Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants

J. Matysik[†,*], Alia[†,#], B. Bhalu[‡] and P. Mohanty[‡]

[†]Biophysical Organic Chemistry, Leiden Institute of Chemistry, Einsteinweg 55, 2300 RA Leiden, The Netherlands
[#]Department of Biophysics, Leiden Institute of Physics, Niels Bohrweg 2, 2300 RA Leiden, The Netherlands
[‡]Jawaharlal Nehru University, New Delhi 110 067, India

Less than 5% of the total pool of free amino acids in plants under stress-free conditions is provided by proline. In many plants under various forms of stress, the concentration increases up to 80% of the amino acid pool. This observation raises the question about the molecular mechanisms, making a high proline concentration favourable under stress conditions. Therefore, the literature about the chemical properties of proline is reviewed, linking it to the plant physiological observations. In addition to its role as an osmolyte and a reservoir of carbon and nitrogen, etc. proline has been shown to protect plants against free radical-induced damage. A recent concept that proline accumulation is linked with the quenching of singlet oxygen has inspired us to look at the molecular mechanism of singlet oxygen quenching by proline. In this review, the key properties and the chemical reactivity of proline with singlet oxygen and other reactive oxygen species are discussed.

Stress induces increase of proline level

Increase of proline concentration

It is known for a long time that the concentration of proline increases in a large variety of plants under stress, up to 100 times the normal level[1], which makes up to 80% of the total amino acid pool. This dramatic increase occurs over several hours[2] or days[3]. The content of proline in plants can be estimated photometrically, in a rather straightforward manner[4]. As recently observed by *in vivo* ^{13}C NMR spectroscopy[5], most of the proline in plants is accumulated in the cytoplasm rather than in the vacuoles. The accumulation and protective effect of proline has been observed in many higher plants and bacteria as well as in protozoa, algae and marine invertebrates (for review, see Delauney and Verma[6]).

Many stresses, same effect

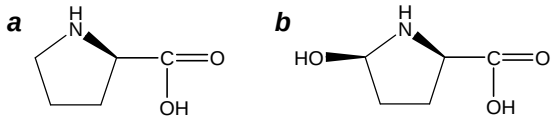
Stresses such as cold[7], heat[8], salt[9], drought[10], UV[11] and heavy metal[12] cause significant increase in the proline concentration in a variety of plants. Interpretations of proline accumulation vary from its role as a useful adaptive response, helping organisms to withstand the effect of stress, to merely a consequence of stress-induced damage to the cells (for review, see Aspinall and Paleg[13]). Transgenic plants which are not able to produce proline, have a significantly lower stress tolerance[14,15]. Therefore, proline may not be just a by-product of stress defence, but a chemically active compound, crucially involved in the physiology of stress protection.

Molecular mechanism

In the literature, the function of proline in stressed plants is often explained by its property as an osmolyte, able to balance water stress[6,11]. In addition, other possible positive roles of proline under stress have been proposed with greater or lesser convictions, which include stabilization of proteins[16], scavenging of hydroxyl radicals[17], regulation of the cytosolic pH[18], and regulation of NAD/NADH ratio[19]. The exact molecular mechanism of proline-induced protection of plants under stress is still unknown.

Searching for a common molecular mechanism of plant protection by proline during various forms of stress, this review tries to link aspects of plant physiology with the knowledge of chemistry. Harman[20] provided a general concept for the molecular basis of ageing, for the first time. According to him, stress is the result of the sum of damages in all cellular components (lipids, proteins and nucleic acids). All stresses induce the production of reactive oxygen species, especially singlet oxygen and free radicals which are known to break DNA[21], destroy the function of proteins and are responsible for lipid peroxidation[22]. Plants have evolved diverse strategies of acclimatization and avoidance to cope with adverse environmental conditions[23]. This includes accumulation of compatible solutes like glycinebetaine, proline and mannitol. Interestingly, among various compatible solutes, proline is the only one which has been shown to protect plants against singlet oxygen and free-radical induced damages[24]. Due to its action as singlet-oxygen quencher[25] and scavenger of OH• radicals[17], proline is able to stabilize proteins[16], DNA[26] and membranes[24]. Accumu-

*For correspondence. (e-mail: j.matysik@chem.leidenuniv.nl)



Scheme 1. Structure of ***a***, L-proline; and ***b***, hydroxyproline.

lation of proline-rich proteins and particularly proline residues in proteins provides additional protection against oxidative stress.

Proline – a special amino acid

The free amino acid

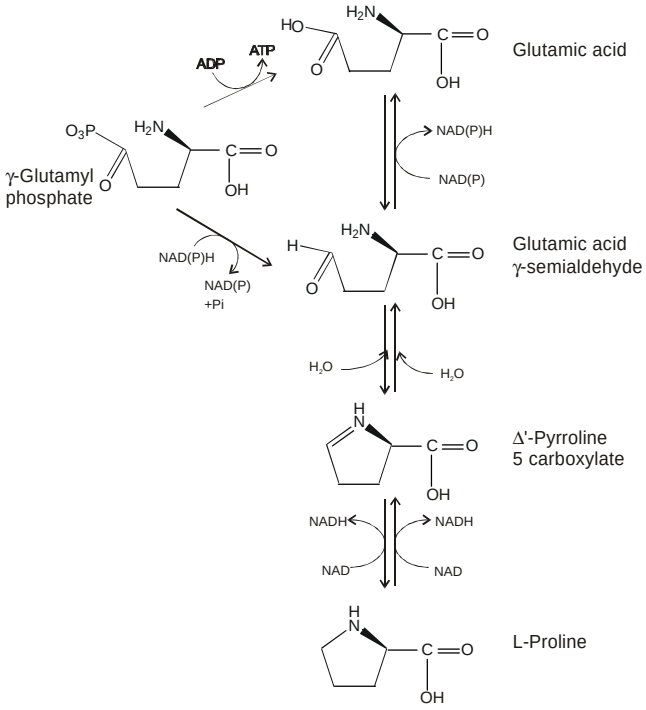
The name 'proline' has been derived from 'pyrrolidine' by Emil Fischer in 1904. The amino acid L-proline (L-pyrrolidine 2-carboxy acid, $C_5H_9NO_2$, Scheme 1 *a*) in its pure form is a colourless substance, highly soluble in water, well (unlike all other amino acids) in alcohols, sparingly in benzene and acetone and insoluble in ether. In aqueous solution at physiological pH, the free amino acid proline occurs in its zwitterionic form, carrying negative charge on the deprotonated carboxyl group and positive charge on the doubly protonated amino function. Therefore it can also be referred to as 'proline betaine'. Due to ring puckering, two conformations of the pyrrolidine ring exist, called *up* and *down*[27].

