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INVITED REVIEW

Reduction of protein radicals by GSH and ascorbate: potential biological significance

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Abstract The oxidation of proteins and other macromolecules by radical species under conditions of oxidative stress can be modulated by antioxidant compounds. Decreased levels of the antioxidants glutathione and ascorbate have been documented in oxidative stress-related diseases. A radical generated on the surface of a protein can: (1) be immediately and fully repaired by direct reaction with an antioxidant; (2) react with dioxygen to form the corresponding peroxyl radical; or (3) undergo intramolecular long range electron transfer to relocate the free electron to another amino acid residue. In pulse radiolysis studies, in vitro production of the initial radical on a protein is conveniently made at a tryptophan residue, and electron transfer often leads ultimately to residence of the unpaired electron on a tyrosine residue. We review here the kinetics data for reactions of the antioxidants glutathione, selenocysteine, and ascorbate with tryptophanyl and tyrosyl radicals as free amino acids in model compounds and proteins. Glutathione repairs a tryptophanyl radical in lysozyme with a rate constant of $(1.05 \pm 0.05) \times 10^5\ M^{-1}\ s^{-1}$, while ascorbate repairs tryptophanyl and tyrosyl radicals ca. 3 orders of magnitude faster. The in vitro reaction of glutathione with these radicals is too slow to prevent formation of peroxyl radicals, which become reduced by glutathione to hydroperoxides; the resulting glutathione thiyl radical is capable of further radical generation by hydrogen abstraction. Although physiologically not significant, selenoglutathione reduces tyrosyl radicals as fast as ascorbate. The reaction of protein radicals formed on insulin, β-lactoglobulin, pepsin, chymotrypsin and bovine serum albumin with ascorbate is relatively rapid, competes with the reaction with dioxygen, and the relatively innocuous ascorbyl radical is formed. On the basis of these kinetics data, we suggest that reductive repair of protein radicals may contribute to the well-documented depletion of ascorbate in living organisms subjected to oxidative stress.

Keywords Protein oxidation · Protein repair · Ascorbate · Glutathione · Selenocysteine · Long range electron transfer

Introduction

Although radical production is part of normal cellular function, excess exposure of macromolecules to oxidising radicals may play a role in ageing and many diseases (Halliwell and Gutteridge 2007). Proteins form by far the largest mass of oxidisable organic components of living matter, and there is a large body of evidence showing that proteins are significant targets of partially reduced oxygen species (PROS) in vivo. Increased levels of protein carbonyls are early markers of damage in biological systems subjected to oxidative stress, even in the presence of endogenous antioxidants. The carbonyls are derived from amino acid residues, principally Arg, Pro and Lys, oxidised in reactions with physiological PROS, where the first products are, in some cases, carbon- and oxygen-centred radicals (Requena et al. 2003; von Sonntag 1987). Additional identified products of reaction between PROS and proteins are protein

