Exploring oxidative modifications of Tyrosine: an update on mechanisms of formation, advances in analysis and biological consequences

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Abstract

Protein oxidation is increasingly recognized as an important modulator of biochemical pathways controlling both physiological and pathological processes. While much attention has focused on cysteine modifications in reversible redox signaling, there is increasing evidence that other protein residues are oxidized in vivo with impact on cellular homeostasis and redox signaling pathways. A notable example is tyrosine, which can undergo a number of oxidative post-translational modifications to form 3-hydroxy-tyrosine, tyrosine crosslinks, 3-nitrotyrosine and halogenated tyrosine, with different effects on cellular functions. Tyrosine oxidation has been studied extensively in vitro, and this has generated detailed information about the molecular mechanisms that may occur in vivo. An important aspect of studying tyrosine oxidation both in vitro and in biological systems is the ability to monitor the formation of oxidized derivatives, which depends on a variety of analytical techniques. While antibody-dependent techniques such as ELISAs are commonly used, these have limitations, and more specific assays based on spectroscopic or spectrometric techniques are required to provide information on the exact residues modified and the nature of the modification. These approaches have helped understanding of the consequences of tyrosine oxidation in biological systems, especially its effects on cell signaling and cell dysfunction, linking to roles in disease. There is mounting evidence that tyrosine oxidation processes are important in vivo and can contribute to cellular pathology.

Keywords :

Tyrosine oxidation; Tyrosine nitration; oxidizing free radicals; redox balance; time resolved techniques, mass spectrometry, antibody-dependent techniques.

Introduction

Biologically relevant free radicals and non-radical oxidants, often collectively known as reactive oxygen and nitrogen species (ROS, RNS), generate a variety of different responses in living organisms, from apoptosis, growth arrest and cell death to activation, differentiation and proliferation [1-3]. At high concentrations, they are hazardous and can damage all major cellular constituents. However, nitric oxide ($^{\bullet}NO$), superoxide radical anion ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2) also play important roles as regulatory mediators in signalling processes at low concentrations. Many ROS-mediated responses protect cells against oxidative stress and re-establish redox homeostasis [2-4]. Redox homeostasis is based on redox-sensitive signalling cascades that cause an increase in the expression of antioxidant or repair enzymes, or result in the activation of the cysteine transport system, which in turn increases intracellular glutathione (GSH) in many cells. A stable redox status in cells or tissues is maintained if ROS/RNS production and scavenging capacity are essentially constant and in balance. Therefore, redox signalling requires a disturbance of this balance, either by an increase in ROS concentrations or a decrease in the activity of one or more antioxidant systems [3,5-7].

Protein oxidation is important in redox-dependent physiological signalling mechanisms [8], and is known to involve a myriad of redox-sensitive enzymes and proteins, including phosphatases, kinases, peroxiredoxins, GSH peroxidases and transcription factors [2,3,9]. While cysteine (Cys) redox switches are likely to be the most common, and are the best understood, there is evidence that oxidation of other residues may also contribute [10]. However, oxidation of residues such as tyrosine (Tyr), histidine (His), tryptophan (Trp) and proline (Pro), can also have deleterious effects. Some oxidative modifications such as disulfide formation or methionine sulfoxide generation can be reversed under normal conditions [11-13]. However, more extensive oxidation of Cys residues to sulfinic or sulfonic acids disrupts signalling and can be irreversible [14]. Inappropriate or excessive oxidation can result in changes (often a loss) of enzymatic activity or function, altered protein-protein interactions, protein aggregation and defective protein turnover via the proteasomal or lysosomal systems [15]. These degradation systems are also protein-dependent and subject to damage themselves [16]. All of these effects can contribute to cellular dysfunction and chronic inflammatory or neurodegenerative diseases.

Tyr residues are prime targets for oxidation by various ROS and RNS during oxidative and nitrative stress [17]. Oxidation of tyrosine, whether present as the free amino acid or as

part of a peptide or protein, yields multiple products, with the materials generated being dependent on both the oxidant and the environment of the residue. Tyr oxidation products have been detected and quantified in a variety of tissues in order to monitor conditions of oxidative stress [18]. Individual proteins can accumulate significant levels of Tyr oxidation products, potentially affecting conformation and activity. Tyr residues are susceptible to photo-oxidative damage, which is mediated by both UVB (λ 280–320 nm) and UVA (λ 320 – 400 nm) radiation [3]; the latter is likely to be more important as the majority (~95%) of UV exposure at the surface of the earth is UVA, with only small amounts of UVB, due to the filtering effect of wavelengths lower than about 295 nm by the earth's upper atmosphere. As a consequence, the photochemistry of Tyr residues is also discussed. Furthermore, Tyr radicals have also been shown to be key intermediates in several enzymatic processes [19-21], and it is known that a number of enzymes contain Tyr radicals with these being essential for catalytic activity [21].

This review focuses on mechanisms of Tyr oxidation, either free in solution or as a protein residue. Methods used to assess these processes are discussed, especially with regard to proteins where technology has improved dramatically over the last few years. We consider both one-electron oxidation processes initiated by free radicals or enzyme systems, as well as two-electron reactions. As there is considerable current interest in biomarkers both for quantification of disease severity or progression, and as a surrogate in drug treatment, we review the known products arising from Tyr oxidation. Finally, the biological consequences of Tyr oxidation are explored.

Mechanisms of Tyr oxidation in vitro

One-electron oxidation

Reactions of Tyr with $^{\bullet}$OH radicals

The $^{\bullet}$OH-induced oxidation of Tyr, either in the presence or the absence of O_2, has been examined in detail using pulse radiolysis. Approximately 85% of the primary $^{\bullet}$OH react to give adducts ortho- (50%), and meta- (35%) to the OH-group, 5% attack the phenolic OH group directly, and the remaining 10% abstract a hydrogen atom from the β-carbon [22-25]. The molar absorption coefficients and the rate constants for their formation and decay have been measured (Table 1). Rate constants for the dimerization of the ortho- and meta-adducts have been reported [22] and are summarized in Table 2. In the presence of O_2, the dihydroxycyclohexadienyl radicals undergo rapid addition of O_2 to yield unstable peroxyl

radicals (Tyr-$O_2^{\bullet}$) (Table 2) [26]. These species decay by eliminating $HO_2^{\bullet}/O_2^{\bullet-}$, giving the hydroxylated products 3,4-DOPA or 2,4-DOPA respectively [23,27]. In contrast to these rapid processes, the direct reaction of the tyrosine phenoxyl radical (TyrO$^{\bullet}$) with O_2 is slow [28].

The only significant product detected by γ-irradiation of Tyr and its glycyl peptides [25] and proteins in aqueous solution at neutral pH has been reported to be 3,4-DOPA, possibly because of the presence of some O_2 (Table 1) [29,30]. The dimeric species 3,3'-diTyr has been detected in γ-irradiated solutions of Tyr, Gly-Tyr and poly-L-Tyr, with the yield of this product dependent on pH and the presence of O_2, with the latter suppressing diTyr formation at low and neutral pH, but not in alkaline solution [31]. 3,3'-DiTyr has also been detected in multiple proteins, including insulin, ribonuclease, papain, collagen, and lysozyme [32-34].

Reactions of Tyr with other one-electron oxidants.

One-electron oxidation of Tyr can be initiated by multiple radicals, but with considerable variation in the rate constants (Table 1**)**. Although not particularly biologically relevant, oxidation by $^{\bullet}N_3$ has been used as a convenient mechanism for generating Tyr phenoxyl radicals, and the rate constants for free Tyr are pH dependent, with an increase at alkaline pH that is greater than for peptides and proteins [35,36]. Interestingly, it has been observed that micelles protect Tyr against oxidation by $^{\bullet}N_3$, which probably relates to localization of Tyr (in aqueous phase) and $^{\bullet}N_3$ (inside micelles), rather than electrostatic effects [37]. The rate constants for halogen and pseudohalogen radicals (e.g. $Cl_2^{\bullet-}$, $Br_2^{\bullet-}$, $I_2^{\bullet-}$, $(SCN)_2^{\bullet-}$) with Tyr also increase with pH due to the increased concentration of the more reactive phenolate anion, $TyrO^-$ [38]. The reaction of chlorine dioxide ($ClO_2^{\bullet}$) is comparable to that of $Cl_2^{\bullet-}$ in alkaline medium, but much slower for the protonated forms of free Tyr and *N*-Ac-Tyr [39-42]. Its reaction with Tyr residues in proteins is very slow compared to other residues [43]. Reaction with a second $ClO_2^{\bullet}$ can lead to poly-hydroxylated products [39,44,45]. Reactions of other inorganic radicals (e.g. $SO_4^{\bullet-}$, $HPO_4^{\bullet-}$, and $PO_4^{\bullet 2-}$) with Tyr and peptides containing Tyr have also been studied, and pH-dependent rate constants and products reported (Table 1).

Nitrogen dioxide ($^{\bullet}NO_2$) has emerged recently as an important biological reactant [46] and is capable of electron (or H atom) abstraction from Tyr to form TyrO$^{\bullet}$ in a pH-dependent manner [41], which subsequently either dimerize, or give rise to 3-nitroTyr on interaction with a second molecule of $^{\bullet}NO_2$ [47]. In contrast, reaction of amino radicals ($^{\bullet}NH_2$) with Tyr

is inefficient and slow [48]. Peroxynitrite ($ONOO^-$), the ionized form of peroxynitrous acid (ONOOH), does not directly react with Tyr [49,50] but promotes Tyr dimerization and nitration via secondary species including $^{\bullet}OH$, $^{\bullet}NO_2$ [51], carbonate radical ($CO_3^{\bullet-}$), and high oxidation state redox-active metal-centers [$Me^{(n+1)+}$=O, Me = Fe, Cu, or Mn] [52]. In the presence of CO_2, peroxynitrite is rapidly converted to $ONOOCO_2^-$, which can mediate Tyr oxidation and nitration via homolytic decomposition to $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ [53-55]. The local environment appears to be important in governing the ratio of nitration to dimerization products, with nitration predominating in membranes and at sites buried in the interior of proteins [56].

Peroxyl radicals ($RO_2^{\bullet}$) react rapidly with Tyr residues [57]. Lipid peroxyl radicals ($LOO^{\bullet}$) mediate one-electron oxidation of Tyr to $TyrO^{\bullet}$ which react further$^{\bullet}$ to give 3,3'-diTyr 3-nitroTyr as described above [58]. Lipid alkoxyl radicals ($LO^{\bullet}$) are more powerful oxidants readily oxidize Tyr residues. Recent measurements of the rate constant for model alkoxyl radical (*tert*-butoxyl radical, *t*-$BuO^{\bullet}$) with Tyr indicate that reaction is enhanced at alkaline pH, consistent with a higher reactivity with $TyrO^-$ [59]. Phenoxyl radicals ($PheO^{\bullet}$) can also oxidize $TyrO^-$, although this reaction is reversible [60].

Photo-induced oxidation

Photo-induced damage to Tyr residues can occur *via* two major pathways [3,4,61,62]. The first of these involves direct absorption by the amino acid and results in the generation of electronically-excited states and photo-ionized species (Figure 1). As Tyr residues only absorb weakly above 280 nm relative to other biological molecules and other protein side-chains (e.g. Trp), direct Tyr photo-oxidation is of limited biological relevance (reviewed in [3]). However, if the triplet state of Tyr is generated, it can undergo electron transfer reactions to yield $Tyr^{\bullet}$ and subsequently $TyrO^{\bullet}$ via rapid deprotonation [3,61].

The second mode of photo-oxidation of Tyr residues involves UV or visible light absorption by endogenous (*e.g.* porphyrins, Trp and its metabolites, vitamins such as riboflavin) or exogenous chromophores (*e.g.,* drugs, polyaromatic compounds and dye molecules), with singlet and subsequent triplet formation (reviewed in [3]). The latter either undergoes decay to the ground state, or further reaction to give Tyr oxidation. Type I damage involves electron or hydrogen abstraction by the triplet state to form $TyrO^{\bullet}$ as above, whereas Type II damage involves energy transfer from the excited sensitizer to molecular oxygen to yield its first excited state ($^1O_2$ $^1\Delta_g$; reviewed in [3,63,64]), which then reacts with Tyr

residues (Figure 1). Indirect photo-oxidation of Tyr residues by $^1O_2$ is facile and fast [5,63-65] due to the relative abundance of Tyr residues on proteins compared to other $^1O_2$-reactive residues (e.g. Trp, Met, Cys and His; reviewed in [5,63]). However, photosensitized reactions typically proceed via a mixture of $^1O_2$ and radical-mediated processes [64].

