RESEARCH COMMUNICATION

Long-lived reactive species on free-radical-damaged proteins

Jeremy A. SIMPSON,* Shigeru NARITA,† Steven GIESEG,* Silvia GEBICKI,† Janusz M. GEBICKI† and Roger T. DEAN*†

*Heart Research Institute, 145 Missenden Road, Camperdown, Sydney, N.S.W. 2050, Australia, and †Biological Sciences, Macquarie University, Sydney, N.S.W. 2109, Australia

We have demonstrated two novel reactive species on radical-modified proteins which are relatively long-lived, one oxidizing and one reducing. The two species are reactive with critical biological components, and so may be of physiological and pathological importance. The oxidizing species, which have been identified as protein hydroperoxides, can consume key cellular reductants, such as ascorbate and glutathione. The reducing species can act on both free and metalloprotein forms of copper and iron ions, which participate in radical generation. These findings suggest that proteins may act as traps for the chemical energy released by free radicals, with the capacity to pass it on to other molecules. The long-lived nature of both the reactive moieties indicates that they may be able to diffuse and transfer damaging reactions to distant sites.

INTRODUCTION

Free radicals are inevitable by-products of biological redox reactions [1] and have been implicated in many pathological states [2–4]. Superoxide radicals ($O_2^{\cdot-}$) and H_2O_2 are known to be produced in biological systems, but these are relatively harmless, as they react with biomolecules at low rates. In the presence of certain iron or copper complexes, however, they can be converted into the highly reactive and damaging hydroxyl radical ($OH^{\cdot}$) and other species. The oxidation state of the catalytic metal is very important for its participation in such radical inter-conversion [5,6]; in general, $OH^{\cdot}$ -generating reactions result from the oxidation of metal, can be limited by availability of reduced metal, and can be accelerated by reductants [7]. Under normal conditions, cells keep metal ions firmly bound in un-reactive forms (e.g. on transferrin) as an antioxidative mechanism.

$OH^{\cdot}$ and other free radicals can damage most important cellular targets. Although much is known about their action on lipids, much less is known about radical attack on proteins. This is unfortunate, in view of the fact that 10–50 % of all antioxidant potential of human plasma challenged with peroxyl radicals seems to be due to proteins [8]. This suggests that protein–free-radical interactions may be common and have considerable significance *in vivo*.

Protein radicals can be either functional or damaging. Transient reactive radicals are produced on protein surfaces as part of the catalytic action of several enzymes [9,10]. In contrast, as mentioned above, proteins are also inevitably exposed to externally generated radicals. These latter cause consequent protein scission and cross-linking, and chemical modification of amino acids. The conformational changes which result from this complex of reactions lead also to increased susceptibility to proteolysis and heat denaturation, and to loss of biological function [1,7, 11–15]. Significantly, some of these changes have also been found in proteins isolated from cells and tissues under conditions resulting in production of free radicals (e.g. [1,4,8,12–14]).

However, in all these studies, modification of the proteins by free radicals was regarded as the significant event, with little

attention given to the possibility that altered protein molecules may contain initiators of further chemical reactions. We have observed two such species: protein peroxides, which can consume important antioxidants; and protein-bound reducing moieties, which can reduce transition metals, and may enhance their activity in radical reactions.

MATERIALS AND METHODS

Materials

Glassware used in handling and storage of solutions was cleaned by heating for 6–7 h in conc. HNO_3 , washing in distilled water and drying. Solutions were made up in distilled water purified by passage through a four-stage Milli Q system (Millipore–Waters, Sydney, Australia) equipped with a $0.2\ \mu m$ -pore-size final filter. BSA (Cohn fraction V, fat free), lysozyme and catalase were supplied by Boehringer–Mannheim (Mannheim, Germany). Superoxide dismutase (bovine blood) was obtained from Sigma (St. Louis, MO, U.S.A.). Chromatographic standard amino acids were from Pierce Chemical Co. (Rockford, IL, U.S.A.); other chemicals, solvents and chromatographic materials were of A.R. or h.p.l.c. grade and obtained from Merck (Darmstadt, Germany), Mallinckrodt (St. Louis, MO, U.S.A.), BDH (Poole, Dorset, U.K.), Pharmacia (Uppsala, Sweden) or Aldrich (Milwaukee, WI, U.S.A.).