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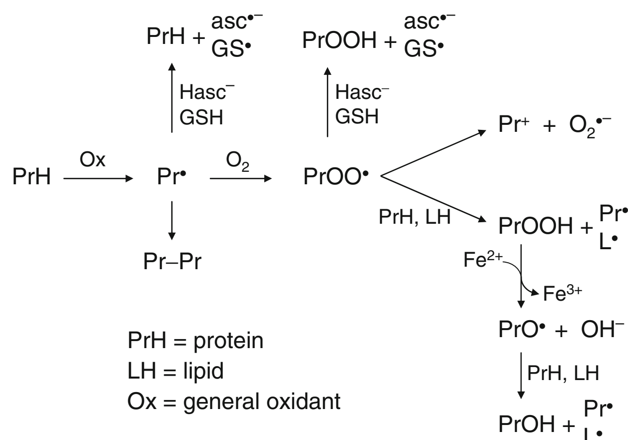
hydroperoxides (Simpson et al. 1992) which, unlike the carbonyls, have the capacity to propagate the PROS-induced damage to other components of living organisms (Davies and Dean 1997; Gebicki 1997). The literature covering the chemistry and biology of protein oxidation in vitro and in vivo is extensive, with the major findings summarised in a number of excellent reviews (Davies 2005; Davies and Dean 1997; Garrison 1987; Pietzsch 2006; Pryor 1986; Requena et al. 2003). Protection against the well-documented damaging effects of PROS in living organisms is normally achieved by a system of antioxidant defences. These defences are multilayered: (1) Superoxide is scavenged by superoxide dismutases, and hydrogen peroxide by catalase, peroxiredoxins and glutathione peroxidases; (2) Since hydroxyl radicals are too reactive to be scavenged in vivo, oxidation of proteins and membranes occurs, but an oxidised protein may be repaired by ascorbate or GSH; and (3) When such repair is not possible, the irreversibly modified protein is marked for recycling by proteases; however, proteins that cannot be processed by proteases accumulate in the cell and may be toxic. Ascorbate ($Hasc^-$) and glutathione (GSH) are widely recognised as important endogenous low-molecular-weight antioxidants (Buettner 1993). Their levels are depleted under conditions known as oxidative stress when oxidative challenge exceeds the capacity of antioxidant defences. This leads to lowering of the organism's ability to resist further stress, unless the levels of ascorbate and GSH are replenished in time to prevent cell and tissue damage. However, decreased levels of both these antioxidants found in disease states associated with oxidative stress—injury, rheumatoid arthritis, ischaemia–reperfusion, trauma, infection, exposure to toxins, ageing, fibrotic diseases and others—demonstrate that such timely replenishment is not always achieved (Buffington and Doe 1995; Drechsel and Patel 2008; Foy et al. 1999; Halliwell and Gutteridge 2007; Kume-Kick and Rice 1998; Liu and Pavia 2010; Locatelli et al. 2003; Misso et al. 2005; Moor et al. 2006).

The reaction mechanisms responsible for the loss of $Hasc^-$ and GSH in vivo under oxidative stress have not been identified, largely because the necessary quantitative data on the potentially responsible reactions are not available. The well-documented loss of GSH and concomitant increase in GSSG, catalysed by glutathione peroxidase (Flohé et al. 1973; Michiels et al. 1994), caused by an increased flux of peroxides is not discussed here, nor is the controversial regeneration of vitamin E radicals by $Hasc^-$ (Bisby and Parker 1995; Burton et al. 1990; Rubbo et al. 2000). We do not consider two-electron processes, e.g. oxidation by HOCl, but instead focus on the one-electron oxidations of these antioxidants by protein radicals. Although the chemistry of the reactions of $Hasc^-$ and GSH with radicals and other oxidants is well understood, the relevance of these reactions to conditions in vivo is questionable. It is highly unlikely that $Hasc^-$ and GSH protect macromolecules in vivo by scavenging the primary PROS directly, as is often suggested, because the in vivo concentrations of the reactants and the corresponding rate constants are not high enough for these reactions to compete with alternative processes (Winterbourn 2008). In contrast, our quantitative estimates of the relative probabilities of reactions of secondary protein radicals with GSH (Nauser et al. 2005) and $Hasc^-$ (Domazou et al. 2009) suggest that these could play a major role in the antioxidant consumption in cells, provided that the rate constants of the relevant reactions are sufficiently high. While there is much information available regarding in vivo concentrations of these antioxidants, rate constant data were for a long time limited to reactions with selected amino acids and lysozyme (Butler et al. 1984; Jovanovic and Simic 1985; Santus et al. 2000). However, the values found, 10^7–$10^8\ M^{-1}\ s^{-1}$, show that oxidation of $Hasc^-$ in cells and tissues by protein radicals may be a significant process. We have confirmed this suggestion by reporting rate constants for reactions of a broader range of amino acid and protein radicals with GSH, as well as with $Hasc^-$ (Domazou et al. 2009; Nauser et al. 2005).

The main question of interest is whether GSH or $Hasc^-$ can reduce an initially formed protein radical fast enough before it oxidises another amino acid or, in the case of a carbon-centred radical, it reacts with dioxygen (Neta et al. 1990); the various possibilities are outlined in Scheme 1. There is the possibility of reaction between two radicals on different proteins, with covalent bond formation between the proteins. If the C-centred radical reacts with dioxygen, the resulting peroxyl radical could be reduced by GSH or $Hasc^-$. The resulting peroxide is not, however, expected to be innocuous as it could react with reduced transition metals to form alkoxyl and other radicals (Rush and Koppenol 1990).