Photo-oxidation of free Tyr by $^1O_2$ initially yields unstable endoperoxides (Figure 2). These rapidly decompose via ring opening to give a C1 hydroperoxide, and cyclized products involving nucleophilic addition of the α-amino group [66-69]. Thermal decay of these intermediates gives rise to a cyclized indolic product, 3a-hydroxy-6-oxo-2,3,3a,6,7,7a-hexahydro-1H-indol-2-carboxylic acid (HOHICA) [66,69]. Decomposition of the initial peroxides and cyclized products is accelerated by metal ions and UV light, and results in radical formation [68,70]. In peptides and proteins, the nucleophilic ring closure reactions of the α-amino group are less favourable (due to its incorporation into a peptide bond), and consequently reaction with other nucleophiles can compete with this cyclization (A. Wright, C.L. Hawkins and M.J. Davies, unpublished data). Such addition reactions may play an important role in protein cross-link formation via Lys, Arg or Cys residues.

One-electron enzymatic oxidation of Tyr

Enzymes play important roles in protein oxidation and multiple enzymes generate radicals during their catalytic cycles, which enable Tyr oxidation [20,71]. In particular, heme-containing enzymes catalyse free radical reactions leading to protein oxidation. Thus free hemin is able to cause oxidative cross-links in LDL, with this involving oxidation of Tyr residues on apoB-100 to 3,3′-diTyr [72,73]. Enzymatic oxidation of proteins by peroxidase enzymes and H_2O_2 also results in extensive cross-link formation involving 3,3′-diTyr and DOPA [74,75]. These reactions generate rigid structures of importance in the fertilization of sea urchin eggs, in the glues exuded by molluscs [76,77], in parasite oocysts [12,78], as well as in ligninolysis [74].

Vascular peroxidase-1 (VPO1), a member of the peroxidase-cyclooxygenase family is present in the artery wall, has been reported to catalyse Tyr oxidation [79]. The peroxidative activity of COX-2 has been reported to generate $TyrO^{\bullet}$ on amyloid beta peptide [80]. On the other hand, protein 3-nitroTyr is formed by peroxidase-catalyzed oxidation of NO_2^-, and by $^{\bullet}NO$ reaction with $TyrO^{\bullet}$ to give nitrosoTyr, that is subsequently oxidized to 3-nitroTyr [81-84]. Nitration of protein Tyr by methemoglobin or hemin has been investigated, and it was concluded that methemoglobin was more effective in oxidizing protein and more toxic [85].

Cytochrome c has also been reported to catalyse the nitration of free Tyr in the presence of H_2O_2 and NO_2^- [86][87]. In metmyoglobin the nitration reaction has been reported to involve formation of a MbFe(IV)=O intermediate that oxidizes NO_2^- to $NO_2^\bullet$ with this then nitrating the phenol ring [88]. Tyr nitration and oxidation is a hallmark of neurodegenerative diseases [89].

There is much interest in myeloperoxidase (MPO), an antimicrobial heme protein released by activated neutrophils and monocytes, and a key enzyme in inflammatory oxidant generation. Although myeloperoxidase is a major source of hypohalous acids, such as HOCl and HOBr, it also generates $TyrO^\bullet$ and $NO_2^\bullet$, which have bactericidal activity against invading organisms [90]. Considerable evidence implicates MPO in promoting oxidative stress in inflammatory diseases including ischemia-reperfusion injury, atherosclerosis, rheumatoid arthritis, periodontal disease and proteinuric glomerulopathies [91]. It has been reported that MPO activity, protein level and 3,3'-diTyr formation are increased in the kidneys of *ad libitum* fed rats during aging [92], and that MPO contributes to Tyr nitration in pulmonary and vascular inflammation [93].

Reactions of $TyrO^\bullet$

Once formed, $TyrO^\bullet$ can react further with other reactive species and radicals, thus expanding the range of products [94-96]; some of these reactions are illustrated in Table 2. Radical-radical reactions of 2 $TyrO^\bullet$ yields the 3,3'-diTyr dimer efficiently [97] with rate constants given in Table 2 [67]. It should however be remembered that the overall rates of all of these reactions are dependent on both the rate constants and the concentration of the reactants. This of particular importance for radical-radical reactions where the rate constants may be very large (and sometimes diffusion controlled) but the reactant species may be present at very low concentrations, resulting in a slow overall rate. Tyr dimerization occurs in protein oxidation processes *in vitro* and *in vivo* provided that the protein conformation(s) allows intra- or intermolecular reaction of the radicals, and sensitive assays have been developed for its quantitation.

$NO^\bullet$ reacts with $TyrO^\bullet$ in free Tyr, its *N*-acetyl derivative and Gly-Tyr (Table 2) [98]. $TyrO^\bullet$ radicals in prostaglandin endoperoxide synthase react with $^\bullet NO$ forming the nitrosocyclohexadienone intermediate, which is further oxidized to 3-nitroTyr [99]. Direct reaction of $TyrO^\bullet$ with $^\bullet NO_2$ represents a key pathway to the formation of 3-nitroTyr [100,101], an important post-translational modification in multiple pathologies and biological

aging [24,102-104]. Radical recombination of $^{\bullet}NO_2$ and Gly-TyrO$^{\bullet}$ is pH dependent [100], while in proteins the reactivity of TyrO$^{\bullet}$ with $^{\bullet}NO_2$ appears to be reduced though there is limited data on these reactions, and they are likely to be protein-dependent; for example, in ribonucleotide reductase, nitration of Tyr121 and other Tyr residues is much slower than in dipeptides (Table 2) [105].

TyrO$^{\bullet}$ reacts readily with $O_2^{\bullet-}$, predominantly via addition to the aromatic ring [67,106,107]. Electron transfer reactions, although thermodynamically feasible [27,28,108], are a minor pathway (<10 %) [67]. $O_2^{\bullet-}$ addition at the *ortho*- and *para*-positions yields cyclohexadienone hydroperoxides [67,109] which rapidly decompose unless they are stabilized through 1,4- or 1,6-additions of the amino group to the cyclohexadienone moiety (Figure 2A). The intermediate formed either releases O_2 to regenerate parent Tyr [110], or is converted into the respective Tyr hydroperoxide [109,111]. The latter process is followed by a Michael-type addition (intramolecular cyclization) and elimination of H_2O_2 [112,113]. The presence of an amino group favours hydroperoxide formation, via Michael-addition [67,106], and therefore Tyr hydroperoxides may be formed either on N-terminal Tyr residues or in basic environments (e.g. where Lys residues are in close proximity) [106], such as in Met-enkephalins [27,67,114] or Boc-Met-enkephalin in which Tyr is the N-terminal residue [114] (Figure 2B). Analogous reactions on peptides or proteins occur in the presence of exogenous amines (e.g. with added ethanolamine), providing a pathway to protein hydroperoxides. Such addition reactions may be of significance when TyrO$^{\bullet}$ is generated on membrane-associated protein domains exposed to lipid peroxidation, as has been demonstrated in a model system involving the hydrophobic Tyr analogue 7-methoxy-2-oxo-2*H*-chromen-4-yl)methyl 3-(4-hydroxyphenyl) propanoate and (6*E*,9*E*)-pentadecadiene exposed to a thermolabile azo-initiator [115]. Analogous addition products have been documented for Tyr derivatives and methyl linoleate, in addition to the formation of 3,3'-diTyr [115,116]. These reactions may not only induce protein conformational changes, but result in the formation of covalent cross-links via subsequent reactions of the protein peroxides [16,117-119], and efficient oxidation of nearby Met residues to Met sulfoxide [111,120].

TyrO$^{\bullet}$ are moderately good oxidants that oxidize thiols to thiyl radicals [121,122], though this reaction is reversible and has an equilibrium constant of 20±4 [123]. Unpaired electron migration between aromatic and sulfur peptide units is a key process in electron transfer in proteins ([123], discussed further below), but in the absence of Cys residues or other good electron donors, such as ascorbate, TyrO$^{\bullet}$ are long-lived and may undergo

reactions with other radicals. Examples include the formation of 3'-(S-cysteinyl)Tyr cross-links in galactose oxidase (EC 1.1.3.9) [124] (Figure 3) and cysteine dioxygenase [125]. These species may be generation via the formation of a Cu^{I}-phenoxyl radical complexes followed by homolytic disulfide cleavage into two thiyl radicals, radical-radical reaction between the phenoxyl radical and a thiyl radical [126], the addition of thiyl radicals to $TyrO^{\bullet}$ followed by one-electron oxidation [127], and/or the reaction of $TyrO^{\bullet}$ with thiolate, followed by one-electron oxidation [128,129].

Two electron oxidation of Tyr

Heme peroxidases such as myeloperoxidase, eosinophil peroxidase, thyroid peroxidase and related species generate halogenating oxidants such as HOCl (hypochlorous acid), HOBr, HOI, chlorine (Cl_2) at low pH, and N-chloro and N-bromo species from reactions of these species with amine and amide compounds, in the presence of H_2O_2 and halide (Cl^-, Br^-, I^-) anions. Similarly, oxidation of the pseudohalide SCN^- yields HOSCN, but other species have also been postulated [130,131]. Reaction of Tyr residues with hypohalous acids yields halogenated ring-derived species, including 3-chloroTyr, 3,5-dichloroTyr, 3-bromoTyr and 3,5-dibromoTyr. The monochlorinated species is formed relatively slowly ($k \sim 26 - 50\ M^{-1}\ s^{-1}$ [40,43,132], with secondary halogenation being more rapid ($k \sim 82\ M^{-1}\ s^{-1}$ [40]). These rate constants increase at higher pH values due to the higher concentration of $TyrO^-$. Bromination by HOBr is much faster than chlorination by HOCl (by a factor of ~5000 [132,133]). The detection of these halogenated products has been used as evidence for biological damage by heme peroxidases *in vivo*. Tyr chlorination by HOCl is proposed to involve intramolecular attack by a chloramine intermediate [40,67,134]. This reaction is optimal under acidic conditions and occurs readily in the phagolysosomal compartment of cells. 3-BromoTyr and 3,5-dibromoTyr are other major oxidative products generated by myeloperoxidase in the presence of Br^- [133,135].

The mechanism of the halogenation reactions has been studied extensively (e.g. [136]) with dispute over whether the attacking species is HOCl, molecular chlorine (Cl_2) (and corresponding trans-halogen species [135]), enzyme-bound species (for the enzyme-catalysed reactions), and amine-derived N-chloro [137] or N-bromo species (in the case of HOBr / eosinophil-mediated reactions [138]). Studies at low pH have provided data supporting an involvement of Cl_2 (and related species) [135,139], but at neutral pH it is likely that HOCl and N-halogen species are involved (i.e. formally Cl^+; though this is unlikely to be a free species [136]). Reaction of protein Tyr residues with HOSCN has also been reported [140]

with this attributed to $(SCN)_2$ rather than HOSCN, although the involvement of $(SCN)_2$ is disputed. For free Tyr alternative pathways predominate, as reaction of the free amine with HOCl and HOBr is more rapid than ring halogenation (cf. rate constants in [132]) with this resulting in the formation of N-chloro / N-bromo species. These semi-stable species undergo dehydrohalogenation reactions to give p-hydroxyphenylacetaldehyde, which can subsequently form adducts with other proteins [139,141-143].