Irradiations

Dilute solutions of proteins or amino acids, usually buffered with 10–20 mM-sodium phosphate at pH 7.4, were irradiated in a ^{60}Co facility. In such a system $OH^{\cdot}$, hydrated electrons [$e^-(aq)$] and $H^{\cdot}$ are the primary species formed. Manipulation of the irradiation system allows specific radical formation [16]. For instance, in air-saturated and anoxic systems the predominant species formed are $OH^{\cdot}$ and $O_2^{\cdot-}$, and $OH^{\cdot}$ and $e^-(aq)$, respectively. The former was achieved by interruption of irradiation and slow stirring of air into samples for 1 min in every 5 min of irradiation. The latter was performed by pre-gassing of samples with N_2 for 15 min and sealing before irradiation. After irradiation, a small volume of catalase ($5\ \mu g/ml$ final concn.) was

Abbreviations used: $O_2^{\cdot-}$, superoxide anion; $OH^{\cdot}$, hydroxyl radical; $e^-(aq)$, hydrated electron; PBRM, protein-bound reducing moieties.

† To whom correspondence should be addressed.

added to the samples to remove radiation-generated H_2O_2 . This did not interfere with any of the assay systems described.

Peroxide assay

A modified spectrophotometric method originally developed for lipid peroxides was used [17–19]. In the procedure adopted here, a spectrophotometer cuvette was flushed with argon for about 5 min, and then 2.0 ml of argon-saturated methanol/acetic acid (2:1, v/v) containing 5% (w/v) KI was added from an Oxford (Monoject Scientific, St. Louis, MO, U.S.A.) constant-volume pipettor. The space above the liquid was flushed briefly with argon and the cuvette closed with a Teflon stopper fitted with an O-ring on the surface contacting the cuvette body. The A_{358} of the solution was measured. The cuvette was then opened, under a stream of argon, and up to 0.5 ml of aqueous solution of the compound under test was added. The stopper was replaced and the cuvette incubated at 50 °C for 15 min. The A_{358} was then read again, after quick cooling to room temperature. The difference between the final and initial readings gave the triiodide (I_3^-) content of the diluted sample. The peroxide content was calculated by subtracting the I_3^- values obtained with controls from those for oxidized samples. This method ensured that each result was independent of any variation in the blank solvent reading, which was determined individually for every sample. The molar absorption coefficient of the I_3^- formed was $3.00 \times 10^4 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The procedure gave results in which duplicate readings differed by less than 0.01 A unit, corresponding to peroxide-concentration differences in the original samples of less than 1.7 μM .

Reductive capacity of radical-exposed samples

Cytochrome *c* reduction. Proteins (0.9 mg/ml final concn.) or amino acids (1.8 mM final concn.) were incubated with cytochrome *c* (80 μM) in 10 mM-sodium phosphate buffer (pH 7.4) for 2 h at room temperature. The reduction of cytochrome *c* was determined at 550 nm by double-beam experiments in which the reference cuvette contained unirradiated BSA with cytochrome *c*.

Neocuproine assay. Samples were tested for their ability to reduce free Cu^{2+} by detecting production of Cu^+ as a complex with neocuproine, which absorbs at 454 nm [20]. The assay conditions were: neocuproine, 1 mM; CuSO_4 , 250 μM ; phosphate buffer, pH 7.4, 10 mM; final volume 2 ml. The reaction proceeded essentially to completion within 1 h, when the absorbance was measured.

RESULTS AND DISCUSSION

We first demonstrated that free radicals derived from γ -radiolysis of oxygenated water generated reactive protein hydroperoxides. When oxygenated solutions of BSA and lysozyme were irradiated [16], hydroperoxides were generated linearly with dose of radicals (Fig. 1). These groups were not affected by catalase, which was used routinely to remove H_2O_2 generated by irradiation. At least 4 mg of protein/ml was necessary to scavenge all the free radicals effective in the peroxidation. The G value (number of hydroperoxide molecules formed per 100 eV of energy absorbed [16]) was 1.0 for BSA and 0.8 for lysozyme. The free radical causing peroxidation was identified by inclusion of solutes competing specifically for $\text{OH}^\bullet$ and $\text{O}_2^{\bullet-}$ free radicals. $\text{O}_2^{\bullet-}$ was ineffective and all of the peroxidation could be accounted for by the $\text{OH}^\bullet$. The addition of small amounts of Fe^{3+} or Cu^{2+} during irradiation did not affect the yields of protein hydroperoxides, showing that site-specific reactions [5,6] made no major contribution. Knowing that the G value for production of the $\text{OH}^\bullet$ radical is 2.7 [16], we can deduce that hydroperoxides

are a major product of the interaction between proteins and $\text{OH}^\bullet$ radicals: 0.4 peroxide group was generated per $\text{OH}^\bullet$ reacting with BSA, as judged by gel filtration and ultrafiltration.