Scheme 1

When $Hasc^-$ or GSH reacts with a protein radical, hydrogen is abstracted to form the $asc^{\bullet-}$ or $GS^{\bullet}$ radical. While $asc^{\bullet-}$ by all accounts is relatively harmless, $GS^{\bullet}$ is not, as is indicated by the electrode potentials at pH 7, $E^{\circ\prime}(asc^{\bullet-}, H^+/Hasc^-) = +0.28$ V (Williams and Yandell 1982) and $E^{\circ\prime}(GS^{\bullet}, H^+/GSH) = +0.92$ V (Madej and Wardman 2007). Further, the cysteinyl radical can abstract hydrogen from the α-carbon of an amino acid (Nauser and Schöneich 2003).

In this review, we examine the evidence supporting the hypothesis that proteins activated by reactions with PROS may be responsible for much of the depletion of $Hasc^-$ and GSH observed under oxidative stress. We focus on these antioxidants because of their importance in vivo and report largely on our own recent work, because earlier literature on the kinetics of the reactions of protein radicals is limited. It is hoped that this work will provide stimulus for further quantitative studies of biologically relevant reactions of protein radicals.

History

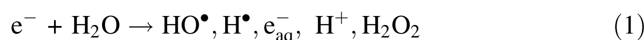
In 1978, it was reported that random attack of $HO^{\bullet}$ with an amino acid on the surface of cytochrome *c* leads to ca. 50% reduction of Fe^{3+} to Fe^{2+} (van Leeuwen et al. 1978). A tunnelling model was developed to explain the transfer of an electron from the surface to the haem iron buried in the interior of the protein. In this case, electron transfer is from a reducing radical to Fe^{3+}. At the end, the amino acid on the surface loses two electrons, most likely via formation of a double bond, although this was never demonstrated.

In landmark papers published during the 1980s, Prütz and co-workers (Butler et al. 1982; Prütz et al. 1980, 1982) showed that radical attack on a protein leads to a cascade of intramolecular electron transfer reactions that usually results in the formation of a tyrosyl radical. Similar intramolecular electron transfers were studied by Bobrowski and coworkers in the 1990s (Bobrowski et al. 1990, 1992), but only a few studies were dedicated to the repair of protein radicals (Hoey and Butler 1984; Jovanovic and Simic 1985; Santus et al. 2000).

Generation of protein radicals

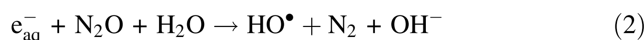
We use radiolysis and photolysis as tools to generate radicals that are identical ($HO^{\bullet}$) or similarly oxidising ($N_3^{\bullet}$) as radicals produced in vivo, e.g. $NO_2^{\bullet}$ and $CO_3^{-\bullet}$ from the reaction of $ONOO^-$ with CO_2 (Bonini et al. 1999; Lymar and Hurst 1995; Meli et al. 1999).

Earlier studies of the kinetics of formation of amino acid and protein radicals demonstrated that the short lifetimes of the radicals require the use of nanosecond laser flash photolysis or pulse radiolysis techniques (von Sonntag 1987). These approaches are capable of generating the radicals commonly produced in organisms subjected to oxidative stress. In flash photolysis, the energy selectively excites the Tyr and Trp residues, converting them to the corresponding Tyr phenoxyl ($TyrO^{\bullet}$) and Trp indolyl radicals ($Trp^{\bullet}$), respectively (Bensasson et al. 1983), and, because the absorbance maxima are sufficiently widely separated ($TyrO^{\bullet}$ at 405 nm and $Trp^{\bullet}$ at 510 nm), the kinetics of formation and decay of these radicals can be studied by spectrophotometry. In pulse radiolysis of dilute aqueous solutions of amino acids and proteins, virtually all of the absorbed energy of the MeV electrons leads to the decomposition of the water:



$$e^- + H_2O \rightarrow HO^{\bullet}, H^{\bullet}, e^-_{aq}, \ H^+, H_2O_2 \quad (1)$$