Proline as protein residue

Incorporated into peptides, the substituents of the pyrrolidine ring can also assume two conformations, *trans* and *cis*, depending on whether the peptide carbonyl oxygen atoms are pointing to each other (*cis*) or not (*trans*). The conformation of a proline residue can affect the protein function, for example, for metal binding[28]. Proline incorporation has a destabilizing effect on the secondary structure of proteins. As a rotationally hindered secondary amine (unique among all neutral amino acids), proline hardly matches the steric requirements needed for forming an *a*-helix. Proline residues are often at the end of an *a*-helix. In the formation of *b*-turns, often proline and hydroxyproline ([2S]-[4R]-hydroxy pyrrolidine 2-carbonic acid, Scheme 1 *b*) are involved. In general, *b*-turn-rich proteins contain a high amount of (hydroxy) proline, e.g. casein (7%) and prolamine (20%). Collagen (26%) forms a special triple helix.

Chemical reactivity and biosynthesis

Primary amino acids R-CH(COOH)(NH_2) react chemically, as expected from a compound carrying an amino and a carboxyl group. They can be oxidized by thermal, chemical or enzymatic decarboxylation. The products are



Scheme 2. Proline biosynthesis and oxidation showing ring-opening from proline to glutamate and prolineglutamate electron sponge system.

amides R-CO(NH_2), aldehydes R-CH-CHO (ref. 29), and amines R-CH_2-NH_2. In case of the secondary amino acid proline, the amide is the cyclic 2-pyrrolidone[30].

In addition to the reactions similar to the primary amino acids, proline can perform reversibly a ring-opening reaction by addition of a molecule of water, forming glutamic acid *g*-semialdehyde (Scheme 2), a derivative of glutamate, which is the most central amino acid in the network of amino acid biosynthesis. Since the semi-aldehyde is reduced two electron equivalents higher as glutamate, proline can be considered as an 'electron-rich glutamate', and hence the redox system proline/glutamate provides a mitochondrial 'electron sponge'[31] (Scheme 2). This system drives, for example, insect flight muscles[32]. In general, proline is formed from glutamate in plants[33]. Alternatively, proline is also enzymatically synthesized from ornithine[34] by exchange of the *d*-amino to a *g*-aldehyde group forming the glutamic acid semi-aldehyde as precursor of proline. Hydroxyproline is produced in the peptide-bound form from proline, by oxidation with an ascorbic acid-dependent monooxygenase.

Protective action of free proline against reactive oxygen species

'Normal' molecular oxygen ($^3O_2$) is in its electronic ground state, which is a triplet state. Due to spin-conservation rules, triplet oxygen is rather inactive to biological material. However, electronically excited singlet

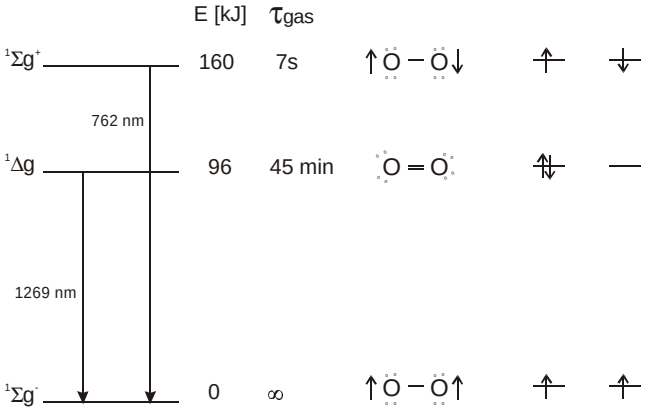
oxygen ($^1O_2$) (Scheme 3)[35] is highly reactive and rapidly oxidizes amino acids, lipids and DNA. Free radicals produced by reaction with $^3O_2$ also react aggressively. The one-electron reduced superoxide radical (HO_2• or O_2^{1-}•) and the hydroxy radical (OH•), which is on the same redox level as the peroxide, are the main oxygen radical compounds (Scheme 4). Reactive oxygen species cause stress to biological systems. Stress in turn induces the production of reactive oxygen species. Therefore, a mechanism to interrupt such an autocatalytic process is required. Under normal circumstances, concentrations of oxygen radicals remain low because of the activity of protective enzymes, including superoxide dismutase, catalase and ascorbate peroxidase[36]. Under stress, accumulation of compatible solutes occurs, in addition to increase in the activities of detoxifying enzymes. As mentioned before, proline accumulates in high amount in several plants under stress. This accumulation of proline has been shown to protect plants against damage by reactive oxygen species. In the following, the chemical reactivity of proline with reactive oxygen species is discussed, with the aim to understand the molecular mechanism of the protective effect of proline under stress in plants.