Role of Tyr residues in long range intra- and intermolecular electron transfer in amino acids, peptides and proteins

Electron transfer (ET) processes involving Tyr residues have been demonstrated both in aqueous and non-aqueous solvents by using model peptides and proteins after selective oxidation of redox-active amino acids residues (e.g. Trp, Met, His, Cys) [144,145]. For example, peroxynitrite and peroxynitrite/bicarbonate-mediated nitration and oxidation of Tyr in Tyr-Met peptide can be stimulated by the presence of a Met residue. Intramolecular electron transfer from the Tyr residue to the Met sulfide radical-cation is proposed to account for enhanced Tyr nitration and oxidation [146].

The reduction potential of the $TyrO^{\bullet}$/Tyr redox couple has been measured to be in the range $E^0(TyrO^{\bullet}, H^+/TyrOH) = (0.85 - 0.94) \pm 0.02$ V vs. NHE at pH 7. It shows a marked pH dependence [147,148] and small differences are observed with substituted Tyr, or Tyr in peptides [149]. Time-resolved studies of Tyr in several model systems and native/modified proteins have provided evidence for the inter- and intramolecular radical transformations (summarized in Figure 4**)** involving Trp [35,150], Cys [123], His [151,152] and various Met-derived radicals [100,153,154]. Many factors influence these reactions, with rate constants for IET ranging from $<10^2$ to $> 10^7$ s^{-1}, but the kinetic diversity can be mostly explained by Marcus theory [155]. However, in some cases the structure of the peptide matrix has to be taken into account [156].

Redox-active Tyr residues play an important role in long-range electron transfer (LRET) in native biological processes [157-159]. $TyrO^{\bullet}$ have been postulated to mediate LRET in enzymes including prostaglandin H synthase (EC 1.14.99.1) [160], galactose and cytochrome c oxidases [161,162], ribonucleotide reductase (RNR; EC 1.17.4.1) [163], photosystem II (PSII) [164] and DNA photolyase [165]. It has also been suggested that electron transfer (ET) involving Tyr residues is responsible for the activation of voltage-sensitive ion channels [166]. In most of these systems, Tyr residues can act as relay amino

acids in ET reactions, since the reduction potential of $TyrO^{\bullet}$ is very low and thus allows facile redox processes [158]. Moreover, the possibility for a proton shuttle should exist in order to regenerate the Tyr radical-cation ($Tyr^{+\bullet}$) needed for the subsequent ET.

Two well-characterized examples are photosystem II (PSII) and ribonucleotide reductase (RNR) [158]. PSII contains two adjacent redox-active Tyr residues, Tyr160 and Tyr161, with the latter directly involved in a proton-coupled ET processes [167]. The other Tyr residue, Tyr160 appears to be indirectly involved in controlling ET in PSII either by the presence of Tyr160-$O^{\bullet}$ which increases oxidizing properties of $P680^{+}$ [168] or by structurally affecting the redox properties of Tyr161 [169]. Two neighboring amino acids, Gln164 and His189, might be involved in a proton shuttle to make Tyr160 a relay amino acid [170]. Involvement of peptide bonds in $TyrO^{\bullet}$-based ET reactions has been also demonstrated in PSII [171,172]. In RNR I, the active cysteine thiyl radical is generated on Cys439 by an ET over a distance of 35 Å to a $TyrO^{\bullet}$ formed at Tyr122 by a diferric cofactor [163]. This LRET can be rationalized by a multistep electron hopping mechanism, where electrons shuttle between the aromatic side-chains of a number of amino acids in a relay system including Tyr356 [163]. A further example of this involves the repair of a $TyrO^{\bullet}$ in some isoforms of superoxide dismutase B (e.g. Fe-SOD-B) by remote Cys residues [173,174]. In order to test such a "relay amino acid" concept in LRET, a family of nonapeptides consisting of a Tyr residue as an efficient electron donor at the N-terminal end, a dialkoxyphenylalanine as an electron acceptor, and a third amino acid (X) with a side chain that might act as a relay amino acid, have been designed and used for the study of intramolecular electron transfer processes and for probing the role of various amino acids residues (Met, Cys, Trp) [158,175-177].

Established and novel detection methods for oxidized Tyr in vitro and in tissues

Time-resolved methods

The time-resolved techniques such as flash photolysis and pulse radiolysis are powerful techniques for obtaining quantitative information concerning reaction kinetics and spectral properties of free radicals in solutions [178]. Free radicals are produced either *via* electronic excitation of molecules by very short pulses of UV/Vis radiation or ionization of solvent or solute molecules by high-energy electrons (> 1 MeV) [4]. Various analytical techniques can be used to quantify and identify these products. For radicals in solution, the most commonly used technique is time-resolved UV-vis spectroscopy [179], however, time-resolved

conductivity [180] , electron paramagnetic resonance [181], vibrational resonance Raman spectroscopy [182], microwave absorption spectroscopy [183,184], polarography, circular dichroism [185], and, infrared spectroscopy [186], can also be used to provide kinetic, spectral, and mechanistic details that are not accessible via absorption measurements.

Spectroscopic methods of quantifying Tyr oxidation products

NMR spectroscopy has been used to analyse and confirm the structure of a number of Tyr oxidation products including DOPA [187] and species generated by singlet oxygen [68], but this method is limited to relatively clean systems, and is not a method of choice for complex systems, or even for some intact proteins, due to the large number of overlapping resonances that prevent identification of low levels of oxidative modifications. This technique has, however, proved to be very useful in other cases, including assessment of the translational incorporation of DOPA residues into proteins [188].

Electron paramagnetic resonance (EPR) spectroscopy (and related techniques such as Electron Nuclear DOuble Resonance, ENDOR) has been used extensively to provide direct evidence for $TyrO^{\bullet}$ formation from free Tyr, and Tyr within peptides and proteins. The wealth of detail afforded by the hyperfine couplings of the (delocalized) unpaired electron has allowed detailed analysis of the electron distribution in these species (and how this is affected by local structure / neighbouring residues [189]), the conformation of the ring relative to the methylene ($-CH_2-$) group linking the ring to the backbone [190,191] and peptide conformations. The *ortho* and *meta* ring proton couplings show only small variation for neutral radicals, but the methylene proton couplings vary dramatically on altering the rotation angle between the (planar) aromatic ring and the C-H bonds allowing exquisite detail to be obtained with regard to the orientation of the phenoxyl radical ring relative to the surrounding protein backbone [190]. The variation in these couplings has allowed multiple different radical sites on different $TyrO^{\bullet}$ to be elucidated in proteins [191]. Examples include Tyr radicals formed on myoglobin [61,192-194], haemoglobin [194,195], leghemoglobin [196,197], cytochrome c [198], prostaglandin synthase [99,160,199-201] , in photosystems [191,202,203], galactose oxidase [161], and various ribonucleotide reductases [72,191,204]. Data on the rates and barriers to rotation have also been obtained for free $TyrO^{\bullet}$ [190]. Tyr-derived radicals have also been detected by EPR spin trapping, in which $TyrO^{\bullet}$ is trapped, using nitroso or nitrone compounds, to give longer-lived radicals adducts where the adducted radical can be identified on the basis of the hyperfine coupling constants [5,193,205,206].

Whilst this technique has some advantages over direct detection, not all radicals are rapidly trapped, and hence the absence of an adduct signal does not necessarily equate to the absence of a radical [5].

Raman (including UV resonance Raman) and infra-red spectroscopy have been used to detect and identify $TyrO^{\bullet}$ on peptides and proteins, particularly in situations where these are relatively long-lived and / or on isolated proteins or in purified fractions. These techniques are of particular value in examining the ionization state of the -OH group, the presence and nature of hydrogen bonding with this function [207-209], and the conformation of the phenol with regard to the surrounding protein / peptide structure [19]. These methods have also been used extensively to examine $TyrO^{\bullet}$ in photosystems (e.g. [210,211]) and plastoquinones [211]. Moreover, these techniques have helped elucidate Tyr oxidation in beta-amyloid peptide [212], the formation of oxidation products that have distinctive bands such as quinones, and metal ion complexes with these species [209,213].

Redox staining methods

DOPA and quinone formation in proteins and tissues has been examined using a redox staining approach using nitroblue tetrazolium and glycine as a reductant at high pH [214,215]. Redox cycling of these quinones, catalyzed by glycine, results in the reduction of the tetrazolium salt to (insoluble) formazan. This process can be applied to proteins separated by 1- or 2-D SDS-PAGE allowing proteins containing quinones to be detected (and subsequently identified by mass spectroscopic sequencing techniques). This approach has also been used to examine proteins with quinone co-factors, and also the biosynthetic incorporation of DOPA in to new proteins by cells [216].

Colorimetric assay of Tyr hydroperoxides

Hydroperoxides and related peroxides can be (at least semi-) quantified using the FOX (Ferrous Oxidation–Xylenol orange) assay. This method utilizes hydroperoxide-mediated oxidation of a Fe(II)-xylenol orange complex to the Fe(III) form, with spectrophotometric quantification at 560 nm [217]. The Fe(II)-xylenol orange complex reacts with many peroxides and there is little distinction, apart from kinetically, between different peroxidic species. It therefore cannot distinguish between different Tyr hydroperoxides (e.g. ring isomers), and care needs to be taken to remove H_2O_2 (e.g. using catalase) that confounds measurements. However, the time period needed to remove H_2O_2 may also result in Tyr hydroperoxide decomposition. Furthermore, the stoichiometry of peroxide reaction with

Fe(II)-xylenol orange is not always well established, and is known to vary [217]. As a consequence, only relative data can be obtained, with values typically reported as H_2O_2 equivalents. This method has been used to examine peroxides formed by $^1O_2$-mediated oxidation of free and protein Tyr residues [68,70], and peroxides from reaction of $O_2^{\bullet-}$ with TyrO$^\bullet$ and related species [106,107,218]. Hydroperoxides can also be assayed by potassium permanganate- and iodometric-titrations [219]. For heterogenous systems (e.g. those containing lipid hydroperoxides), assays have been developed in which the lipid and protein components are separated before quantification [220].

Antibody-based analytical techniques

Antibody-based methods such as ELISA and Western blotting are widely employed due to their ease of use, but they depend on the availability of antibodies selective for the analyte of interest. Tyr oxidation products are typically detected by use of antibodies that recognize them directly, though not all antibodies are validated for all procedures. For example, some antibodies developed initially for immunocytochemistry (ICC), immunohistochemistry (IHC) or Western blotting are not suitable for immunoassays or immunoprecipitation. Immunostaining approaches such as IHC and ICC provide information on spatial localization and cellular or tissue occurrence of Tyr oxidation, while Western blotting offers more information on the proteins modified (though see also below). However, these methods are semi-quantitative at best, dependent on scoring of stain intensity or densitometry. In contrast, immunoassays such as ELISAs generate a numerical output and use of appropriate standard curves allows assessment of total levels of Tyr oxidation, although there may be a number of complicating factors.

One of the earliest reports of an ELISA for 3-nitroTyr was a competition assay involving immobilized antigen (nitrated BSA) and a polyclonal anti-3-nitroTyr from rabbit (TCS Biologicals, Bucks, UK) in solution with the sample [221]. The polyclonal antibody was reported to have low affinity for 3-chloroTyr, phosphoTyr, 4-nitrophenylalanine or free 3-nitroTyr; it also discriminated against 3-nitroTyr in different peptides, but consequently was only semi-quantitative with mixtures of proteins. However, a sandwich ELISA using two mouse monoclonal IgG clones (HM.11 and HM.12) was subsequently reported to be more specific [222]. The HM.11 clone is commercially available, and all of these antibodies are compatible with immunoblotting. Franze et al. have compared several anti-3-nitroTyr antibodies for use in immunoassays for nitrated proteins, and concluded that sandwich assays were better than one-sided assays, owing to less interference from native proteins [223].

These assays are very sensitive, with limits of detection in the picomolar range. A solid phase ELISA, involving binding to a nitrocellulose membrane and detection by ^{125}I-labelled IgG as the secondary antibody and beta-counting for quantification has also been used in studies on infants with bronchopulmonary dysplasia [224] and carbon monoxide-exposed endothelium [225]. More recently, a custom ELISA microarray platform for 3-nitroTyr analysis in clinical plasma samples has been reported, which uses a biotinylated antibody (from Hycult Biotechnology) [226]. These applications are summarized in **Table 3**.