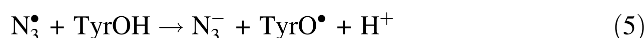
When the sample solutions are previously saturated with N_2O, the e^-_{aq} is converted to $HO^{\bullet}$ (Eq. 2), a powerful oxidant that reacts with virtually all organic compounds. To prevent the generation of too wide a range of protein radical residues, each with its own reactivity, we also include N_3^- to convert $HO^{\bullet}$ to $N_3^{\bullet}$ (Eq. 3), which selectively oxidises Trp and Tyr residues (Eqs. 4, 5) (Land and Prütz 1979):



$$e^-_{aq} + N_2O + H_2O \rightarrow HO^{\bullet} + N_2 + OH^- \quad (2)$$

$$HO^{\bullet} + N_3^- \rightarrow OH^- + N_3^{\bullet} \quad (3)$$

$$N_3^{\bullet} + TrpH \rightarrow N_3^- + Trp^{\bullet} + H^+ \quad (4)$$



$$N_3^{\bullet} + TyrOH \rightarrow N_3^- + TyrO^{\bullet} + H^+ \quad (5)$$

The rate constants of these reactions at near neutral pH are $k_3 = 1.2 \times 10^{10}$, $k_4 \approx 2 \times 10^9$ and $k_5 = 1.0 \times 10^8$ M^{-1} s^{-1} (Butler et al. 1982, 1984; Buxton et al. 1988). These values were used as a guide to choose the concentrations of solutes in the irradiated solutions to ensure completion of Eqs. 3–5 and to exclude competing processes, such as radical–radical reactions.

Reaction of free and lysozyme-bound $TyrO^{\bullet}$ and $Trp^{\bullet}$ with glutathione

The absorbance of solutions of 140 μM lysozyme (Lyz), 10 mM phosphate buffer pH 7.4 and 0.1 M sodium azide saturated with N_2O after a 20-ns radiation pulse in the absence and presence of glutathione (GSH) is shown as a function of time in Fig. 1; second-order rate constants for the reactions of GSH are collected in Table 1. The 510 nm absorbance due to the Lyz($Trp^{\bullet}$) develops within 200 μs after the pulse, while the subsequent decay of the radical is slower. In contrast, the Lyz($TyrO^{\bullet}$) (405 nm peak)

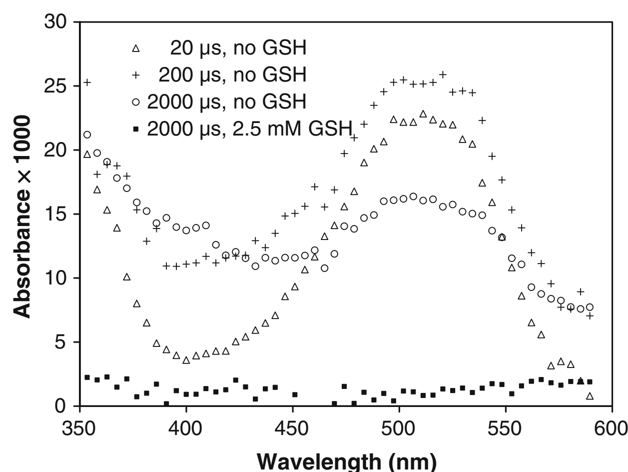


Fig. 1 Time-dependent absorbance changes in pulse-irradiated lysozyme (LZ). The peaks at 510 and 410 nm are due to the Lyz(Trp$^\bullet$) and Lyz(TyrO$^\bullet$) radicals, respectively (reproduced from Nauser et al. 2005 with permission of the authors and the Biochemical Society)

generated by intramolecular long-range electron transfer (LRET) to Lyz(Trp$^\bullet$) forms more slowly, as reported in earlier studies (Butler et al. 1982). Irradiation in the presence of 2.5 mM GSH leads to complete loss of the absorbance at 510 nm due to Trp$^\bullet$ within 2 ms, which indicates that all Lyz(Trp$^\bullet$) radicals are effectively repaired by H transfer. Under conditions of excess GSH, the reaction follows pseudo-first-order kinetics, and the first-order rate constant (k_{obs}) is linearly dependent on the concentration of GSH, which allows the second-order rate constant, $k_6 = (1.05 \pm 0.05) \times 10^5\ M^{-1}\ s^{-1}$ for the bimolecular reaction to be derived