Peroxidation of BSA was also induced by the classical Fenton system (Fe^{2+} with H_2O_2), leading to $\text{OH}^\bullet$ free radicals [5,6], and by the thermal decomposition of the bis-azo compound 2,2'-azobis-(2-amidinopropane) dihydrochloride ('AAPH'), which gives relatively weakly oxidizing peroxy radicals [21,22]. We confirmed the identity of the hydroperoxides by reductive removal with NaBH_4 , GSH, ascorbate, triphenylphosphine and dithionite (Fig. 2; [17–19,23]). Additional evidence for the identification was the absolute requirement for oxygen for their production, and there are other chemical grounds for favouring hydroperoxides rather than peroxides. The only other study of protein peroxidation known to us also proposed the generation of hydroperoxides in aerobic irradiation [24]. As would be predicted, they are rapidly destroyed upon addition of reduced iron or copper. Protein hydroperoxides were otherwise moderately stable at room temperature, with approx. 30% remaining after 1 week of storage. Lower temperatures slowed the decay.

When we irradiated separately the 21 common free amino acids, only six were peroxidized by free radicals: proline, glutamate, lysine, valine, leucine and isoleucine acquired hydroperoxide groups with G values of 0.8–1.4 for the process. Another four (alanine, arginine, glutamine and tryptophan) gave G values of 0.3–0.5, but the rest were below 0.2. This broadly confirms more limited earlier findings [24]. The most readily peroxidized amino acids have either a tertiary carbon atom or a segment containing at least two methylene groups, which may stabilize the carbon-centred free radical generated by the reaction with $\text{OH}^\bullet$ [16] with the free amino acids. This permits the intermediate peroxy radical then to be reduced to the hydroperoxide by a mechanism dependent on the system under study.

The possibility that peroxidized proteins may react with normal tissue biomolecules was tested by incubating a series of compounds with BSA peroxides (BSA-OOH). NADH and NADPH were not oxidized, but approximately equimolar amounts of

Fig. 1. Generation of protein hydroperoxides by exposure of BSA and lysozyme to $\text{OH}^\bullet$ radicals

BSA (■) and lysozyme (□) (5 mg/ml) in 20 mM-sodium phosphate buffer, pH 7.4, were irradiated (^{60}Co source) at 63 Gy/min (1 Gy = 1 J/kg). Air was stirred into the samples during irradiation, catalase was added afterwards, and hydroperoxides present were measured. A single experiment representative of five is shown, where the points are means of duplicates, which were within 1.5% of the mean.

Fig. 2. Reduction of protein hydroperoxides by GSH, and its concomitant oxidation

BSA (5 mg/ml) was oxically irradiated as described in Fig. 1 for 20 min, to generate protein hydroperoxides (BSA-OOH). The product was then added at a final concentration of $43 \mu\text{M}$ -hydroperoxide to GSH ($42 \mu\text{M}$) in 20 mM-sodium phosphate buffer, pH 7.4, containing $50 \mu\text{M}$ -diethylenetriaminepenta-acetic acid ('DETAPAC'), and incubated at 37°C for the specified times. The reaction mixtures were then filtered on an Amicon Centricon (cut-off 30 kDa) before measurement of protein hydroperoxides and GSH. Control incubations lacking hydroperoxide (and replacing it by unirradiated BSA), or lacking GSH, showed that some GSH was probably consumed in reactions additional to the reduction of hydroperoxides. A single experiment is shown. It was conducted with duplicates which were within 5% of the mean, and is representative of three experiments. Loss of: ■, BSA-OOH; □, BSA-OOH (+GSH); ●, GSH (+BSA); ○, GSH (+BSA-OOH).

Fig. 3. Generation of protein-bound reducing moieties (PBRedM) by exposure of BSA and lysozyme to $\text{OH}^\cdot$ radicals

BSA (■) and lysozyme (□) (10 mg/ml) in 10 mM-sodium phosphate buffer, pH 7.4 (total volume 10 ml), were irradiated (^{60}Co source) at 63 Gy/min under anoxic conditions. A single experiment representative of five is shown, and the data are means of duplicate irradiations, which were in turn assayed in duplicate. Values shown represent the total reductive capacity of 1 ml of the irradiated samples and were within 1.5% of the mean.

Fig. 4. Generation of protein-bound reducing moieties by exposure to autoxidizing glucose

BSA (10 mg/ml) was incubated under sterile conditions at 37°C for the specified periods, in the presence of 100 mM-phosphate buffer, pH 7.4, with the following additions: ■, glucose; □, Cu^{2+} ; ○, glucose + Cu^{2+} . The concentrations used were: CuSO_4 , $100 \mu\text{M}$; glucose, 100 mM. At the chosen times, 0.5 ml samples were removed from the incubations and tested in the neocuproine assay. Values shown are the reductive activity of 5 mg of incubated BSA. The experiment was done in triplicate (and is representative of three), and s.d. values are shown where they exceed the size of the graph point.

ascorbate (results not shown) and GSH (Fig. 2) both reacted with the hydroperoxide groups and were themselves oxidized. The potential biological significance of such reactions is that they may cause a depletion of cellular reductants, and thus constitute an aspect of oxidative stress. When the same experiment was conducted with ascorbate ($46.1 \mu\text{M}$) as reductant, the hydroperoxide remaining after 60 min incubation was 33% of the initial, and correspondingly 56% of the initial ascorbate remained. When ascorbate was incubated under identical conditions, but in the absence of protein hydroperoxides, 78% remained after 60 min. Thus ascorbate could also reduce the hydroperoxides.

