentage conversion of the parent to products is low, the error is low, but with high extents of modification (*i.e.* only low levels of parent remain), any missing modified peptides can have a major impact. Inherent in such analyses is the assumption that the native and modified peptides behave identically under the MS conditions (*e.g.* ionization), and this is clearly not always the case (*e.g.* where suppression of ionization occurs or the modified peptide has a different number of charges). In theory, these effects can be discounted by use of standards, but this is rarely done as specific modified peptides can be costly and tricky to prepare, especially for complex samples where many parent and modified species would need to be generated and examined. Instability of modifications, under the conditions used for MS sample preparation and analysis, is another potential confounding factor as this can perturb the extent of modification detected. Thus, although direct LC/MS can be used to detect hydroperoxides, sulfenic acids, chloramines/bromamines, and *S*-nitrosated species present on amino acids and peptides (see above), these materials are often too labile to survive digestion and processing when present on intact proteins. Information on some of these materials can be obtained by derivatization (see above), but in some cases this is not possible without loss of information (*e.g.* hydroperoxides are refractory to derivatization, and reduction of the alcohol

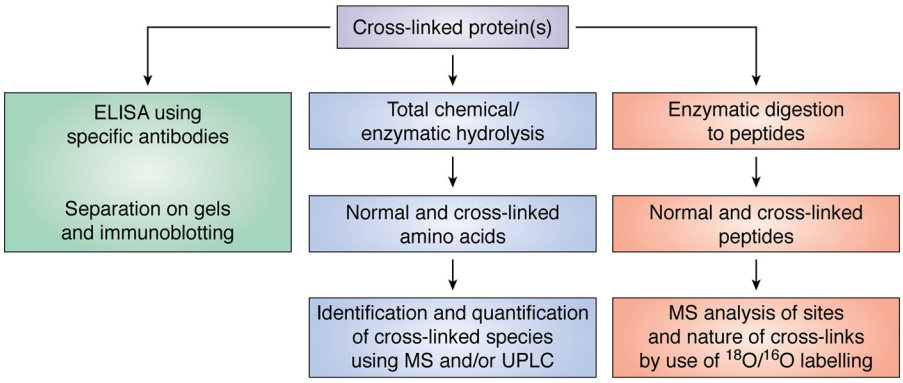


Figure 10. Overview of approaches to detect cross-links on proteins.

eliminates knowledge whether the species came from a hydroperoxide or another pathway).

Detection, identification, and quantification of protein cross-links—The generation and subsequent accumulation of cross-linked or aggregated (noncross-linked) proteins have been linked to a number of human pathologies, and hence there is considerable interest in the detection, characterization, and quantification of these species (41). Tyr–Tyr is a well-established protein cross-link, but other cross-linked species have been identified, involving DOPA quinone (from further oxidation of DOPA), Trp–Trp, Trp–Tyr, His–His, His–Lys, His–Arg, thioethers (lanthionine; from reaction of dehydroalanine with Cys), lysinoalanine (from reaction of dehydroalanine with Lys), sulfonium species derived from Met, carbonyl-Lys, and Schiff base-related species (*e.g.* involving allysine), and some advanced glycation end products (reviewed in Ref. 41). Some of these are generated enzymatically and deliberately (*e.g.* allysine species formed by lysyl oxidase and related species (239)), and Met sulfonium cross-links by peroxidasin enzymes in extracellular matrix assembly (240, 241), and some quinone and Tyr–Tyr related cross-links in insect exoskeletons and glues (242, 243), but others appear accidental and driven by oxidation (reviewed in Ref. 41).

Multiple methods have been developed to analyze for these species (Fig. 10). Direct UV absorbance, and particularly fluorescence, detection have been used for Tyr–Tyr (λ_{ex} ~280 nm or 305–315 nm, λ_{em} ~410 nm) (54), but this is problematic if other fluorophores are present (*e.g.* Trp and Trp-derived products, AGEs). Thus, prior isolation and separation are often required (see above). As with other modifications, data can be obtained at the free amino acid, peptide, and protein levels, with the workflow depending on sample, the species being analyzed, and the desired information (identity, quantity, and location).