Western blotting has been used extensively to examine protein-bound 3-nitroTyr, [226-228]. Detailed protocols have been published [229], and the efficiency of separation can be increased by use of 2D gel electrophoresis followed by MS detection, as described below. Appropriate choice of conditions for immunoblotting, and in particular the absence of reducing agents such as DTT, is important, as 3-nitroTyr can be converted to 3-aminoTyr in the presence of DTT and heme proteins, and this may contribute to signal loss [230]. Interestingly, Dragusanu *et al*. investigated the nitropeptide binding affinities of the mouse mAb MAB5404 from Chemicon International (Switzerland), and found that binding was enhanced by the presence of arginine or lysine positioned 2 to 3 residues away from the 3-nitroTyr on the N-terminal side, showing that antibody responses to proteins may be sequence-dependent [231].

In contrast to many studies on 3-nitroTyr, much less attention has been paid to other Tyr modifications. A monoclonal antibody against KLH conjugated to 3(*p*-hydroxyphenyl) propionic acid dimer (i.e. an analogous cross-link to 3,3'-diTyr) has been and used to demonstrate the presence of 3,3'-diTyr in atherosclerotic plaques by Western blotting [232]. This antibody has also been used in immunoblotting experiments to show that copper can induce 3,3'-diTyr cross-links in β-amyloid [233], and in RANTES and IL-8 [234]; the mAb and ELISA kit are commercially available from JaICA.com. More recently, a mAb that recognizes protein-bound 3,5-dihalogenated Tyr, and especially 3,5'-dibromoTyr, has been reported; this species does not recognize mono-halogenated forms and other modifications such as 3-nitroTyr [235,236]. These antibodies have been used both in ELISAs and in IHC of mouse liver following exposure to lipopolysaccharide [236].

Mass spectrometry

A major challenge in analysing Tyr oxidation is to determine the exact sites of modifications in proteins, with the only routine method being mass spectrometry. This is critical, because otherwise the protein modified cannot be identified with confidence. For

example, a band or spot on a gel may stain positively with anti-oxPTM antibodies in Western blot experiments, but parallel analysis of the band or spot by peptide mass fingerprinting may identify several proteins present at that location, and the modified protein may not necessarily be that with the highest abundance. Therefore most other methods, such as Western blotting or chromatography, are at some stage interfaced or integrated with MS analysis.

The procedure is briefly summarized in Figure 5. Most work has been carried out on Tyr nitration, although research has been carried out on chlorination and hydroxylation. For 3-nitroTyr, the methods can be divided into label-free and label-dependent approaches; the latter can be used to both selectively separate 3-nitroTyr containing species and to provide fragmentation patterns to facilitate MS identification. The most routine methodology involves identifying proteins present in bands of 1D gels, or spots in 2D gels by peptide mass fingerprinting, usually carried out by MALDI-TOF MS, and confirming the presence of 3-nitroTyr by the addition of +45 a.m.u. to the peptide mass, relative to the mass of the unmodified peptide containing Tyr. However, the laser excitation used in MALDI can lead to loss of either 1 or 2 oxygen atoms from the nitro group to form 3-nitrosoTyr or 3-nitreneTyr respectively. While this has potential as a diagnostic signal for 3-nitroTyr, it also reduces the signal intensity of the +45 species and therefore the sensitivity of the method [81,237]. This fragmentation is not observed with electrospray ionization, where nitrated peptides or fragment ions containing 3-nitroTyr are only observed at +45 a.m.u compared to the native peptide and fragment ions. This approach is relatively straightforward for purified proteins, such as in a study of $ONOO^-$-oxidized GroEL where nitrated peptides were confirmed as containing 3-nitroTyr using collision-induced dissociation MS/MS [238]. Vana's group carried out detection of 3-nitroTyr in isolated tau and mutant tau proteins nitrated *in vitro* by this approach, with MS/MS sequencing [239-241]. It has also been used to study protein nitration in *Arabidopsis thaliana* [242]. The delayed elution in reverse phase chromatography of nitrated peptides, which are more hydrophobic than the non-modified peptides, can be useful in identifying 3-nitroTyr-containing peptides.

Analogous label-free but untargeted approaches have been used to detect both 3-chloroTyr and DOPA on the apo-AI protein of HDL [243,244]. In the former case, 3-chloroTyr was detected on human apo-AI protein treated with MPO-H_2O_2-Cl^-; tryptic peptides were analysed by LC-ESI/MS/MS and Tyr chlorination was identified by a mass increase of 34 a.m.u., with chlorination reported to occur preferentially at Tyr 192 [245,246]. More recently, selected reaction monitoring using specific transitions for the peptide containing Tyr192 (LAEYHAK) have been used to demonstrate the presence of 3-nitroTyr on

apoA-1 isolated from human plasma HDL and atherosclerotic lesions. DOPA, and its oxidation product dopaquinone, were detected in mouse brain and heart tissues by LC-MS/MS analysis [244]. An initial fractionation using a strong cation exchange column was carried out before nano-LC-MS/MS, and the data were searched for 3-nitroTyr, DOPA, and dopaquinone by applying dynamic modifications of 44.9851, 15.9949, and 13.9793 Da on Tyr residues, respectively. DOPA and dopaquinone modifications were found to be more abundant than 3-nitroTyr. DOPA modified peptides were also detected in *E. coli* and HeLa cell mitochondria using similar methods [247,248]. PTMap, a sequence alignment software for unrestricted, accurate, and full-spectrum identification of post-translational modification sites, was used to identify DOPA-containing peptides before manual validation [247]. These authors also reported a high abundance of proteins containing DOPA (2.5% in E. coli and 0.5% in HeLa mitochondria), whereas 3-nitroTyr containing peptides only represented 0.00001-0.001% of total proteins [249].

In label-free approaches for protein nitration, the occurrence of the 3-nitroTyr immonium ion at m/z 181.06 is often used as an additional marker in the b and y ion series spectrum to confirm the presence of 3-nitroTyr. It can also be used for targeted scanning routines such as precursor ion scanning. Such an approach is important for analysis of complex mixtures, as it is very difficult to undertake manual sequencing to confirm nitro-PTMs on numerous proteins. Precursor ion scanning involves peptides containing the modification of interest fragmenting to give a diagnostic MS^2 daughter ion whose detection indicates the presence of the oxPTM in the parent ion. Petersson et al. nitrated angiotensin II and bovine serum albumin in vitro with tetranitromethane, and the formation of 3-nitroTyr was detected by precursor ion scanning for the 3-nitroTyr immonium ion at *m/z* 181.06 [250]. The site of modification was confirmed by peptide sequencing, which can also identify the source protein. However, it should be noted that precursor ion scanning at low resolution may result in false positives owing to the occurrence of isobaric ions and a protocol for confirming 3-nitroTyr is recommended [251]. Similar problems with MS^2 precursor ion scanning for 3-nitroTyr have been reported [10,252], but an MS^3 strategy could be used to obtain more specific diagnostic fragment ions for 3-chloroTyr, DOPA, 2-hydroxytryptophan and 5-hydroxytryptophan. Alternatively, high resolution selection of product ions in pseudo-precursor ion scanning on accurate mass instruments offer improvements for the analysis of both 3-chloroTyr and 3-nitroTyr [253].

In view of the possibilities for misidentification, data analysis software tools for stringent data handling and avoidance of false positives are important especially with label-

free methods [254]. One example is Peptizer software, which works on "agents" constructed from diagnostic ion and MS parameter rules [255]. The use of a set of 3-nitroTyr peptides from 48 proteins as standards to optimize methodology has been proposed [256]. It is important to note that the fragmentation method used influences the ability to detect modifications. ECD fragmentation is not as good for nitrated peptides as slow heating fragmentation methods (e.g. CID), especially for 2+ charge states, although it has advantages for top-down analysis of proteins [257,258].

Even with targeted MS methods, the detection of Tyr modifications is difficult due to their low abundance and the high background of unmodified proteins. Many groups have attempted to enrich peptides and proteins containing oxidative modifications, with most effort (and success) directed at 3-nitroTyr. Although immunoprecipitation (IP) methods with anti-3-nitroTyr antibodies have been used, these have not always proved effective [259]. Other enrichment methods have been developed, involving the specific labelling of 3-nitroTyr with tags that allow separation of nitropeptides by solid phase extractions. The first stage in all of these procedures is the reduction of 3-nitroTyr to 3-aminoTyr, a more chemically reactive group that can be derivatized with different reagents [260]. Many different labelling strategies have been tested, and are summarized in Table 4. These methodologies are reported to improve the detection levels and sensitivity, and often reduce the false discovery rate, as reported for example in mouse brain homogenate [261].

Overall, label-free MS methods for various oxPTMs have the advantage of simplicity, but depend on targeted scanning methods. Enrichment methods have only been developed to a major extent for 3-nitroTyr, but these do increase coverage and detection in complex mixtures, and some also provide convenient diagnostic fragment ions for targeted MS methods [262-264]. With all approaches, meticulous examination of the sequence data is required, standards are ideally required [256] and approaches for verification, at least for 3-nitroTyr, have been suggested [254]. A final caveat that needs to noted, when using reduction of 3-nitroTyr to 3-aminoTyr, is the assumption that the proteins containing 3-aminoTyr correspond to those that contained 3-nitroTyr *in vivo*. This may not always be the case. For example, 3-aminoTyr in plant extracts was identified using a label-free approach, suggesting that this material may occur naturally [242].

Tyr oxidation and nitration *in vivo*

The availability of antibodies against 3-nitroTyr, together with the MS techniques described above, have resulted in many studies on the contribution of Tyr oxidation,

halogenation and nitration to pathologic processes (Table 3), including neurodegenerative diseases [265,266], cardiovascular dysfunction [267,268] and cancer [269-272]. Some proteomic studies have provided extensive lists of protein targets for Tyr oxidation and nitration *in vivo* [81,244]. A key question arising from many of these studies is whether Tyr modification is causal in these processes, or merely a consequence (bystander) without biological function. Oxidants which modify Tyr (e.g., peroxynitrite and HOCl) usually also react with other amino acids (e.g., Cys and Met) and it is not sufficient to merely document oxidation or nitration of Tyr on a protein in order to correlate Tyr modification with biologic function. An interesting example is the controversy surrounding apoA-I activation of lecithin:cholesterol acyltransferase (LCAT), where separate studies reported on the critical role of Tyr chlorination and Met oxidation [273]. However, while the extent to which the oxidation and/or nitration of individual amino acids contributes to protein dysfunction in specific cases may be uncertain, the accumulation of oxidized and nitrated proteins *per se* may be of clinical relevance. Several studies have evaluated Tyr nitration in specific cancers, and revealed a correlation between nitration and clinical prognosis, including long-term survival [271]. One possible rationale for this association may be nitration of specific chemokines, which negatively impacts on leukocyte recruitment to tumors, i.e. a weakening of the immune response towards cancer cells [272].

Repair of oxidized Tyr compounds

Repair of TyrO$^\bullet$ by various systems in vitro

Repair of TyrO$^\bullet$ by reductants such AscH$^-$, GSH, Cys, GSeH, SeCys, urate, and Trolox have been observed for both free Tyr, and Tyr in peptides and proteins [34,128,274-278]. Ascorbate anions reduce free TyrO$^\bullet$ effectively and the rate constants for reaction with GSH, urate and Trolox have been determined both for model compounds and proteins [274-276]. The variations are typically small, indicating that the location of TyrO$^\bullet$ in the protein chain is not a significant barrier to reaction with ascorbate [34].

Table 5 summarizes data for a range of species including *N*-Ac-Tyr-NH_2 and insulin, with GSH, Cys, GSeH and SeCys. For protein TyrO$^\bullet$ (e.g. in insulin) there is a significant reduction in reactivity (3 and 2 orders of magnitude) compared to free TyrO$^\bullet$ [278]. Dimerization of TyrO$^\bullet$ was inhibited by cysteine or O_2, consistent with TyrO$^\bullet$ repair by cysteine (TyrO$^\bullet$ + Cys $\rightarrow$ TyrOH + Cys$^\bullet$) or $O_2^{\bullet-}$ (either via addition to give a hydroperoxide or electron transfer; see above) [108]. Cys and GSH react more slowly (3 and 5 orders of

magnitude slower) than their selenium analogues with TyrO$^{\bullet}$ [278]. Conversely, the rate constants for repair of TyrO$^{\bullet}$ by urate do not vary significantly between proteins [277]. In contrast, Hoey *et al*. showed that repair of protein-bound TyrO$^{\bullet}$ varies strongly with the protein [275]. The quantitative data in Table 5 are in agreement with numerous qualitative studies.