$$\text{Lyz(Trp}^\bullet) + \text{GSH} \rightarrow \text{Lyz(TrpH)} + \text{GS}^\bullet \qquad (6)$$

thereby preventing formation of Lyz(TyrO$^\bullet$) by intramolecular LRET:

$$\text{Lyz(Trp}^\bullet) \rightarrow \text{Lyz(TyrO}^\bullet) \qquad (7)$$

which proceeds with a first-order rate constant $k_7 = (5.3 \pm 1.3) \times 10^2\ s^{-1}$. Measurements of the kinetics of the reaction between GSH and Lyz(Trp$^\bullet$) show that the reaction is complete within 1.5 ms. GSH is also effective in vitro at accelerating the decay of the Lyz(Trp$^\bullet$) generated by flash photolysis of Lyz (28 μM in10 mM phosphate buffer at pH 7.4 in the presence of 100 mM *t*-butanol). Table 1 also lists the rate constants for reaction of GSH with the protein amino acid analogues *N*-Ac-Trp amide and *N*-Ac-TyrOH amide at pH 7.4 measured in similar experiments.

The value of the rate constant for LRET in Lyz agrees well with an earlier published rate constant (Bobrowski et al. 1997). It should be noted that, although the reactions of Lyz(Trp$^\bullet$) radicals with GSH are only moderately fast, the result indicates that reaction with oxidised protein could account for much or for at least some of the documented loss of GSH under conditions of oxidative stress in vivo. Further, when residues other than Trp and Tyr become oxidised to form C-centred radicals, these radicals subsequently form peroxyl radicals, which greatly enhances the amount of GSH oxidised; under air, enhancement of GSH oxidation can be as high as 20-fold, and we have suggested that the peroxyl radicals and hydroperoxides generated can constitute a new source of biological damage (Nauser et al. 2005).

Table 1 Rate constants for reactions of GSH, Sec and GSeH with proteins and protein amino acid analogues

Reaction	Rate constant ($M^{-1}\ s^{-1}$)	Reference
GSH + Lyz(Trp$^\bullet$)	$(1.05 \pm 0.05) \times 10^5$	Nauser et al. (2005)
GSH + *N*-Ac-Trp$^\bullet$ amide	$(1.1 \pm 0.3) \times 10^5$	Nauser et al. (2005)
GSH + *N*-Ac-TyrO$^\bullet$ amide	$(2.4 \pm 1.6) \times 10^2$	Nauser et al. (2005)
Sec + *N*-Ac-TyrO$^\bullet$	$(8 \pm 2) \times 10^8$	Steinmann et al. (2008)
Sec + Ins(TyrO$^\bullet$)	$(1.6 \pm 0.4) \times 10^8$	Steinmann et al. (2008)
GSeH + *N*-Ac-TyrO$^\bullet$	$(5 \pm 2) \times 10^8$	Steinmann et al. (2008)

Repair of tyrosyl radicals by selenocysteine and selenoglutathione

We have also found that selenocysteine (Sec) repairs TyrO$^\bullet$ radicals (Steinmann et al. 2008). Sec reduces *N*-Ac-TyrO$^\bullet$-amide with a rate constant of $(8 \pm 2) \times 10^8\ M^{-1}\ s^{-1}$ and TyrO$^\bullet$ in insulin (Ins) with $(1.6 \pm 0.4) \times 10^8\ M^{-1}\ s^{-1}$ (Table 1). The rate constant for the reduction of *N*-Ac-TyrO$^\bullet$-amide by selenoglutathione (GSeH) is slightly lower $(5 \pm 2) \times 10^8\ M^{-1}\ s^{-1}$ than that for Sec. In contrast, Cys and GSH react 3 and 5 orders of magnitude, respectively, more slowly with *N*-Ac-TyrO$^\bullet$-amide than Sec and GSeH; Cys and GSH react 3 and 2 orders of magnitude, respectively, more slowly with TyrO$^\bullet$ in Ins than their selenium analogues.