Both GC-MS and LC-MS^n have been used to quantify *in situ* and released cross-linked species. Free Tyr–Tyr levels in urine and plasma have been quantified by both LC-MS^n and GC-MS, with LC-MS^n having a higher sensitivity (244–247), and also have been used with tissue samples such as eye lens proteins (248). Chemical hydrolysis followed by MS analysis has been employed to detect and quantify protein cross-links, with isotope-labeled standards added before acid or alkaline hydrolysis depending on the cross-link (249). For di-Trp cross-links, alkaline hydrolysis is typically used, due to the sensitivity of indoles to acid-mediated cleavage (250). Enzymatic hydrolysis has also been employed (250), although this is less efficient, and autodigestion of the protease can confound data. These methods allow (approximate) quantification of the total cross-link yield, but not positional data. The low levels of Tyr–Tyr detected in some studies with high levels of oligomerization indicate that other species must also play a major role in the detected aggregation (252).

Analysis at the peptide level typically involves proteolytic digestion and subsequent LC-MS^n analysis, but this is challenging as linear (noncross-linked) peptides usually constitute the majority of the peptides, and the cross-linked species are easily missed (41). A number of different strategies have therefore been applied to isolate or enrich cross-linked species, at the protein or peptide level, to reduce complexity and facilitate analysis. For oligomers containing intermolecular (but not intramolecular) cross-links, size-exclusion chromatography (SEC) (253, 254) or SDS-PAGE can be used to isolate cross-linked from noncross-linked species. SEC can be advantageous, as it avoids in-gel digestion that can be inefficient for cross-linked species. Strong cation-exchange and charged-base fractional diagonal chromatography can also be used for intermolecular cross-link enrichment (255, 256), as these species often have a larger number of protonatable sites (*e.g.* two N termini) than linear peptides. Multistep enrichment methods have also been reported (257).

The abundance limitation has also been partly overcome by use of ^{18}O labeling with trypsin (or GluC, LysC, or pepsin (258–260)) as the cleavage enzyme. This utilizes the capacity of these enzymes to incorporate *two* ^{18}O atoms from $H_2{}^{18}O$ into the C terminus of the generated peptides. The first ^{18}O arises from the peptide–backbone cleavage reaction, and the second is from enzyme-mediated carbonyl–oxygen exchange (261). This results in cross-linked peptides with two C termini having *four* ^{18}O atoms incorporated compared with *two* for linear peptides. Thus, proteins are digested separately in $H_2{}^{16}O$ and $H_2{}^{18}O$, and then mixed in a 1:1 ratio prior to MS analysis (Fig. 11). As the isotope analogues co-elute, a mass difference of 4 Da is observed for the linear peptides from the two ^{18}O atoms incorporated, but an 8-Da mass difference is detected for cross-linked peptides; this +8-Da shift is diagnostic for the cross-linked species (Fig. 11) (41, 262). This method has been used to identify His–His links in IgG (263), Trp–Trp links in superoxide dismutase (264), Tyr–Tyr cross-links in tropoelastin (82), and Tyr–Tyr, Tyr–Trp, Tyr–Lys, His–Arg, and His–Lys cross-