Proline quenches singlet oxygen

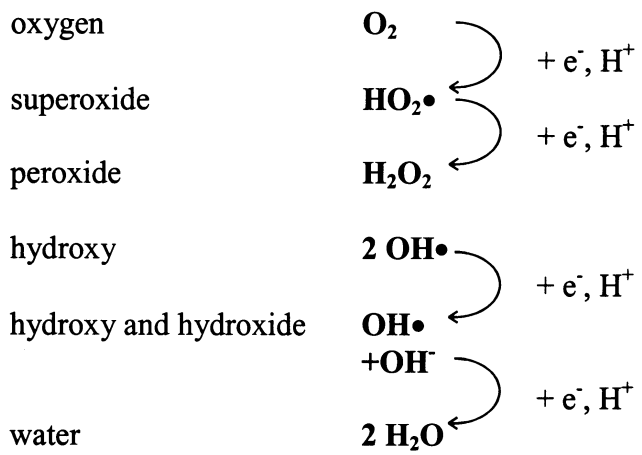
First some important properties of singlet oxygen will be introduced. Subsequently, the electronic state of singlet oxygen, its production (*in vitro* and in plants) and its chemical reactivity with proline and other amines are discussed.

Electronic state of singlet oxygen: Since chemical properties depend on the electronic structure, electronically excited molecules undergo different chemical reactions as the same molecules in their electronic ground state. Therefore, molecules in different electronic states have to be regarded as different chemical species. Most molecules have a singlet state (S) as electronic ground state, i.e. all electrons are paired and there is no electronic spin. There are, however, important exceptions. One of them is molecular oxygen (O_2) which has triplet state (T) as electronic ground state. 'Normal' molecular oxygen (Scheme 3), which is in the air we are breathing, is therefore in triplet state ($^3O_2$) (denoted as $^1\Sigma g^-$). Other examples for compounds with T-ground state are given by carbene compounds (CR_2). Due to quantum mechanical spin-conservation rules, chemical reactions between species in triplet and singlet states are spin-forbidden, and therefore have a high kinetic reaction barrier. This is the reason why atmospheric oxygen does not react easily with organic matter, although such reaction would be energetically very favourable. In the electronically excited state (denoted as $^1\Delta g$), oxygen becomes an electronic singlet (Scheme 3). Reactions of singlet oxygen with organic molecules are not spin-forbidden, and have much less activation energy. One way to circumvent the spin-dependent selection rules is provided by catalysis with transition metals (via spin-orbit coupling of their d-orbitals). Hence, nature uses transition metals as the heme iron in cytochrome-*c* oxidase[37,38] or a tetra-manganese cluster in photosystem II (ref. 39) for undergoing oxygen chemistry. Lifetime and action radius of singlet oxygen depend on the surrounding medium. In aqueous solution singlet oxygen has a lifetime of ca. 3 μs and can diffuse almost 0.2 μm (ref. 40). In D_2O, the lifetime of singlet oxygen is 10 to 15 times higher than in water[41,42]. In gas phase, singlet oxygen molecules can diffuse up to a radius of 1 mm (ref. 43). This is a rather long lifetime, compared to other reactive oxygen species. Consequently, chemical reactions of singlet oxygen are much more specific compared to OH• radicals. Singlet oxygen reacting with DNA interacts selectively with guanine, since it has the lowest redox potential among the nucleotides constituting DNA[44].

The decay of the singlet state to the triplet ground state can be observed by its chemiluminiscence emission.



Scheme 3. Structure and lifetime (t) of electronically excited oxygen molecules. The $^1\Sigma g^-$ ground state is a triplet. The two singlet states $^1\Delta g$ and $^1\Sigma g^+$ are electronically excited. (Adapted from Adam, W.[35]).



Scheme 4. Oxidation level of reactive oxygen species.

There are single-molecule emissions (1269 and 1588 nm) as well as double-molecule emissions (634 and 703 nm). The latter can be observed only at high concentrations. Another method to detect singlet oxygen is by observing spin-trapped reaction products by EPR spectroscopy[45–48].

Production of singlet oxygen: The usual way to produce singlet oxygen *in vitro* is by irradiation of photosensitizers. These are dyes with high quantum yield for triplet formation (Scheme 5, formulae [1]–[3]). In nature, singlet oxygen is produced in the same photochemical way. For example P_{680}, the primary electron donor in reaction centres of photosystem II in plants[49], acts as photosensitizer and is the main source of singlet oxygen in plants[50,51]. Another cellular source of singlet oxygen are heme proteins such as peroxidases[52]. In plants under high light irradiation, the likelihood for the production of singlet oxygen increases, because photons are absorbed faster than electrons pumped. The direct involvement of singlet oxygen in photobleaching of photosynthetic pigments, D1 protein degradation and protein cross-linking has been reported[53]. Also, various stress conditions produce singlet oxygen, which affects the structural integrity of the photosystem II membrane protein complex, and destabilizes proteins and membranes further. Plants have developed several methods for dealing with singlet oxygen. For example, carotenoids in photosystem II are efficient singlet-oxygen quenchers. The long conjugated chain of a carotenoid allows fast thermalization of excitation energy. The degradation of D1 by singlet oxygen is tackled by the fast turn-over of D1 polypeptide without deconstruction of the rest of the enzyme complex[54].

Chemical reactivity of singlet oxygen with proline: Our recent *in vitro* experiments[55,56] on singlet oxygen quenching by proline have shown that it is an excellent quencher for singlet oxygen (Figure 1). Singlet oxygen ($^1O_2$) was produced photochemically by irradiating a solution of sensitizer and detected by following the formation of stable nitroxide radical (TEMPO) during the reaction of $^1O_2$ with the sterically hindered amine (2,2,6,6-tetramethylpiperidine, TEMP). Figure 1 *a* shows the production of singlet oxygen as detected by the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) by EPR spectrometry. Interestingly, the production of TEMPO was decreased in the presence of 5 and 10 mM proline (Figure 1 *b* and *c*) and completely stopped by the presence of proline at concentration as low as 20 mM (Figure 1 *d*). These results show that proline is a very effective singlet-oxygen quencher. Other singlet-oxygen generating photosensitizers such as hemeatoporphyrin and fluorescein also produced identical results with proline (data not shown). This is in line with the low ionization potential (IP) of proline, as discussed below. Glycine or different types of sugar have shown no effect on the singlet oxygen concentration. These experiments suggest that the increase in proline concentration in stressed plants is due to the efficient singlet-oxygen quenching properties of proline. In the following paragraphs, the molecular mechanism of the chemical reaction of proline with singlet oxygen is discussed in detail.