Biochemical detoxification of 3-nitroTyr

For a PTM to have significance as a regulatory mechanism, there must be a biochemical process for reversal of the modification, ideally a rapid one. For many years, Tyr oxidation products were considered as stable PTMs because many are relatively chemically inert, though DOPA is a notable exception. However, a number of studies have provided evidence for cellular detoxification of modified proteins, with most studies focusing on denitration, though dechlorination has also been reported. Some early work suggested that nitrated proteins, generated by peroxynitrite, were preferentially degraded proteolytically [279]. Later, the hypothesis that denitration could occur stemmed from the observation that 3-nitroTyr detection by immunoassay decreased after incubation of nitrated protein with human plasma or homogenates of rat brain, liver or lung, and appeared to be time- and temperature-dependent and could occur without protein degradation [280]. This observation has been confirmed in other tissues, and it has been reported that the process is inhibited by heat or protein filtration, but is insensitive to proteasomal inhibitors such as lactacystin (reviewed in [81]), providing support for the concept of 3-nitroTyr metabolism.

Enzymatic removal of 3-nitroTyr could result either from denitration or from reduction to 3-aminoTyr (see above), and evidence has been reported for both processes. Chen et al. reported that lipoyl dehydrogenase could reduce free 3-nitroTyr to 3-aminoTyr [281], and subsequently showed using CID MS, that 3-nitroTyr in proteins can be reduced to 3-aminoTyr by metalloproteins, GSH and ascorbate [281]. It has also been reported that free nitrate can be released from protein 3-nitroTyr by an *E. coli* nitrate reductase, or a mammalian cytochrome P450 [282], based on Western blotting for 3-nitroTyr, though this was not confirmed by MS. Similar approaches subsequently identified mitochondrial nitration and denitration activity [283] and a lipoprotein-associated denitration activity [284]. These immunoassay studies are not necessarily conclusive, as loss of antibody recognition could be due to further protein modification rather than denitration. Consequently FTICR MS was used to confirm nitration and denitration of calmodulin during macrophage activation, providing

more robust evidence for denitration [285]. It appears that there are both O_2-dependent and independent denitration processes. It has been suggested that in mitochondria the cycle of nitration and denitration is target specific, and could act as a signalling mechanism responsive to O_2 levels [283]. Similarly, denitration of an L-type Ca channel by LPS-activated RAW264.7 cell lysates has been suggested as a physiological mechanism to regulate cellular excitotoxicity [286].

GC-MS studies have shown that free 3-chloroTyr can be metabolized in rats to 3-chloro-4-hydroxyphenylacetic acid via further oxidation of the amino group [143], and dechlorination of modified albumin by liver homogenates was also reported [287]. Thus whilst evidence for metabolism of oxidatively-modified Tyr is increasing, a critical question remains with regard to whether denitration and dechlorination restores native protein activity, which would be critical for a regulatory cycle [281].

Biological consequences of oxidation of Tyr in proteins

Structural and functional consequences of Tyr oxidation

Modification of Tyr in proteins may result in alterations of protein structure and dynamics, potentially accompanied by changes in protein turnover (depending on the degree of modification), protein-protein interaction, polymerization, and/or protein activity [18,103,288]. Crystallographic studies have reported slight to significant structural alterations when Tyr residues are converted to 3-nitroTyr. Thus, nitration of Tyr34 in human manganese superoxide dismutase (MnSOD; SOD2; EC 1.15.1.1) results in a new hydrogen bond between an oxygen atom of the nitro group and the side chain amide of Gln143 [289]. However, comparison of the structures of nitrated and native human MnSOD reveals that the aromatic rings of Tyr34 and 3-nitroTyr34 nearly overlap, indicating little conformational change. In contrast, for human glutathione reductase, Tyr nitration results in a significant conformational change with the aromatic ring of 3-nitroTyr 114 rotated by ca. 60^o compared to the parent residue in the native protein [290].

An unusual Tyr modification, 3'-(*S*-cysteinyl)Tyr, is present in galactose oxidase (EC 1.1.3.9) [124] (Figure 3) and human cysteine dioxygenase (EC 1.13.11.20) [125]. This modification may be formed via reaction of TyrO$^\bullet$ with Cys thiyl radicals [126], the addition of thiyl radicals to Tyr followed by one-electron oxidation [127] and/or the reaction of Tyr radicals with thiolate, followed by one-electron oxidation [129]. The significance of the thioether bond in 3'-(*S*-cysteinyl)Tyr has been addressed in a series of studies [126,291-293].

It appears that the thioether ligand affects both the O-H homolytic bond dissociation energy (BDE) and the reduction potential of the substituted phenol, and affords significant delocalization of the spin of the oxidized amino acid, the 3'-(S-cysteinyl)Tyr radical, onto the sulfur. Two conformers of model 3'-(*S*-alkyl)phenols showed different O-H BDEs, with this depending on whether the alkylthio substituent is oriented in-plane or out-of-plane relative to the aromatic ring system [291], with this having has significant consequences for the phenoxyl radical-stabilizing propensities of the thioether ligand. It has therefore been proposed that galactose oxidase can fine-tune its catalytic properties through minor conformational changes around the thioether substituent [291].

Ca^{2+}-ATPases, especially the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA; EC 3.6.3.8), offer good examples to illustrate the effects of Tyr nitration and oxidation. The physiological role of SERCA is to sequester cytosolic Ca^{2+} into sarco/endoplasmic reticulum (SR/ER), which plays a key role in maintaining intracellular Ca^{2+} levels within the physiological range [294,295]. There is evidence that redox regulation of SERCA is important in vascular relaxation and in ageing heart and skeletal muscle; nitration of vicinal Tyr294 and Tyr295 (Figure 6) on the lumenal side of the membrane-spanning helix M4 was observed, which correlated with partial inhibition [296,297]. It has been concluded that peroxynitrite inactivates SERCA1 via both Cys oxidation and Tyr nitration [298]. The predominant nitration of Tyr122 was confirmed in the SERCA sequence at a molar ratio of 3-nitroTyr to protein of 0.23 [299]. Tyr nitration induces a dramatic decrease in the pKa of Tyr phenolic group (from 10 to 7.2) and Tyr294 and Tyr295 are in close proximity to a negatively-charged amino acid side chain. When nitrated, this cluster of three negative charges may distort helix-helix interactions at the M4-M5 interface and further hinder the coordinated movements of membrane helices required for optimal rates of active transport by the Ca^{2+}-ATPase. This nitration of Tyr and resulting changes in conformation are consistent with a partial decrease of ATPase activity, and suggests that nitration has a regulatory effect rather than merely reflecting protein damage [300].

One notable feature of SERCA2a nitration is the limited number of the 18 Tyr residues that are nitrated both *in vivo* and *in vitro*. It was shown that lipid-soluble Tyr are nitrated ~10-fold more efficiently than those in the aqueous phase [301]. Nitration of Tyr has been observed to be more frequent in the vicinity of positive charges, on Lys, Arg, and the amino terminus, than in the absence of these residues [302], consistent with electrostatic attraction between basic amino acids and nitrating species such as nitrosoperoxycarbonate or $CO_3^{\cdot-}$ [303].

Interference with phosphorylation pathways

Tyr phosphorylation is a key process in signal transduction, and modification of Tyr residues in receptor molecules has been shown to impair signalling pathways and cell regulation processes [304]. Nitration can therefore potentially compromise the phosphorylation / dephosphorylation cycle of Tyr [276]. Evidence for this has been obtained from studies on the nitration/oxidation of Tyr in model substrates, with this preventing phosphorylation by protein Tyr kinases [280,305]. Nitration may lock some enzymes into inactive forms (although this may be reversible, see above), but the overall extent of Tyr nitration appears to be rather low. Another protein thought to be affected by Tyr nitration is the platelet-derived growth factor (PDGF) receptor, which has five Tyr autophosphorylation sites [306,307]. Mutation or modification of these residues can block signalling; in particular, nitration of Tyr1009 and Tyr1021 prevents the binding and activation of phospholipase C-gamma (PLC-gamma), an important signalling protein [308]. If PLC-gamma does not bind to the Tyr residues, the inositol phospholipid signalling pathway is not activated. Conversely nitration and dimerization (via 3,3'-diTyr) of epidermal growth factor induced by peroxynitrite, did not significantly affect its autophosphorylation [309]. Furthermore, the phosphorylation of the dimers was more extensive. Other important receptors, such as the insulin receptor also contains key Tyr residues that can be phosphorylated and which may become inactivated by nitration [310]. Intermolecular cross-linking of enzymes involved in signal transduction via 3,3'-diTyr formation has also been detected in cells [309]. On the other hand, there has also been a report that 3-nitroTyr substitution into Tyr-containing peptides mimicking the C-terminus of src kinases or N-terminus of erythrocyte band 3 resulted in activation of the src kinase lyn, suggesting that 3-nitroTyr can mimic phosphoTyr in some signalling pathways [311]. Thus the signalling effects of Tyr nitration appear to be complex and pathway-dependent.

Effects of protein cross-linking and aggregation on proteasomal and lysosomal degradation

In normal cellular conditions oxidised proteins are either repaired, which is limited to a small number of specific modifications such as methionine sulfoxides [312,313], disulfide bonds [314-316] and possibly Tyr nitration (see above), or removed by proteolytic degradation (Figure 7). Cells have highly-regulated intracellular proteolytic systems for the removal of non-functional proteins, such as membrane proteases, lysosomal cathepsins,

calcium-activated calpains, caspases, mitochondrial proteases and the proteasomal system [317-321]. There is evidence that oxidised proteins, and particularly those containing hydroperoxide groups including Tyr-derived species, can modulate the activity of Cys-dependent lysosomal cathepsin enzymes and thereby perturb normal cellular turnover of materials [16]. This inhibition has been shown to involve oxidation of the active site Cys residue by Tyr (and other) hydroperoxides, with inhibition of other structurally-related cathepsins without Cys residues in their active sites being less affected. This may result in a accumulation of damaged materials and consequent toxicity [322,323]. Aggregated/cross-linked materials can undergo autophagocytosis resulting in a major accumulation of the material in lysosomes [324,325].

The major proteolytic system responsible for the removal of oxidised proteins is the proteasome [326-328]. The 20S core proteasome is the main structure of this system, though regulatory units such as 19S (PA700) and 11S (PA28) can attached to the core to give further structures such as the 26S proteasome [326,329]. It has been reported that aconitase and other proteins oxidized by peroxynitrite are preferentially degraded by the proteasome [279], and further work has demonstrated that protein Tyr nitration is sufficient to induce accelerated protein degradation [18]. More recently it has been reported that nitration of Tyr46 and Tyr48 in cytochrome c results in cellular degradation [330]. While mildly modified proteins, including limited Tyr nitration, are preferentially degraded, heavily oxidised and cross-linked proteins are poor substrates for the 20S proteasome and are able to inhibit proteasome activity. Cross-linked proteins arising from 3,3'-diTyr formation have been investigated in particular [331]. Lipofuscin, ceroid or AGE (advanced glycation end products)-pigment-like fluorophores, insoluble cross-linked materials found at elevated levels within aged cells [322,332,333] may also inhibit this activity. Since the proteasome is a relatively narrow barrel-shaped structure, aggregates may not be capable of being unfolded to enter the central core where the multiple chymotryptic, tryptic and caspase-like active sites are located, leading to poor degradation, accumulation and inhibition of proteasomal activity. Furthermore, aggregate formation is a continuous process and can be accompanied by a time-dependent enlargement of aggregate size.

