Crosslinking of DNA and proteins induced by protein hydroperoxides

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Exposure of DNA to several proteins peroxidized by radiation-generated hydroxyl free radicals resulted in formation of crosslinks between the macromolecules, detected by retardation and broadening of DNA bands in agarose gels. This technique proved suitable for the study of crosslinking of DNA with peroxidized BSA, insulin, apotransferrin and α casein, but not with several other proteins, including histones. The crosslinking depended on the presence of intact hydroperoxide groups on the protein, on their number, and on the duration of the interaction with DNA. All DNA samples tested, pBR322, pGEM, λ/*Hin*dIII and pUC18, formed crosslinks with the peroxidized BSA. Sodium chloride and formate prevented the crosslinking if present during incubation of the peroxidized protein and DNA, but had no effect once the crosslinks had formed. The gel shift of the crosslinked DNA was reversed by proteolysis, indicating that the DNA mobility change was due to attachment of protein and that the crosslinking did not induce DNA strand breaks. The metal chelators Desferal and neocuproine reduced the extent of the crosslinking, but did not prevent it. Scavengers of free radicals did not inhibit the crosslink formation. The DNA–protein complex was not disrupted by vigorous agitation, by filtration or by non-ionic detergents. These observations show that the crosslinking of DNA with proteins mediated by protein hydroperoxides is spontaneous and probably covalent, and that it may be assisted by transition metals. It is suggested that formation of such crosslinks in living organisms could account for some of the well-documented forms of biological damage induced by reactive oxygen species-induced oxidative stress.

Key words: DNA gel shift, free radical, oxidation, radiation, reactive oxygen species.

INTRODUCTION

Free radicals and other reactive intermediates derived from oxygen are inevitable products of biological redox reactions [1,2]. In addition, they are known to form in living organisms subjected to oxygen stress from exposure to exogenous agents such as drugs, toxins, pollutants and radiation, or from conditions accompanying a wide range of diseases, malnutrition, ageing, medical procedures or occupational hazards [3,4]. Whereas the formation of reactive oxygen species (ROS) does not inevitably result in biological damage, current evidence suggests that their subsequent reactions are, in the main, potentially harmful.

The biochemical pathways linking the formation of ROS with their observable effects in cells and tissues are not clear. The first step must be direct reaction of a short-lived reactive oxygen derivative with a biomolecule, usually resulting in its oxidation. The product may be a new free radical or reactive molecule, which can in turn oxidize or otherwise alter other biomolecules. Irreversible damage to molecules vital to the survival or propagation of cells will lead to impairment of biological activity, apoptosis, transformation, or death of the cell or organism. The identification of these vital target molecules has been a major challenge for some time. Until recently, proteins were not held to be biologically significant targets for ROS. On the contrary, their main role in the biochemical events initiated by ROS was believed to be benign, with the proteins constituting passive targets for the ROS, protecting more vital molecules by acting as radical scavengers, anti-oxidants and metal chelators [5,6]. This view was challenged by our demonstration that proteins exposed to a range of ROS can acquire hydroperoxide and other redox-active groups [7,8]. Subsequent work has shown that many proteins can be peroxidized by physiologically important ROS, that the resultant protein hydroperoxides can oxidize some biological anti-oxidants and that they can be a source of secondary free radicals [9–12]. These findings suggest that protein hydroperoxides may constitute important links in the chain of damage initiated by the ROS under physiological conditions [13]. Evidence for this hypothesis has begun to appear only recently, because of lack of techniques for direct detection of protein hydroperoxides in tissues and the past failure to appreciate their potential to cause biochemical damage. At present, the best proof of their formation in tissues is provided by the finding of unique hydroxylated derivatives of the six amino acids known to be highly susceptible to peroxidation [8]. These are derived from the parent amino acid hydroperoxides generated by the action of ROS on proteins. Thus the recent finding of hydroxyleucine and hydroxyvaline in human atherosclerotic plaques [14] provides strong evidence for the formation of protein hydroperoxides under physiological conditions in tissues subjected to oxidative stress.

In this study, we investigated the ability of protein hydroperoxides to react with DNA. Such reactions could lead to various forms of damage to the nucleic acid, including DNA–protein crosslinking. Formation of such crosslinks in a variety of systems exposed to ROS has been reported in numerous studies, whose common features were simultaneous exposure of DNA and protein to highly reactive and short-lived oxidants and close association between the macromolecules [15]. These conditions resulted in formation of short-lived free radicals or active centres on the macromolecules, whose proximity enhanced the possibility of crosslinking. The DNA samples used ranged from isolated purified molecules [15–17] to cell lysates [18]. In some studies, purified proteins were added to DNA solutions, but most involved natural DNA–protein complexes in the form of nucleohistones [19,20], phage DNA [21,22] or chromatin from cultured cells [23,24]. Some crosslinks were also found in DNA isolated from cells not deliberately subjected to oxidant stress [23]. However, in most cases they were induced by agents generating

Abbreviations used: OOH, hydroperoxide group; HO$^{\bullet}$, hydroxyl free radical; *t*-butyl-OOH, *t*-butyl hydroperoxide; ROS, reactive oxygen species.