The most characteristic feature of the reactivity of singlet oxygen is its electrophilicity. Therefore, C=C or

$^1D + h\nu \rightarrow {}^1D^*$ (photochemical excitation) [1]

$^1D^* \rightarrow {}^3D$ (inter-system crossing) [2]

$^3D + {}^3O_2 \rightarrow {}^1D + {}^1O_2$ (photo-sensitization) [3]

$^1O_2 + A \leftrightarrows {}^1[O_2^{\delta-} \leftarrow A^{\delta+}]$ (reversible formation of a charge-transfer complex) [4]

$^1[O_2^{\delta-} \leftarrow A^{\delta+}] \rightarrow {}^3[O_2^{\delta-} \leftarrow A^{\delta+}] \rightarrow {}^3O_2 + A$ (physical quenching) [5]

$^1[O_2^{\delta-} \leftarrow A^{\delta+}] \rightarrow$ products (chemical quenching) [6]

Scheme 5. Photochemical production of singlet oxygen ($^3O_2$) by a photosensitizer (D) and its reaction with amines (A).

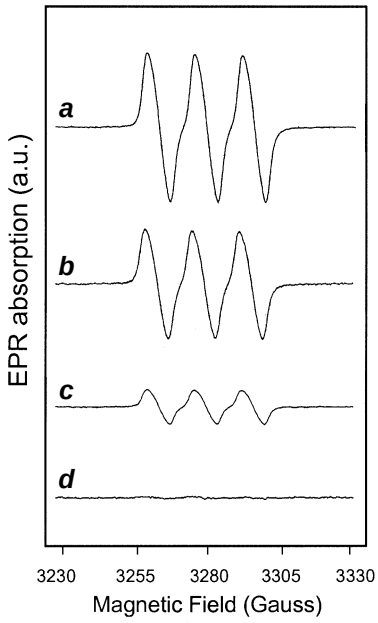


Figure 1. *In vitro* quenching of singlet oxygen by proline: Nitroxide radical (TEMPO) was formed due to reaction of sterically hindered amine (TEMP) with singlet oxygen ($^1O_2$) generated by photoirradiation of toluidine blue in the absence (***a***) or presence of 5 mM (***b***), 10 mM (***c***) and 20 mM (***d***) proline. Sample contained 1 mM toluidine blue and 10 mM TEMP was irradiated with white light (1200 μE m^{-2} s^{-1}) for 20 min. EPR spectra were recorded with an X-band EPR spectrometer at modulation amplitude 1 Gauss; modulation frequency 100 kHz; microwave power 15 mW; temperature 25°C.

C=O double bonds as well as the lone pairs of sulphur and amine compounds are preferably the targets of singlet-oxygen attack. Investigations of the reaction with amines have revealed that the kinetics of the reaction is controlled by the IP (i.e. the capability to provide an electron) of the amine. Therefore, the formation of a charge-transfer (CT) complex in a reversible reaction is assumed to be the initial step[57] (Scheme 5, formula [4]).

An amine, having a low IP, is easily capable of forming a CT complex, and can therefore quench singlet-oxygen faster. IPs can be determined either by photo-electron spectroscopy (PES) or electrochemically. The first electron removed from an amine can be assigned to the lone pair of nitrogen atom. The value of IP depends on the chemical structure of the amine. Due to the electron-donating character of alkyl substituents, tertiary amines have lower IP than secondary amines, and secondary amines have lower IP than primary amines. Therefore, charge transfer complexes of tertiary amines are more stable than secondary amines and secondary amines more than primary amines (Scheme 6). Also cyclic compounds are easier to ionize as open-chained amines. The larger the ring, the lower is the IP[58,59]. The IP of enamines is lower than the IP of tertiary amines[60]. IPs of peptide-bound amino acids are similar to those of isolated amino acids, since interactions among amino and carboxyl end groups are weak[61].

Pyrrolidine, which forms the 5-membered ring of proline, has a remarkable low IP of 8.0 eV. The substitution of the carboxyl group increases the IP slightly, by withdrawing electrons from the ring[62]. A comprehensive body of IP data of amino acids at gas phase has also been reported[62–64]. Only amino acids with aromatic systems (Trp, 7.9 eV; Tyr, 8.5 eV; Phe, 9.4 eV), a sulphur-centred lone pair (Met, 8.65 eV) or a pyrrolidine ring structure (Pro, 9.0 eV) have an IP lower than 9.5 eV. Aromatic amino acids, however, are only poorly soluble in aqueous solution compared to proline (at 25°C solubility in 100 g water: 0.045 g tyrosine, 1.06 g tryptophan, 4.2 g histidine, 162 g proline)[65]. Hence, their use as singlet-oxygen quenchers in biological systems is low in comparison with proline.

The decay of the initial CT complex of the amine with the singlet oxygen can occur either by physical (Scheme 5, formula [5]) or chemical (Scheme 5, formula [6]) quenching. For amines, both pathways are competing. Due to its low IP, proline forms CT complexes with singlet oxygen, and due to its structure, chemical and physical quenching is possible. Physical quenching works via a mechanism involving spin-orbit coupling, restoring the original amine compound in its singlet ground state. For instance, azide (N_3^-) is known to quench via the physical pathway. In the case of chemical quenching, the structure of the amine is changed and a reaction product is formed. One crucial aspect for the feasibility of a chemical pathway is the existence of α-hydrogen atoms and the possibility to form a $C{=}NH^+$ group. For highly constrained amines like 2,2,6,6-tetramethylpiperidine (TEMP), another chemical pathway producing stable nitroxide radicals is possible. This reaction is used for the detection of singlet oxygen by EPR spectroscopy (see above). Hence, the experimental finding of the high capability of proline to quench singlet oxygen can be well understood by its chemical properties.