We also detected a second type of reactive species, protein-bound reducing moieties (PBRedM); these were generated (to approximately the same extent) in both oxic and anoxic (Fig. 3) irradiations. PBRedM were detected initially by their capacity to reduce cytochrome *c*, and are more stable than protein hydroperoxides, remaining reactive over several weeks. PBRedM are also preserved on proteins from which hydroperoxides have been removed by reduction by GSH or ascorbate. BSA or lysozyme (10 mg/ml) was required to scavenge all radiolysis products responsible for the formation of PBRedM, and under such conditions the *G* values for PBRedM generation (under anoxic conditions) were 0.2 (BSA) and 0.6 (lysozyme). PBRedM (but not native protein) reduced cytochrome *c*, in a reaction not inhibited by superoxide dismutase, and not requiring oxygen. Experiments involving the addition of various scavengers [16] to the radiolysis system indicated that hydroxyl radicals were the main agent of formation of PBRedM. About 0.1 and 0.3 molecule of PBRedM was produced on BSA and lysozyme respectively per hydroxyl radical reacting with protein.

PBRedM can also reduce free copper (Fig. 4) and iron. Protein irradiated as in Fig. 3 could also reduce Cu^{2+} , with a *G* value for

production of reducing equivalents of around 1.2 for both BSA and lysozyme. In other experiments the reduction of free iron was measured by formation of Fe^{2+} /ferrozine complexes (results not shown). Free thiol groups on proteins also possess this capacity to reduce the free metals. Thus native BSA (zero-time samples) reduces copper, and this reaction can be prevented by prior reaction of its thiol group with Ellman's reagent. Some PBRedM can be due to the generation of further protein-thiol groups during anaerobic radiolysis in the presence of formate, but not in most other conditions. PBRedM remain protein-bound, as judged by non-denaturing gel filtration and ultrafiltration. Furthermore, peptides obtained by trypsin digestion of the proteins still contain PBRedM.

The individual amino acids on which the metal reductants could be generated were different from those producing hydroperoxides. Thus tyrosine, and to a lesser degree the other aromatic amino acids, were active. The *G* value for production of cytochrome *c*-reducing equivalents on tyrosine was 0.4. Valine, on which the hydroperoxide production is greatest, was inert in respect of generation of the reducing species. Other studies have shown that dityrosine is not the reducing moiety, though it is generated under several of the conditions in which the reducing moiety is formed (such as u.v. irradiation at 265 nm [25]). Nor is dityrosine a good starting material for generation of the reducing moiety. We envisage that a tyrosine hydroxylation pathway produces catechols such as 3,4-dihydroxyphenylalanine ('DOPA')-like molecules, which are known to be able to reduce transition metals [9,10,23] but which remain protein-bound, and constitute the main PBRedM.

PBRedM can also be formed in free-radical-generating biochemical systems, such as autoxidizing sugars [4,26] at concentrations which occur in uncontrolled diabetics (Fig. 4: the sugar autoxidation is catalysed by copper), and the Fenton system. Thus even though there are many competing radical reactions on protein surfaces, PBRedM can be generated in conditions relevant to the physiological milieu.

There has been much emphasis on site-specific radical reactions on proteins [5,6], envisaged as dictated by the presence of iron and copper bound to amino acids such as histidine [12,27]. The metal is thought to localize the radical-generating, transforming and hence damaging, reactions. Our new observations of protein-bound reactive moieties indicate further factors influencing the localization of reactions due to radical-protein interactions. Firstly, the localization of the oxidative and reductive species on a protein may facilitate subsequent reactions on the same protein molecules and in their vicinity, constituting a new category of site-specific reactions. Secondly, the reducing species may transiently bind oxidized metal and release it after reduction, as indicated by the ease of access of the reduced metal to the organometallic ligands that we have used. Thus the reduced metal might be available in the bulk phase for further action [28]. Thirdly, the stability of the PBRedM should permit them to diffuse through biological systems, transferring reactions from organelle to organelle, in contrast with the transient radicals

themselves or with the relatively fixed lipid hydroperoxides. The implications in radical-induced damage of the reactive species that we have described are thus substantial.

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