The 26S proteasome is responsible for turnover of most undamaged proteins in an ATP- and ubiquitin ligation-dependent manner, and was assumed not to play a major role in the degradation of oxidized proteins [327]. However, evidence has been presented for inhibition of the tryptic and chymotryptic activity of the 26S proteasome by oxidized proteins, at least in part via reaction of hydroperoxides present on proteins, including those present on

Tyr residues. This inhibition appears to involve oxidation of critical Cys residues on the regulatory subunits [118] and may have significant consequences for the turnover of regulatory proteins and transcription factors, resulting in the potential initiation or continuation of signalling events and the induction of apoptosis [334].

Immunogenicity of Tyr oxidation products

Post-translational modification of natural amino acids can generate neoepitopes with potential consequences for immunogenicity (reviewed in [335]). The formation of such neoepitopes on autologous proteins can result in antibody formation towards the modified protein, and cross-reactivity of these antibodies towards the unmodified protein, breaking tolerance and potentially triggering an autoimmune response. Several post-translational protein modifications have been associated with autoimmune diseases, including systemic lupus erythematosus (SLE), multiple sclerosis, rheumatoid arthritis and Type 1 diabetes [336,337]. For post-translational modifications of Tyr, only a few reports have been published, such as the demonstration that immunization of B6 mice with modified epidermal growth factor (EGF), in which Tyr29 was mutated to either nitrated or sulfated Tyr, with this resulting in autoantibody formation against native EGF [338]. This clearly shows that Tyr modification can break tolerance against native proteins, and suggests that oxidative modification of Tyr residues may contribute to the etiology of autoimmune disorders. Such cross-reactivity towards non-modified peptide sequences was not observed following the nitration of Tyr97 in cytochrome c [339], indicating that potential autoimmune responses may be sequence- and protein-specific. Immunization of rabbits with nitrated histone H2A resulted in the formation of antibodies, which showed a limited cross-reactivity with native H2A [340], and there is evidence for cross-reactivity of antibodies resulting from the immunization of MRL/lpr mice with nitrated IgG towards single-stranded DNA (ssDNA) [341]. Anti-DNA antibodies are a feature of autoimmune diseases such as SLE [342], and it was suggested that the immunogenicity of nitrated autologous proteins such as IgG may trigger anti-DNA antibody formation [342,343]. A robust immune response was also observed after systemic administration of nitrated ovalbumin to mice in a search for a potential role of protein nitration in the induction of food allergies [344]. However, no immune response was detected after oral administration of nitrated ovalbumin, probably due to rapid gastric digestion of the protein. It has also been reported that immunoglobulins have been detected against 3-nitroTyr epitopes in both acute lung injury and coronary artery disease [345,346]. Clearly further studies are needed to document a role of specific Tyr modifications on selected proteins in the

stimulation of the immune system and in autoimmune disorders. Some results are available on the immunogenicity of brominated proteins, specifically containing 3,5-dibromoTyr [236], but the potential for cross-reactivity with native, non-brominated proteins was not evaluated.

Summary and perspectives

Tyr oxidation results in the formation of multiple oxidation products, with the nature of the products formed being critically dependent not only on the oxidant, but also on the protein structure and environment. In the majority of cases, oxidation occurs preferentially at the aromatic ring, and in these situations the products are often very similar between the free amino acid and those detected on proteins. Some of these products (e.g. dimers, hydroxylated species) can be formed by multiple processes, whereas others can be diagnostic of particular reactive species. Thus nitrated products indicate the involvement of RNS such as $ONOO^-$ and $^{\bullet}NO_2$, while 3-haloTyr are markers for exposure to oxidizing hypohalous acids or $ClO_2^{\bullet}$. 3-nitroTyr formation has been widely examined in both aging tissues and diseases, and used as a marker of oxidative stress in human atherosclerosis and diabetes [347-349], hypercholesterolemia in rabbits [350] and aging [351].

A wide range of analytical techniques is available to detect oxidized Tyr, and the appropriate approach depends on whether free Tyr products or protein-bound Tyr are being studied, and the information required. Techniques such as pulse radiolysis and flash photolysis are ideal to study the kinetics of reactions in vitro for free Tyr or small peptides, but are not readily applicable to biological samples. Similarly, data from NMR or EPR analysis is harder to interpret for proteins and complex samples, whereas antibody-based techniques and redox-staining are ideal for visualization of Tyr oxidation products in tissues and proteins. Other methods, such as colorimetric assays and mass spectrometry, can readily be applied to different types of sample, although the sample preparation would vary accordingly.

The development of sensitive and specific techniques is critical to enable study of the mechanisms of formation of Tyr oxidation products and their occurrence in vivo, which may contribute to disease and pathology as there is increasing evidence for their biological effects. Although Tyr oxidation can be repaired and there is evidence that cellular detoxification systems exist, modification of Tyr residues is known to have both structural and functional consequences for the proteins and systems in which they are formed. In many cases, oxidation and nitration interferes with the catalytic mechanism and it can also affect recognition of

signalling proteins, resulting in disrupted cellular responses. Finally, there is evidence that Tyr oxidation products on proteins are immunogenic, providing a clear link to inflammatory outcomes. The number of biological and biomedical studies on 3-nitroTyr indicates the importance attributed to this modification already, while understanding of other oxidative modifications of Tyr lags behind and represents an important potential target for the future.

Abbreviations

Apo-AI, apolipoprotein-AI; Apo-B: apolipoprotein B; BSA, bovine serum albumin; CID, collision induced dissociation; Co-fradic, combined fractional diagonal chromatography; DOPA, 3,4-dihydroxyphenylalanine; DTT, dithiothreitol; ECD, electron capture dissociation; ELISA: Enzyme-Linked Immunosorbent Assay; ENDOR: Electron-Nuclear DOuble Resonance; EPR, Electron paramagnetic resonance; ET, electron transfer; FBA: 4-formylbenzoylamido tag; FITC: fluorescein isothiocyanate; FTICR, Fourier Transform ion cyclotron resonance; GSH, reduced glutathione; HeLa cells: Henrietta Lacks immortalized cells; HDL, high-density lipoproteins; Huh-7: human hepatoma cell line; iTRAQ: Isobaric tags for relative and absolute quantitation; KLH, Keyhole limpet hemocyanin; LC-ESI/MS/MS, liquid chromatography coupled to electrospray ionisation tandem mass spectrometry; LRET: long range electron transfer; LTQ-orbitrap: Linear ion Trap Quadrupole mass filter orbitrap; MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometry; MnSOD: manganese superoxide dismutase; MPO, myeloperoxidase; oxPTM, oxidative post-translational modification; RONS, reactive oxygen and nitrogen species; RNS, reactive nitrogen species; SERCA: sarco/endoplasmic reticulum Ca^{2+}-ATPase; src kinase: sarcoma Tyr kinase; SPAER: Solid Phase Active Ester Resin; $TyrO^{\bullet}$, Tyrosine phenoxyl radical (also called tyrosyl radical); RIGhT: reporter ion generation tag.

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Table 1. Rate constants for oxidation of free or protein-bound Tyr by free radicals (R•).

R•	k(R• + Tyr) mol^{-1} L s^{-1})	Radical formed	Final product(s)	References
$OH^•$	pH 2.2: $(5.8 \pm 0.3)\times10^9$ pH 4.6: $(3.7 \pm 0.6)\times10^9$ pH 7.5: 1.3×10^{10} pH 10.6: $(2.2 \pm 0.5)\times10^{10}$	Mostly OH-adducts on o- or m-positions	Mostly dihydroxyphenylalanine (3,4 DOPA) [a], DiTyr [b]	[22-25,296]
$N_3^•$	Neutral: 3.6×10^9 Alkaline: 1×10^8	TyrO•	DiTyr	[35]
	N-Ac-Tyr-amide: $1.3–1.7\times10^8$ Chymotrypsin-Tyr $(3\pm0.5)\times10^8$ BSA-Tyr $(4.7 \pm 0.3)\times10^8$			[36]
$Cl_2^{•-}$ $Br_2^{•-}$ $I_2^{•-}$ $(SCN)_2^{•-}$	2.7×10^8 2.0×10^7 $< 1.0\times10^6$ 5.0×10^6	TyrO•		[38]
$SO_4^{•-}$	5.8×10^8 [c] $(0.4–1.2)\times10^9$ [d] $(1.2–2.7)\times10^{10}$ [e]	$SO_4^{•-}$ adducts		[150]
$HPO_4^{•-}$,	4.0×10^8 [c] $(2.2– 5.9)\times10^8$ [d] $(2.0 – 2.1)\times10^9$ [e]			[352,353]
$PO_4^{•2-}$	$(1.0 – 1.4)\times10^8$ [c, d]			[352,353]
$CO_3^{•-}$	pH 7: 4.5×10^7 pH 11: 1.4×10^8	TyrO• Adduct		[354,355] [355,356]
$ClO_2^{•-}$	Tyr: 1.8×10^5 N-Ac-Tyr: 3.2×10^4 Basic medium: $(8.2\text{-}18)\times10^7$ [c] 7.6×10^7 [f]	TyrO•	3,4-dihydroxyphenylalanine (DOPA) and 2,4,5-trihydroxyphenylalanine (TOPA)	[39-42]
$NO_2^•$	$(2.9 \pm 0.3)\times10^7$ [c] pH 7.5: 3.2×10^5 2.0×10^7 [g]	TyrO•	3,3'-diTyr or 3-nitroTyr	[41,47]
$^{•}NH_2$	8×10^6			[356]
Lipid Peroxyl radicals (LOO•)	N-t-Boc-L-Tyr-tert-butyl ester in liposomes: 4.8×10^3	TyrO•		[58]
RO•	*t*-$BuO^•$ in neutral medium: $\sim3\times10^5$ In basic medium: $\sim2\times10^8$	TyrO•		[59]
Phenoxyl radicals ($PhO^•$)	In ionized state: 4.9×10^8			[60]

[a] In glycyl peptides and in various proteins
[b] In Tyr, Gly-Tyr and poly-L-Tyr and in proteins (insulin, ribonuclease, papain, collagen, and lysozyme)
[c] In Tyr amino acid in solution
[d] In Val-containing peptides
[e] In Gly containing peptides
[f] In N-Ac-Tyr; g) in Gly Tyr.

Table 2. Rate constants for reaction of Tyr-derived radicals (TyrO•, Tyr-OH adduct) with various radical or molecular reactants.

Reactants	Products	Rate constant (mol^{-1} L s^{-1})	Reference
$TyrO^{\bullet}$ + itself	o-DiTyr m-DiTyr	3.0×10^8 [a] 2.0×10^9 [a] 4.5×10^8 [a] 4.7×10^8 [b] 3.6×10^8 [c] 5.1×10^8 [d]	[22,23,26,67]
Tyr-OH adduct + O_2	Peroxyl radical	5×10^8 to 10^9	[26]
$TyrO^{\bullet}$ + O_2		$< 10^3$	[28]
$TyrO^{\bullet}$ + phenoxide (PheO-)	$TyrO^-$ (tyrosine anion) + $PheO^{\bullet}$	2.8×10^7	[60]
$TyrO^{\bullet}$ + NO	3-nitrosoTyr	$(1\text{-}2) \times 10^9$ [e]	[98,99]
$TyrO^{\bullet}$ + $NO_2^{\bullet}$	3-nitroTyr	*Ca.* $3x10^9$ [a] 3.0×10^7 [b] (at pH 9.3) 3×10^4 [f] 9×10^2 [g]	[100,101]
$TyrO^{\bullet}$ + $O_2^{\bullet-}$	Adduct at o- or p-positions	$(1.6\pm0.1) \times 10^9$ [a] $(1.5 - 1.7) \times 10^9$ [c] 2.5×10^9 [d]	[27,67,114]

[a] With Tyr amino acid in solution
[b] With Gly Tyr
[c] With Met enkephalin
[d] With Boc Met enkephalin
[e] With Tyr, N-acetyl Tyr and Gly Tyr
[f] Y121 in ribonucleotide reductase
[g] In ribonucleotide reductase, Y residues other than Y121.