Primary amine:	Ethyl amine $CH_3CH_2NH_2$	**8.9 eV**
Secondary amine:	Dimethyl amine $(CH_3)_2HNH_2$	**8.2 eV**
Pyrrolidine:		**8.0 eV**
Tertiary amine:	Trimethyl amine $N(CH_3)_3$	**7.8 eV**

Scheme 6. Ionization potentials of some amines measured in the gas phase.

Reactivity of proline toward hydroxy radicals

Compared to singlet oxygen, OH• radicals react much faster and therefore with less selectivity. Both reactive species can react as strong oxidants and are able to abstract hydrogen atoms. The involvement of hydroxyl OH• and superoxide O_2^-• radicals in oxidative stress is well known. The protective capability of proline against free radical-induced plant stress was also reported[17].

Formation of hydroxyl radicals: OH• radicals can be formed in several ways. Fenton's reaction for production of hydroxyl radicals by oxidation of Fe^{2+} ions is well known:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^-.$$

Also other metal ions as Cu^{1+} are able to reduce hydrogen peroxide (H_2O_2). In the presence of primary carbonate HCO_3^-, Mn^{2+}, which occurs in high concentration in plants, can cause the disproportionation of H_2O_2 (ref. 66). Hydrogen peroxide also can form two OH• radicals, either by heating or by photolyzation under UV irradiation. Organic peroxides as tertiary butyl hydroperoxide provide OH• radicals. Heme proteins undergoing oxygen redox chemistry can release free radicals[67]. Another method for the production of OH• radicals (together with H• radicals and solvated electrons e_{aq}) are γ-rays from a cobalt-60 source[68,69]. Superoxide radicals are formed by the reaction of H• or e_{aq}^- with molecular oxygen. OH• radicals can be detected with EPR spectroscopy by using trapping compound *p*-nitrosodimethylaniline[70]. The same strategy can also be applied to superoxide radicals[71].

Reactions of hydroxy radicals with proline: Smirnoff and Cumbes[17] have assessed the hydroxy-radical sca-

venging activity of various compatible solutes, including proline, which accumulate in plants under stress and they have found that sorbitol, mannitol, myo-inositol and proline are effective hydroxy radical scavengers. According to Rustgi *et al.*[72], proline reacts with OH• under hydrogen abstraction by forming the most stable radical, which carries the spin on the C-5 atom since it is far from the carboxyl group and close to the nitrogen. In case of hydroxyproline, the radical with spin localization on the C-4 is more stable. The rate constant of the reaction with proline is lower ($6.5 \times 10^8\ M^{-1}\ s^{-1}$) than that for the aromatic and sulphur-containing amino acids, but exceeds that of most other amino acids[73]. In Fenton-type or tertiary butyl hydroperoxide-containing systems, a long-lived proline–nitroxyl radical $R_2N–O•$ has been observed[74].

Most of the OH• radicals generated *in vivo*, except during excessive exposure to ionizing radiation, come from Fenton's reaction by oxidation of metals[75]. Whether this reaction occurs and OH• radicals are released, depends on the state of complexation of the redox-active metal. Normally, metal ions in aqueous solution do not exist 'naked', but are either hydrated or ligated by other molecules such as hydrogen carbonate[66] or chelating compounds as EDTA, which have a strong effect on the chemical reactivity of the metal. Hepes or MES buffers inhibit Fenton's reaction[76]. Oxygen rapidly oxidizes $Fe(OH)_2$ to Fe(III). Since Fe(III) exists largely as insoluble polymeric $Fe(OH)_3$ at physiological pH, substoichiometrical amounts of chelating compounds can keep a trace of iron soluble, catalysing Fenton's reaction. Under these conditions, the reaction may occur in the complexation shell of the redox-active metal and may only affect the ligands. Such a 'caged reaction'[77] can effectively protect the surrounding biological material, since the OH• radical cannot attack outside the cage. This means that binding of proline to redox-active metal ions can protect the surrounding biological tissue from damage by OH• radicals.

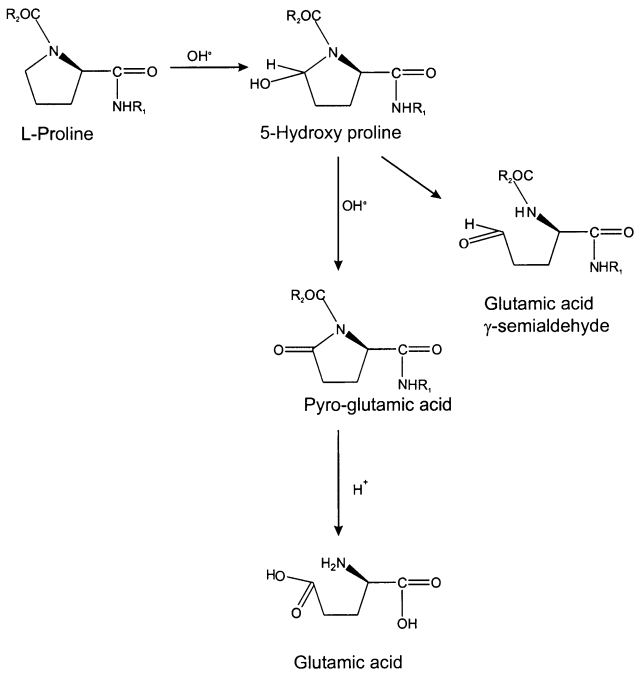
Proline residues in proteins

Singlet-oxygen quenching

Proline incorporated into a peptide backbone becomes a tertiary amide. The IP of peptide-bound proline residues is similar to free proline, and therefore the capability to quench singlet oxygen is also similar. It has been shown that proline-rich proteins, stabilizing cell walls, are secreted by salt-adapted bean cells[78]. Also in animal and human cells, production of proline-rich proteins has been described. Examples are the salivary proline-rich proteins (PrPs)[79] and the epithelial small proline-rich proteins (SPRRs)[80,81]. Both classes of proteins can be cross-linked by transglutaminases[82,83]. PrPs are part of the salivary pellicle covering the tooth surface and SPRRs, also called cornifins, are precursor proteins of the cornified cell envelope of the epidermal skin[84,85]. Interestingly, the expression of SPRRs is strongly induced after exposure of epidermal keratinocytes[80] or skin[86] to UV radiation. Whether this regulation reflects the role of proline to protect against singlet oxygen-induced skin damage, is not yet known.