Reactions of free and protein-bound TyrO$^\bullet$ and Trp$^\bullet$ radicals with ascorbate

The kinetics for repair of the Trp$^\bullet$ and TyrO$^\bullet$ radicals by ascorbate (Hasc$^-$) were studied by pulse radiolysis with the *N*-acetylated amino acid amides and with the proteins Pep, Ins, chymotrypsin (Chym) and *β*-lactoglobulin (*β*-Lact) (Domazou et al. 2009). Radicals were generated by reaction with $N_3^\bullet$; repair of Trp$^\bullet$ was followed at 510 and of TyrO$^\bullet$ at 405 nm. The conditions were chosen to ensure

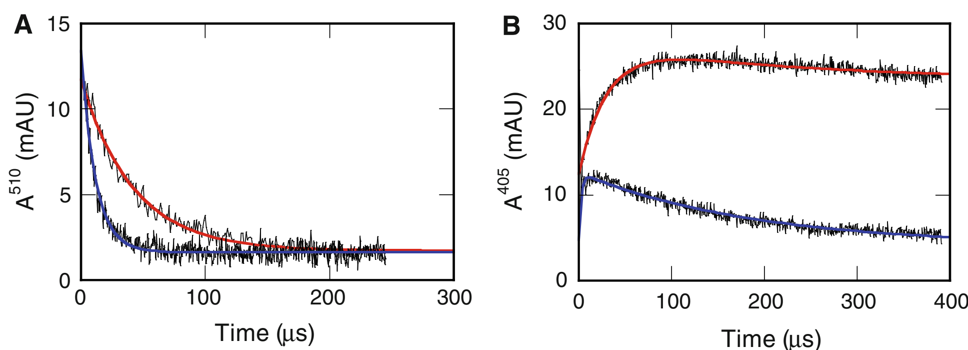
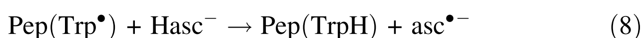


Fig. 2 The spontaneous decay (*red*) of **a** Pep(Trp$^{\bullet}$) and **b** Pep(TyrO$^{\bullet}$). The radicals were generated by pulse radiolysis of solutions that contained pepsin and sodium azide, and were saturated with N_2O. The *blue curves* show the accelerated decay of Pep(Trp$^{\bullet}$) and Pep(TyrO$^{\bullet}$) in the presence of 0.2 mM $Hasc^-$

conversion of e^-_{aq} to $HO^{\bullet}$ (Eq. 2) and complete scavenging of $HO^{\bullet}$ by $N_3{}^-$ (Eq. 3) to guarantee formation of stoichiometric amounts of $N_3^{\bullet}$. Furthermore, low radiation doses were used to limit radical–radical recombination, and the amino acid or protein was present in excess to prevent direct reaction of $N_3^{\bullet}$ with $Hasc^-$. In all cases, spontaneous rates of decay of radicals were accelerated in the presence of $Hasc^-$. The overall result is the formation of the relatively inert ascorbyl radical and repair of the amino acid. The results for pepsin, as summarised in Eqs. 8 and 9, are representative of all results with proteins and are shown in Fig. 2.



$$\text{Pep(Trp}^{\bullet}) + \text{Hasc}^- \rightarrow \text{Pep(TrpH)} + \text{asc}^{\bullet -} \quad (8)$$

$$\text{Pep(TyrO}^{\bullet}) + \text{Hasc}^- \rightarrow \text{Pep(TyrOH)} + \text{asc}^{\bullet -} \quad (9)$$

The collected rate constants for Eqs. 8 and 9 and for the corresponding reactions in proteins other than pepsin are listed in Table 2. The rate constants for reaction of $Hasc^-$ with *N*-Ac-TyrO$^{\bullet}$ amide are comparable to those for the reduction of TyrO$^{\bullet}$ by Sec and GSeH. It can be seen that, in general, $Hasc^-$ reacts 10–50 times faster with protein-bound Trp$^{\bullet}$ than with protein-bound TyrO$^{\bullet}$ radicals. Interestingly, the protein environment apparently does not affect the kinetics of Eq. 8, but lowers the rate constant for protein-bound TyrO$^{\bullet}$ (Eq. 9) by about a factor of 10, compared to free *N*-Ac-Trp$^{\bullet}$ amide. Presumably, these effects depend on variables such as the nature of the protein, the location of the reacting residue and potential stabilisation of the radical by neighbouring amino acids. Preliminary evidence suggests that peroxyl radicals of *N*-Ac-Pro-amide, *N*-Ac-Ala-amide and *N*-Ac-Gly-Ala-amide react with $Hasc^-$ with rate constants of ca. 10^7 M^{-1} s^{-1} (Zelenay and Domazou 2009, unpublished).