Table 3. Detection of nitrotyrosine in various pathologies

Pathology /tissue/fluid	Method of detection	References
Plasma of infants with bronchopulmonary dysplasia	Solid phase ELISA	[224]
Carbon monoxide-exposed endothelium	Solid phase ELISA	[225]
Bipolar disorders & Schizophrenia	ELISA	[357]
Type 2 diabetes with obstrusive sleep apnea	ELISA	[358]
Rat brain following high salt loading	ELISA for free 3-nitro-Tyr	[359]
Nervous tissue of streptozotocin diabetic rats	Double-label fluorescent immunohistochemistry	[360]
Neurodegenerative diseases (Alzheimer's disease & ALS)	Western blotting	[265,266,361,362]
Coronary vessel atherosclerotic plaques & pancreatic adenocarcinoma	Western blotting, IP and MS sequencing techniques	[304,363]
Mouse models of cardiovascular dysfunction	Western blotting, IP and MS sequencing techniques	[267,268]
Rat Cerebellar Purkinje cells	Fluorogenic tagging strategy with MS	[364]
Mouse model of heart failure (LONP1 nitration)	Immunoblotting	[365]
Murine endotoxin-induced retinal inflammation (Sirt6 nitration)	Western blotting	[366]

Table 4. Labelling and enrichment strategies for detection of oxidized or nitrated tyrosine

Label	Enrichment or detection method	Tissue / samples assayed	References
N-succinimidyl *S*-acetylthioacetate (SATA)	Removal of the *S*-acetyl group after labelling exposed free thiol groups, for binding to a thiopropyl sepharose.	Mouse brain homogenate	[261]
4-formylbenzoyl-amido (FBA)	Chemoprecipitation, captured on a Solid Phase Active Ester Resin (SPAER)	Human plasma nitrated with tetranitromethane	[367]
(3R,4S)-1-(4-(amino-methyl) phenylsulfonyl) pyrrolidine-3,4-diol (APPD)	Strong retention on sulfonamide phenylboronic acid columns compared to native proteins ; fluorescent tag gave diagnostic m/z increase and fragment ions	Total protein lysate of cultured C2Cl2 cells ; cerebellar Purkinje cells	[264,364]
Biotinylation	Enriched on avidin immobilized agarose beads ; a simple 1-buffer system approach	Peroxynitrite-treated angiotensin in a mixture of BSA peptides	[260]
Pyridine-2-carboxaldehyde + cyanoborohydride labelling	Labelled peptides were enriched by immobilized metal ion affinity chromatography (Ni^{2+} nitrilotriacetic acid magnetic beads)	*E. coli* lysate and HeLa cell mitochondria	[248]
N-succinimidyl 3-(perfluorobutyl) propionate	Combined with fluorinated solid phase extraction (FSPE)	Proteins in a Huh7 cell lysate, in the absence of induced nitrative stress	[259]
Dansyl chloride	Used as a “reporter ion generation tag” (RIGhT) owing to diagnostic fragment ions / transitions	Nitrated BSA as a model protein	[262]
iTRAQ labelling reagents	Labels were released in MS^2 and detected at *m/z* 114/115/116/117	*E. coli* and bovine milk extract	[263]

Table 5. Rate constants for reaction of $TyrO^{\cdot}$ with reducing agents.

Repair agent	Rate constant (mol^{-1} L s^{-1})	References
Ascorbate	$(0.9\text{-}4.4) \times 10^7$ [a] $(2.6 \pm 0.2) \times 10^8$ [b] $(2.9 \pm 0.1) \times 10^7$ [c] 4.0×10^7 [d] $(3.5 \pm 0.9) \times 10^7$ [e] $(4.0 \pm 0.6) \times 10^5$ [f] 1.1×10^7 [g]	[28] [275] [34]
GSH	2×10^6 [a] $(2.4 \pm 1.6) \times 10^3$ [b] $(1.0 \pm 0.6) \times 10^4$ [c]	[274] [128]
Cysteine	$(6 \pm 3) \times 10^5$ [b] $(1.0 \pm 0.6) \times 10^5$ [c]	[128]
Selenoglutathione	$(5 \pm 2) \times 10^8$ [b] $(4.0 \pm 3) \times 10^6$ [c]	[278]
Selenocysteine	$(8 \pm 2) \times 10^8$ [b] $(1.6 \pm 0.8) \times 10^8$ [c]	[278]
Urate	2.4×10^8 [a] $< 1 \times 10^6$ [d] 3×10^8 [e] 5.4×10^6 [g]	[28,275,277]
Trolox	3.1×10^8	[28]

[a] For Tyr free amino acid in solution
[b] For N-Ac-Tyr-NH_2
[c] For insulin
[d] For chymotrypsin
[e] For pepsin
[f] For lactoglobulin
[g] For lysosyme in the presence of micelles

Figure Legends

Figure 1. Photo-oxidation of Tyr by direct absorption of energy to produce electronically excited states and photo-ionized species. The electrically excited state can react with oxygen or disulfides to give TyrO$^{\bullet}$, which may then react together to give different cross-linked products. Reaction of free Tyr with singlet oxygen following photo-sensitizationcan lead to the formation of endoperoxides, followed by rearrangement and generation of cyclization products.

Figure 2. Reactions of TyrO$^{\bullet}$ with the superoxide radical anion. (A) For a residue which is within a polypeptide chain or close to the C-terminal; (B) for a residue which is either N-terminal or a free tyrosyl radical.

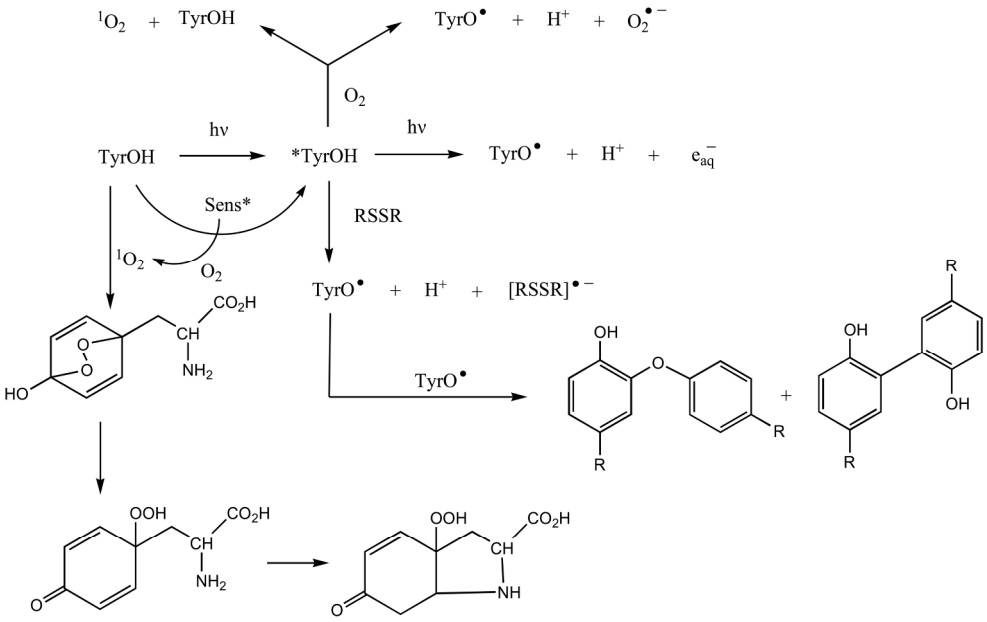
Figure 3. The thioether bond between Cys228 and Tyr272 in galactose oxidase (PDB 1GOF) [124].

Figure 4. Examples of protein free radicals that can be reduced by a Tyr residue, leading to formation of a tyrosine phenoxyl radical (TyrO$^{\bullet}$) and regeneration of the other amino acids. Top row, left to right: tryptophan-derived indolyl radical ; cysteine-derived thiyl radical; histidine-derived imidazole radical-cation. Bottom row, left to right: methionine-derived sulfur-centered radical-cation; three-electron-bonded sulfur-bromine radical species from methionine; three-electron-bonded cyclized sulfur-nitrogen species from methionine.

Figure 5. Mass spectrometry approaches to analysis of Tyr oxidation. Proteins containing oxidized Tyr can be separated by immuno-enrichment with specific antibodies, and subjected to label-dependent or –independent methods for mass spectrometry analysis. Label-free methods use the change in mass-to-charge ratio (m/z) resulting from the modification to characterize the oxidation, while label-dependent methods are mostly used for 3-nitroTyr following reduction to 3-aminoTyr. They can then be used either for targeted enrichment, or label-dependent MS analysis, or both. In most cases, peptide fragmentation by MSMS is used to determine the sequence of the modified peptide with search engines such as Mascot by reference to a database of protein sequences.

Figure 6. The two Tyr residues in SERCA that have been reported to be nitrated are Tyr294 and Tyr295.

Figure 7. The possible fates of oxidized Tyr in proteins.



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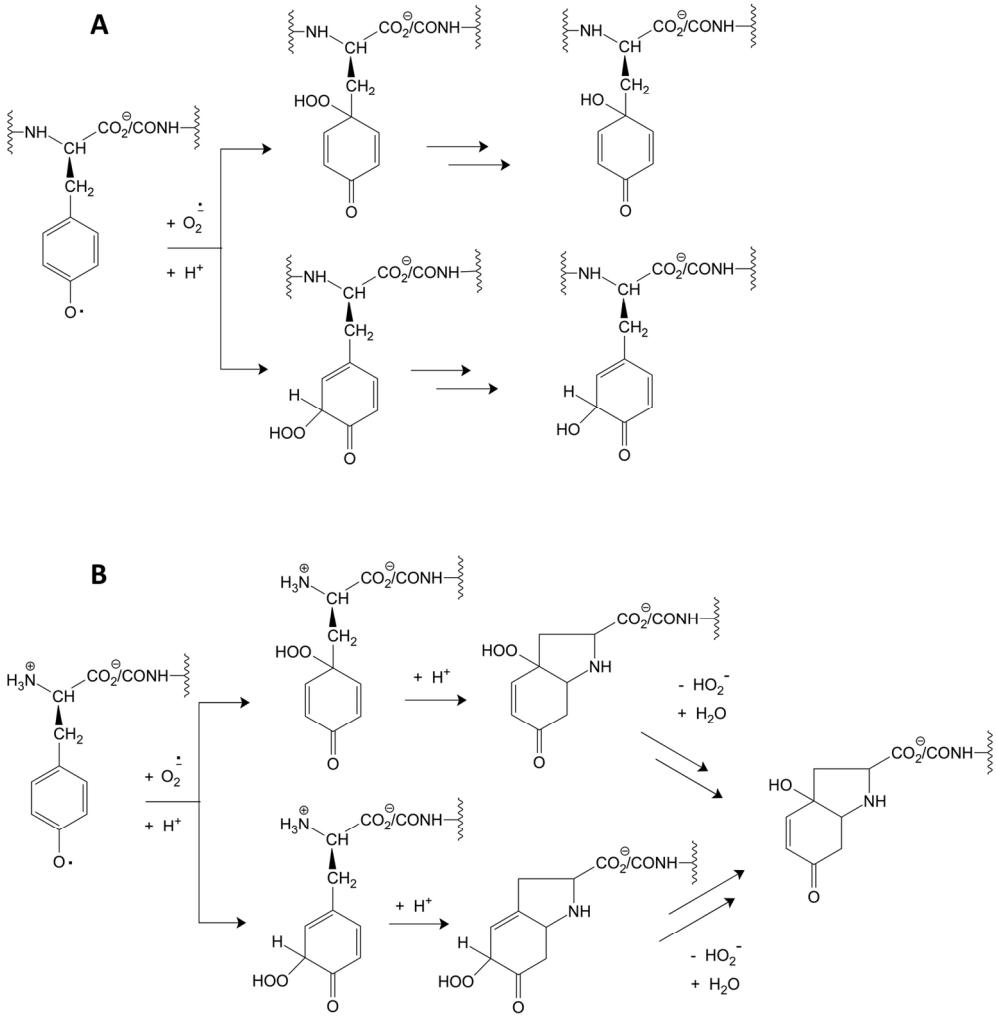