Reactions with hydroxyl radicals

Attack by OH• and HO_2• radicals is known to destabilize proteins[87]. The peptide backbone can be cleaved[88] by forming a carbon-centred radical after *a*-hydrogen abstraction. Furthermore, side chains can be attacked. Schuessler and Schilling[89] proposed that proline residues are preferred sites of radical attack. Proline can be oxidized to various compounds (Scheme 7)[77]. Formation of 4-hydroxyproline[90] will not cause a cleavage of the polypeptide. Also the formation of 5-hydroxyproline, which can hydrolyse to glutamate *g*-semialdehyde[91], does not damage the amino-acid backbone. The observation of *g*-aminobutyric acid, glutamic acid[92] and 2-pyrrolidone[93,94], however, has to be explained by backbone cleavage. Oxidation of proline to 2-pyrrolidone provides a unique mechanism for peptide backbone cleavage that is not associated with the formation of a reactive carbonyl group. The question arises whether the very high content of proline and hydroxyproline in collagen is advantageous for the maintenance of tissue structures under metabolic conditions. Collagen degradation is a hallmark of photodamaged connective tissue[95]. X-ray irradiation,



Scheme 7. Proline residues in polypeptides/proteins. Prolyl residues are selectively oxidized by OH^o to glutamic acid.

applied in lung cancer, is known to induce fibrosis, i.e. an excessive accumulation of collagen, limiting the irradiation level in radiation therapy of thoracic tumours (for review, see ref. 96).

Caged reactions in the enzyme

If the redox-active metal is bound to the protein, oxidation can be site-specific. The Fenton-type generation of the OH• radical is followed by the formation of a more stable carbon-centred radical. Just as guanine becomes selectively oxidized[44] within nucleic acids, 'hole hopping'[97] may also occur among amino acids, forming the most stable amino-acid radical. Such 'caged' enzymatic reactions can be important for the function of an enzyme, and several proteins with oxidative, modified amino acids in the reaction centre are known. Cross-linked amino acid residues are known (e.g. the reactive centres of cytochrome-*c* oxidase[98] and galactose oxidase[99]). In both cases, the two covalently bound amino acids are functionally important and are in direct neighbourhood of the metal. Site-directed oxidation of proline, histidine, arginine, lysine, threonine, tyrosine and cysteine has been reported (for review, see ref. 77). Hence, proline residues, which are very good singlet-oxygen quenchers and traps for OH• radicals, stabilize proteins endogenously. The heavy-metal binding properties of carpenter's glue, containing proline-rich proteins as glutin or casein from animal bones, used in order to prevent unwanted by-reactions, are well known among synthetic chemists[100].

Concluding remarks

The high capability of proline to quench singlet oxygen and hydroxy radicals can be well understood by its chemical properties. Pyrrolidine, which forms the 5-membered ring of proline, has a remarkably low IP, and therefore proline is capable of forming charge-transfer complex and can quench singlet oxygen effectively. Proline reacts with OH• under hydrogen abstraction by forming the most stable radical, which carries the spin on the C-5 atom. Therefore, proline accumulation in high amounts in plants under stress could be well understood by its property to scavenge reactive oxygen species. In the future, genetic engineering of proline biosynthesis in important crop plants which do not accumulate proline under stress would be an important strategy to tackle the high level of reactive oxygen species generated during stressful conditions. Furthermore, dermatology may profit from understanding the action of proline-rich proteins in order to protect tissues from radiation-induced damages.