Potential biological significance of radical oxidation of proteins and long-range electron transfer

We have presented evidence that the antioxidants GSH and $Hasc^-$ react with TyrO$^{\bullet}$ and Trp$^{\bullet}$ as free amino acids, protein residue analogues and protein radicals. Both antioxidants inhibit the transfer of electrons from Tyr to Trp$^{\bullet}$ in proteins in vitro, but GSH does not prevent the formation of protein peroxyl radicals or hydroperoxides. The repair of protein C-centred radicals by $Hasc^-$ competes with the reaction with O_2. Both reactions may contribute to the loss of GSH and $Hasc^-$ in living organisms under conditions of oxidative stress. It should be noted that, while the radicals formed in a protein can be efficiently repaired by GSH and $Hasc^-$, repair does not necessarily restore biological function, which may be irreversibly altered by the initial radical reaction and/or subsequent formation of other radical species by LRET.

Certain cellular signalling pathways, e.g. the signal transducers and activators of transcription (STAT) family of transcription factors, function via reversible phosphorylation of tyrosine residues by tyrosine kinases and phosphorylases (Ihle 2001), which are modulated by the redox status of the cell (Monteiro and Stern 1996), likely due to the impact of LRET on ultimate formation of TyrO$^{\bullet}$ radicals in proteins.

Table 2 Rate constants of reactions ascorbate with Trp$^{\bullet}$ and TyrO$^{\bullet}$ as free amino acids, peptide models and proteins

Amino acid or protein radical	Rate constant	Reference
TyrO$^{\bullet}$	9.3×10^7	Hoey and Butler (1984)
N-Ac-TyrO$^{\bullet}$ amide	$(2.6 \pm 0.2) \times 10^8$	Domazou et al. (2009)
Trp$^{\bullet}$	1×10^8	Jovanovic and Simic (1985)
N-Ac-Trp$^{\bullet}$ amide	$(1.4 \pm 0.1) \times 10^8$	Domazou et al. (2009)
Ins(TyrO$^{\bullet}$)	$(2.9 \pm 0.1) \times 10^7$	Domazou et al. (2009)
Chym(TyrO$^{\bullet}$)	4×10^7	Domazou et al. (2009)
Chym(Trp$^{\bullet}$)	$(1.6 \pm 0.1) \times 10^8$	Domazou et al. (2009)
Pep(TyrO$^{\bullet}$)	$(3.5 \pm 0.9) \times 10^7$	Domazou et al. (2009)
Pep(Trp$^{\bullet}$)	$(1.8 \pm 0.1) \times 10^8$	Domazou et al. (2009)
β-Lact(TyrO$^{\bullet}$)	$(4.0 \pm 0.6) \times 10^5$	Domazou et al. (2009)
β-Lact(Trp$^{\bullet}$)	$(2.2 \pm 0.2) \times 10^7$	Domazou et al. (2009)
Lyz(TyrO$^{\bullet}$)	1.1×10^7	Hoey and Butler (1984)
Lyz(Trp$^{\bullet}$)	8×10^7	Santus et al. (2000)
Lyz(Trp$^{\bullet}$)	8.3×10^7	Hoey and Butler (1984)

Finally, radical reactions in proteins are not uniformly harmful. Numerous enzyme reaction mechanisms rely upon transient formation of an organic radical, e.g. the relatively stable tyrosyl radical formed during the reaction of ribonuclease reductase I (Stubbe and van der Donk 1998). Iodination of tyrosine residues on thyroglobulin during thyroid hormone biosynthesis synthesis also involves transient tyrosyl radical formation (Nunez 1984). Phosphorylation of free tyrosine has been shown to block tyrosine iodination (Gmeiner and Seelos 1996) and dimerisation, both of which are radical reactions.

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