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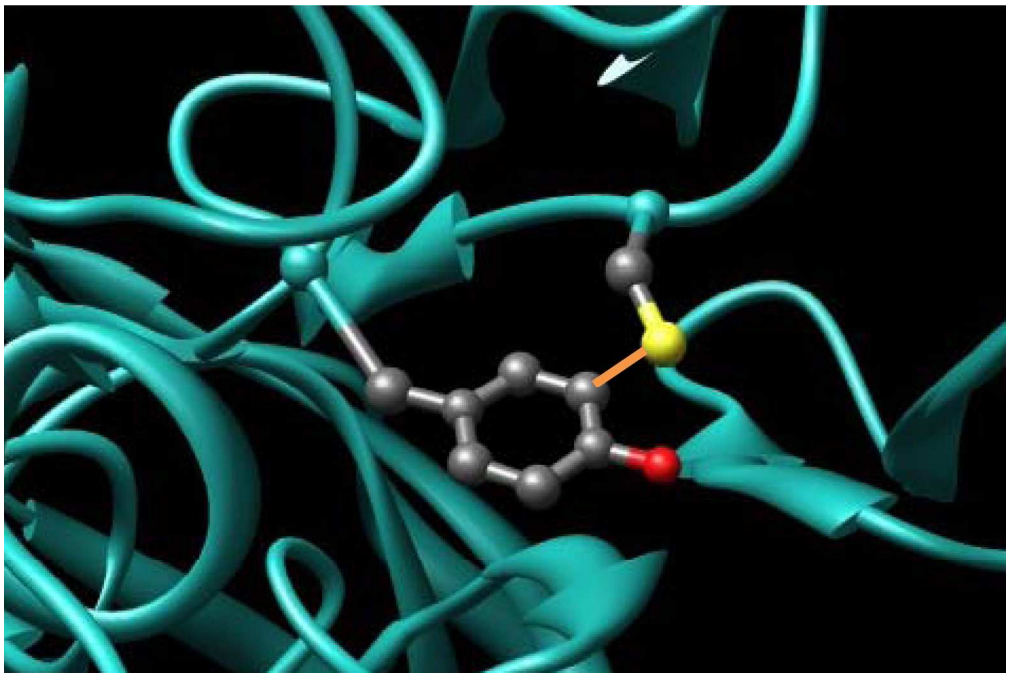

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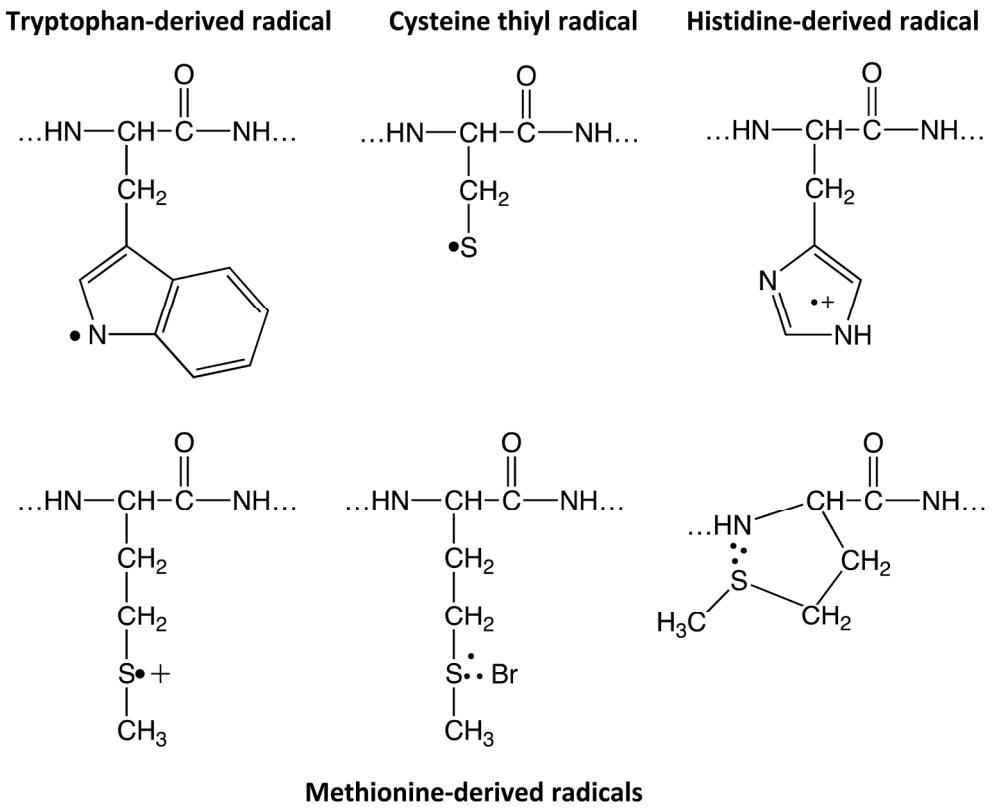

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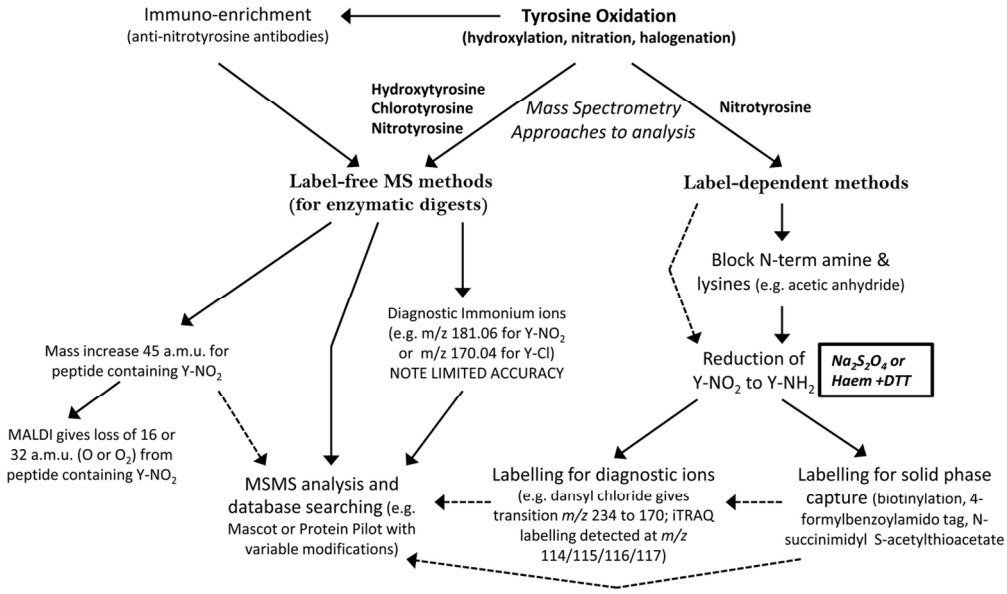

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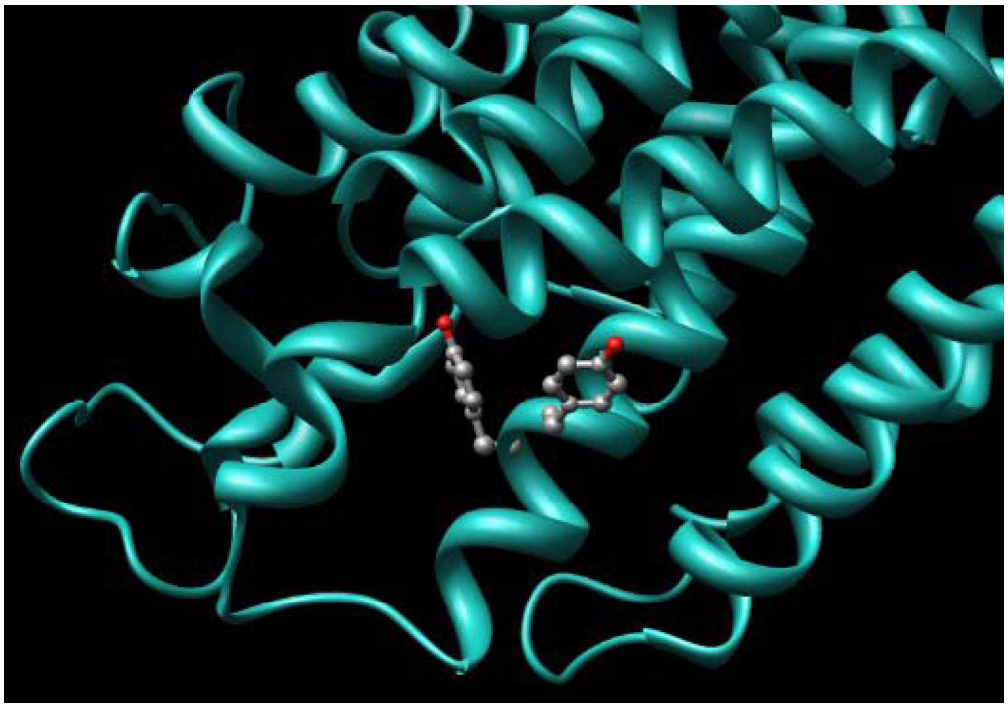

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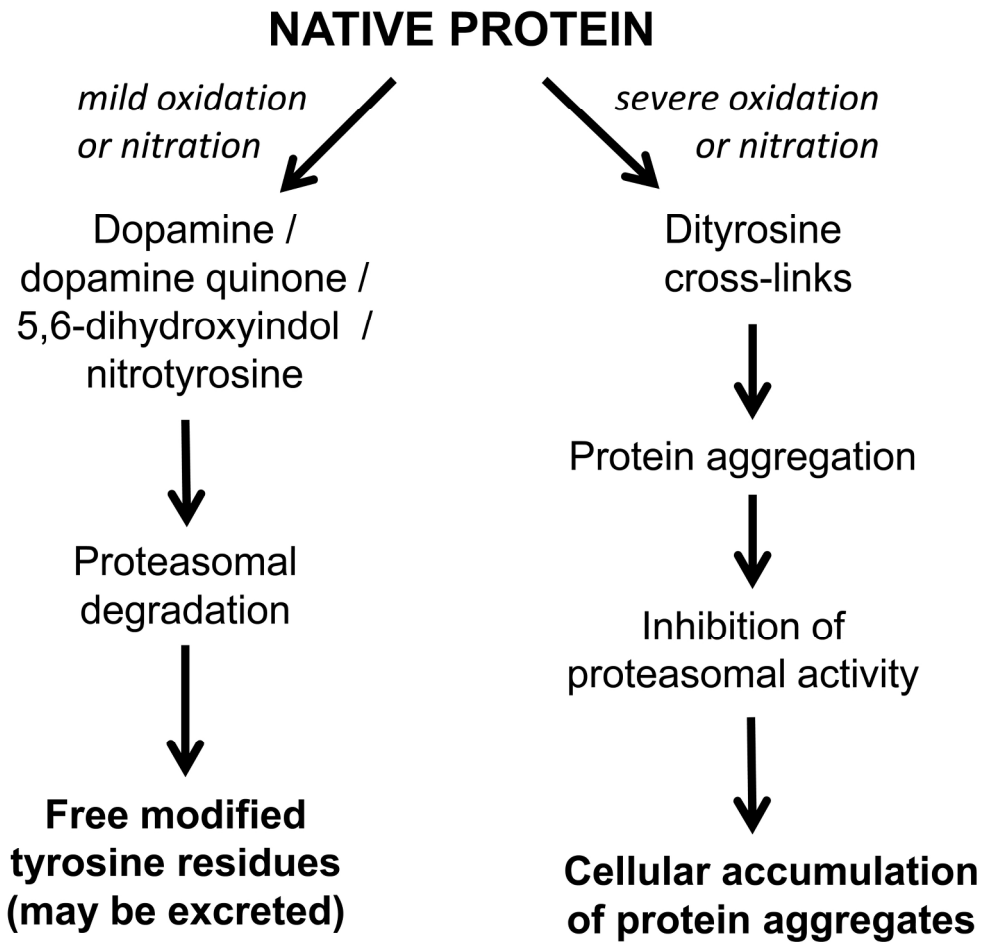

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