1. Barnett, N. M. and Naylor, A. W., *Plant Physiol*., 1966, **41**, 1222–1230.
2. Aziz, A., Martin-Tanguy, J. and Larher, F., *Physiol. Plant*., 1998, **104**, 195202.
3. Lee, T. M. and Chang, Y. C., *J. Phycol*., 1999, **35**, 8488.
4. Bates, L. S., Waldren, R. P. and Teare, I. D., *Plant Soil*, 1973, **39**, 205207.
5. Aubert, S., Hennion, F., Bouchereau, A., Gout, E., Bligny, R. and Dorne, A. J., *Plant Cell Environ*., 1999, **22**, 255259.
6. Delauney, A. J. and Verma, D. P. S., *Plant J*., 1993, **4**, 215–223.
7. Wanner, L. A. and Junttila, O., *Plant Physiol*., 1999, **120**, 391–399.
8. Chang, Y. C. and Lee, T. M., *Bot. Bull. Acad. Sin.*, 1999, **40**, 289–294.
9. Ali, G., Srivastava, P. S. and Iqbal, M., *Biol. Plant.*, 1999, **42**, 89–95.
10. Stewart, G. R. and Larhar, F., in *The Biochemistry of Plants* (eds Stumpf, P. K. and Conn, E. E.), Academic Press, New York, 1980, vol. 5, pp. 609635.
11. Saradhi, P. P., Alia, Arora, S. and Prasad, K. V. S. K., *Biochem. Biophys. Res. Commun*., 1995, **209**, 15.
12. Alia and Pardha Saradhi, P., *J. Plant Physiol*., 1991, **138**, 554–558.
13. Aspinall, D. and Paleg, L. G. (eds), in *Physiology and Biochemistry of Drought Resistance in Plants*, Academic Press, Sydney, 1981, pp. 243259.
14. Nanjo. *et al*., *Plant J*., 1999, **18**, 185193.
15. Xin, Z. and Browse, J., *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 77997804.
16. Anjum, F., Rishi, V. and Ahmed, F., *Biochim. Biophys. Acta*, 2000, **1476**, 7584.
17. Smirnoff, N. and Cumbes, Q. J., *Phytochemistry*, 1989, **28**, 1057–1060.
18. Venekamp, J. H., *Physiol. Plant.*, 1989, **76**, 112117.
19. Alia, Saradhi, P. P., *Biochem. Biophys. Res. Commun*., 1993, **193**, 5458.
20. Harman, D., *J. Gerontol.*, 1956, **11**, 298300.
21. Wei, H. C., Ca, Q. Y., Rahn, R., Zhang, X. S., Wang, Y. and Lebwohl, M., *Biochemistry*, 1998, **37**, 64856490.
22. Heath, R. L. and Packer, L., *Arch. Biochem. Biophys.*, 1968, **125**, 189198.
23. Levitt, J., in *Physiological Ecology* (ed. Kozlowski, T. T.), Academic Press, New York, 1980, pp. 2356.
24. Alia, Saradhi, P. P. and Mohanty, P., *Biochem. Biophys. Res. Commun*., 1991, **181**, 12381244.
25. Alia, Pardha Saradhi, P. and Mohanty, P., *J. Photochem. Photobiol. B*, 1997, **38**, 253257.
26. Iakobasshvili, R. and Lapidot, A., *Nucleic Acids Res*., 1999, **27**, 15661568.
27. Milner-White, E. J., Bell, L. H. and Maccallum, P. H., *J. Mol. Biol*., 1992, **228**, 725734.
28. Perera, L., Darden, T. A. and Pedersen, L. G., *Biochemistry*, 1998, **37**, 1092010927.
29. Ram Reddy, M. G., Sethuram, B. and Navaneeth Rao, T., *Z. Phys. Chem*., (*Leipzig*), 1975, **256**, 880884.
30. Ochiai, M., Inenaga, M., Nagao, Y., Moryarty, R. M., Vaid, R. K. and Duncan, M. P., *Tetrahedron Lett.*, 1988, **29**, 69176920.
31. Guignard, R., Michea-Hamzehpour, M. and Turian, G., *FEMS Microbiol. Lett*., 1984, **25**, 265269.
32. Bursell, E. and Slack, E., *Insect Biochem.*, 1976, **6**, 159167.
33. Yang, C. W. and Kao, C. H., *Plant Growth Regul*., 1999, **27**, 189–192.
34. Kenklies, J., Ziehn, R., Fritsche, K., Pich, A. and Andreesen, J. R., *Microbiology*, 1999, **145**, 819826.
35. Adam, W., *Chem. Unserer Zeit*, 1981, **15**, 190196.
36. Asada, A., *Methods Enzymol*., 1984, **105**, 422428.
37. Matysik, J., *Indian J. Exp. Biol*., 1997, **35**, 679684.
38. Matysik, J., Alia, in *Biophysical Processes in Living Systems* (ed.