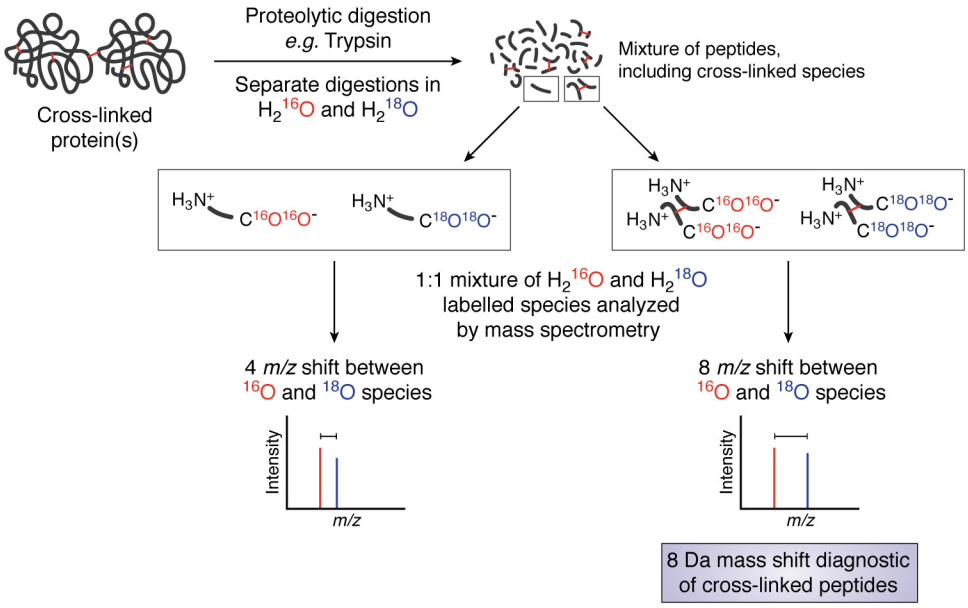


Figure 11. Outline of isotope-labeling method to detect and characterize protein cross-links using MS.

links in glucose-6-phosphate dehydrogenase, RNase, and lysozyme (42, 227, 262). This approach has also been shown to work in protein extracts from Gram-positive bacterium with Tyr–Tyr, Tyr–Trp, and Trp–Trp cross-links detected (262). This approach is cost-effective, widely applicable, and able to be used on clinical materials, as the labeling is carried out after tissue sampling and occurs only in the protease substrates (41). The method does however have some challenges, including the difficulty in achieving complete ^{18}O incorporation as the exchange reactions are sometimes slow (with this resulting in mixed isotopic isoforms, although this can be minimized with longer incubation times (262)), and protease-catalyzed back-exchange of ^{16}O, which can occur after the $H_2{}^{16}O$ and $H_2{}^{18}O$ digests are mixed (41). The latter can be minimized by combining the samples immediately prior to analysis.

MS/MS spectra of cross-linked peptides can be highly complex, due to the presence of fragment ions from both peptide chains, even though one is typically underrepresented in the fragment ion spectra (83). The reasons for this have been partly elucidated (peptide length, with longer peptides dissociating more effectively; fragmentation efficiency; and the presence of specific amino acids (251)). The fragmentation pattern can be further complicated by cross-linked cleavage (*e.g.* the C–N bonds seen in some Tyr–Trp cross-links and the N–S bonds of sulfilimines (240, 253, 264)) but not others (*e.g.* the C–C bonds of Tyr–Tyr). Electron-transfer dissociation and higher-energy collisional dissociation have been shown to generate abundant ions (262), with detection of ions from both peptides allowing the precise site of the cross-link to be determined, with some of the ions typically arising from species that retain the cross-link (42, 82, 227, 262). The detection and identification of cross-links are, however, still in its infancy, and there is likely to be significant scope for further development in this area and also with regard to the quantification of these species.

Conclusions

Over the last few years considerable progress has been made in the development of new and powerful techniques to detect, identify, and quantify modifications on proteins, but it is clear that there is still much to be done. There is a pressing need for reliable and robust methods for the absolute quantification of oxidation products formed from amino acids, peptides, and proteins, which can be applied to complex biological systems.

As many current methods are less than optimal, it is highly advisable to use multiple different analytical methods or approaches, as these are more likely to give an accurate representation. Data obtained using single methods can be misleading, even when this involves highly-sophisticated technologies such as MS. In addition, *quantitative* methods are preferable when compared with qualitative approaches, as the *absolute magnitude* of a change is much more compelling and informative than "fold differences," when there is no absolute reference value available. Thus, a 10-fold change may be of little biological consequence when it corresponds to a change from (for example) 0.1 to 1% in intact protein levels, whereas a 10-fold change from 10 to 100% is likely to be of major significance. In either case, it is critical to try and address whether the observed modification(s) and their quantitative changes are consistent with the observed biological, structural, or functional effects and to therefore address the question of causality.

Only with the development of better and complementary methodologies, as well as more accurate methods to quantify absolute changes, will the relative importance of protein oxidation/modification become clear when compared with other targets (*e.g.* to lipids, carbohydrates, and DNA) and whether protein alteration is a cause, or merely a consequence, of injurious processes.

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