Pardha Sardhi, P.), Science Publishers, Inc., Enfield (NH), USA, 2001, pp. 227–244.
39. Debus, R. J., *Biochim. Biophys. Acta*, 1992, **1102**, 269–352.
40. Rodgers, M. A. J. and Bates, A. L., *Photochem. Photobiol*., 1982, **35**, 473–477.
41. Rodgers, M. A. J., *Photochem. Photobiol.*, 1983, **37**, 99–103.
42. Rodgers, M. A. J., *J. Am. Chem. Soc*., 1983, **105**, 6201–6205.
43. Midden, W. R. and Wang, S. Y., *ibid*, 4129–4135.
44. Piette, J., *J. Photochem. Photobiol. B*, 1990, **4**, 335–342.
45. Lion, Y., Delmelle, M. and van de Vorst, *A*., *Nature*, 1976, **263**, 442–443.
46. Kraljic, I. and El Mohsni, S., *Photochem. Photobiol*., 1978, **28**, 577–581.
47. Kraljic, I. and Sharpati, V. A., *ibid*, 583–586.
48. Harbour, J. R., Issler, S. L. and Hair, M. L., *J. Am. Chem. Soc*., 1980, **102**, 7778–7779.
49. Matysik, J., Alia, Gast, P., van Gorkom, H. J., Hoff, A. J. and de Groot, H. J. M., *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 9865–9870.
50. Macpherson, A. N., Telfer, A., Barber, J. and Truscott, T. G., *Biochim. Biophys. Acta*, 1993, **1143**, 301–309.
51. Hideg, E. and Vass, I., *Photochem. Photobiol*., 1995, **62**, 949–952.
52. Kanofsky, J. R., in *Peroxidases in Chemistry and Biology* (eds Everse, J., Everse, K. E. and Grisham, M. G.), CRC Press, Boca Raton, 1992, vol. II, pp. 219–237.
53. Mishra, N. P., Franke, C., Van Gorkom, H. J. and Ghanotakis, D. F., *Biochim. Biophys. Acta*, 1994, **1186**, 81–90.
54. Aro, E-M., Virgin, I. and Andersson, B., *Biochim. Biophys. Acta*, 1993, **1143**, 113–134.
55. Alia, Matysik, J. and Mohanty., P. in *Toxicology and Environmental Health* (eds Vohora, S. B. and Agarwal, V. P.), Society Biosciences, Jamia Hamdard University/Aptech Publications, New Delhi, 1999, pp. 121–124.
56. Alia, Mohanty, P. and Matysik, J., *Amino Acids*, 2001, **21**, 195–200.
57. Lissi, E. A., Encinas, M. V., Lemp, E. and Rubio, M. A., *Chem. Rev*., 1993, **93**, 699–723.
58. Yoshikawa, K. M., Hashimoto, M. and Morishima, I., *J. Am. Chem. Soc*., 1973, **96**, 288–289.
59. Morishima, I., Yoshikawa, K., Hashimoto, M. and Bekki, K., *J. Am. Chem. Soc*., 1975, **97**, 4283–4288.
60. Colonna, F. P., Distefano, G., Pignataro, S., Pitacco, G. and Valentin, E., *Perkin Trans*., 1975, 1572–1576.
61. Richer, G., Sandorfy, C. and Nascimento, M. A. C., *J. Electron Spectrosc. Relat. Phenom*., 1984, **34**, 327–335.
62. Aue, D. H., Webb, H. M. and Bowers, M. T., *J. Am. Chem. Soc*., 1976, **98**, 311–317.
63. Cannington, P. H. and Ham, N. S., *J. Electron Spectrosc. Relat. Phenom*., 1983, **32**, 139–151.
64. Cannington, P. H. and Ham, N. S., *ibid*, 1979, **15**, 79–82.
65. *The Merck Index*, Whitehouse Station, New Jersey, 1996, 12th edn.
66. Stadtman, E. R. and Berlett, B. S., *J. Biol. Chem*., 1991, **226**, 17201–17208.
67. Matysik, J., Hildebrandt, P. and Ludwig, B., *Biochim. Biophys. Acta*, 2000, **1459**, 125–130.
68. Garrison, W. M., *Curr. Top. Radiat. Res*., 1968, **4**, 43–94.
69. Farhataziz and Rodgers, M. A. J. (eds), *Radiation Chemistry*, Verlag Chemie, New York, 1987.
70. Kraljic, I. and Trumbore, C. N., *J. Am. Chem. Soc*., 1965, **87**, 2547–2550.
71. Rigo, A., Argese, E., Stevanato, R., and Orsega, E. F., *Inorg. Chim. Acta*, 1977, **24**, L71–L73.
72. Rustgi, S., Joshi, A., Moss, H. and Riesz, P., *Int. J. Radiat. Biol*., 1977, **31**, 415–440.
73. Masuda, T., Nakano, S. and Kondo, M., *J. Radiat. Res*., 1973, **14**, 339–345.
74. Floyd, R. A. and Zs-Nagy, I., *Biochim. Biophys. Acta*, 1984 **790**, 94–97.
75. Halliwell, B. and Gutteridge, M. C., *Methods Enzymol*., 1990, **186**, 1–85.
76. Van Dyke, B. R., Clopton, D. A. and Saltman, *P*., *Inorg. Chim. Acta*, 1996, **242**, 57–61.
77. Stadtman, E. R., *Annu. Rev. Biochem*., 1993, **62**, 797–821.
78. Esaka, M. and Hayakawa, H., *Plant Cell Physiol*., 1997, **36**, 441–446.
79. Bennick, A., *Mol. Cell. Biochem*., 1982, **45**, 83–99.
80. Kartasova, T. and van de Putte, P., *Mol. Cell. Biol*., 1988, **8**, 2195–2203.
81. Cabral, A., Voskamp, P., Cleton-Jansen, A. M., South, A., Nizetic, D. and Backendorf, C., *J. Biol. Chem*., 2001, **276**, 19231–19237.
82. Yao, Y., Lamkin, M. S. and Oppenheim, F. G., *J. Dent. Res*., 1999, **78**, 1696–1703.
83. Steinert, P. M. and Marekov, L. N., *J. Biol. Chem*., 1995, **270**, 17702–17711.
84. Backendorf, C. and Hohl, D., *Nature Genet*., 1992, **2**, 91.
85. Marvin, K. W., George, M. D., Fujimoto, W., Saunders, N. A., Bernacki, S. H. and Jetten, A. M., *Proc. Natl. Acad. Sci., USA*, 1992, **89**, 11026–11030.
86. Yaar, M., Eller, M. S., Bhawan, J., Harkness, D. D., DiBenedetto, P. J. and Gilchrest, B. A., *Exp. Cell Res*., 1995, **217**, 217–226.
87. Stadtman, E., *Methods Enzymol*., 1995, **258**, 379–393.
88. Garrison, W. M., *Chem. Rev*., 1987, **87**, 381–422.
89. Schuessler, H. and Schilling, K., *Int. J. Radiat. Biol*., 1984, **45**, 267–281.
90. Poston, J. M., *Fed. Proc., Fed. Am. Soc. Exp. Biol*., 1988, **46**, 1979.
91. Amici, A., Levine, R. L., Tsai, L. and Stadtman, E. R., *J. Biol. Chem*., 1989, **264**, 3341–3346.
92. Cooper, B., Creeth, J. M. and Donald, A. S. R., *Biochem. J.* 1985, **228**, 615–626.
93. Uchida, K. and Kawakishi, S., *Bioorg. Chem*., 1989, **17**, 330–343.
94. Uchida, K., Kato, Y. and Kawakishi, S., *Biochem. Biophys. Res. Commun*., 1990, **169**, 265–271.
95. Scharffetter-Kochanek, K., Brenneisen, P., Wenk, J., Herrmann, G., Ma, W., Meewes, C. and Wlaschek, M., *Exp. Gerontol*., 2000, **35**, 307–316.
96. Herrmann, T., Baumann, M., Voigtmann, L. and Knorr, A., *Radiother. Onkol*., 1997, **4**, 35–40.
97. Steenken, S. and Jovanovic, S. V., *J. Am. Chem. Soc*., 1997, **119**, 617–618.
98. Yoshikawa, S. *et al.*, *Science*, 1998, **280**, 1724–1729.
99. Ito, N. *et al.*, *Science*, 1991, **350**, 879–0.
100. Jander, G. and Blasius, E., Lehrbuch der analytischen und präparativen anorganischen Chemie, S. Hirzel Verlag, Stuttgart, 1983, p. 471.

ACKNOWLEDGEMENTS.We thank Dr C. Backendorf and Prof. P. Pardha Sardhi for fruitful discussions. Our thanks to Prof. H. J. M de Groot, Prof. A. J. Hoff and Dr H. J. van Gorkom for their interest and support. J. M. acknowledges a Casimir-Ziegler award of the Academies of Sciences in Amsterdam and Düsseldorf.

Received 22 June 2001; revised accepted 2 November 2001