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excited states or free radicals, such as UV [16,25–28], X and γ radiation [15,17–24,29–31], and H_2O_2 in the absence or presence of added metal ions [23,24].

There are three reports of crosslinking between DNA and protein molecules after the removal of the source of ROS. Interaction between UV-irradiated BSA and DNA led to reduced extractability [25] and increased filter binding of the DNA [26], indicating probable formation of covalent intermolecular bonds. Irradiation of the protein was more effective, suggesting that radiation converted it into an activated form. There was evidence that the crosslinks were covalent, but the chemistry of the process was not elucidated. In a subsequent study, Minsky and Braun [29] found that the extent of intermolecular crosslinking of DNA irradiated with X rays in the presence of BSA continued to increase slowly after the X rays were turned off. As the process was inhibited by catalase, the authors concluded that it was caused by protein hydroperoxides, being apparently not aware that protein hydroperoxides are not substrates of this enzyme [7]. A more likely explanation lies in the presence of radiation-generated H_2O_2 during the post-irradiation incubation, which could provide hydroxyl free radicals (HO$^{\bullet}$) known to generate DNA–protein crosslinks [19,20,23,24].

We now provide a first account of the formation of DNA–protein crosslinks in the absence of ROS, which depends on intact hydroperoxide groups (OOH) on the protein. Our results, summarized in a preliminary report [32], indicate that the resultant DNA–protein crosslinks are probably covalent. Since the formation of protein hydroperoxides in biological systems exposed to ROS is a highly probable event [13,14], their subsequent interaction with DNA may constitute a significant step in the development of ROS-initiated damage.

MATERIALS AND METHODS

Materials

Water was purified by passage through a 4-stage Milli Q system (Millipore-Waters, Sydney, Australia) with a 0.2 μm final filter. BSA, Cohn fraction V (fatty acid free), bovine liver catalase, proteinase K, calf thymus histone type III SS, lysozyme, insulin, α casein, apotransferrin, haemoglobin, urease, ribonuclease, turkey egg albumin, amino acids, pBR322 and pUC18 plasmid DNA, λ/*Hin*dIII marker DNA and Desferal were obtained from Boehringer Mannheim (Sydney, Australia) or Sigma (St. Louis, MO, U.S.A.). Small amounts of pGEM plasmid were extracted from *Escherichia coli* with a Wizard Miniprep (Promega, Madison, WI, U.S.A.) kit. Agarose was from International Biotechnologies Inc. (New Haven, CT, U.S.A.). Glacial acetic acid, AR grade, was obtained from Ajax Chemicals (Sydney, Australia) and PD-10 (Sephadex G-25) chromatography columns were from Pharmacia (Sydney, Australia). Centricon microfiltration units were from Amicon (Beverly, MA, U.S.A.). All other chemicals were of analytical or HPLC grade and supplied by Merck (Kilsyth, Victoria, Australia).

Irradiation of proteins and amino acids

The proteins (usually at 2 mg/ml in water), or amino acids (10 mM in water), were irradiated with γ rays under a slow stream of oxygen in a ^{60}Co source, usually to a dose of 2000 Gy (2000 J/kg). Radiation-generated H_2O_2 was removed by 10 min incubation with 260 units of catalase per ml of solution. In some experiments the irradiated solutions were saturated with nitrogen or N_2O gas instead of oxygen.

Measurement of peroxides

Hydroperoxides were measured by a modified tri-iodide assay that produces one I_3^- for every OOH reacting [33]. The I_3^- has an absorbance peak at 358 nm, with an absorption coefficient of 29000 $M^{-1} \cdot cm^{-1}$. Exactly 2 ml of a pre-saturated nitrogen-bubbled solution of acetic acid and water (1:1, v/v) containing 10 % KI (w/v) was added to a spectrophotometer cuvette under a stream of oxygen-free nitrogen. The cuvette was stoppered and the absorbance measured at 358 nm (initial). Up to 0.5 ml of the solution under test was then added, again under a stream of nitrogen, the cuvette re-stoppered and incubated in a water bath at 50 °C for 15 min. The A_{358} was measured again after cooling to room temperature (final absorbance) and the peroxide concentrations were calculated from the difference between the two readings.

Incubation of DNA and electrophoresis

An aliquot of 3 μl of 0.1 μg/μl DNA in water was gently diluted with 27 μl of water, 2 mg/ml protein or 10 mM amino acid solution and placed in a water bath at 37 °C for the required time. In some experiments the protein concentrations were varied. At the end of incubation, 4 μl of tracking dye was added to each sample. The solutions (32 μl) were loaded on to a 0.7 % (w/v) agarose gel containing ethidium bromide and run in electrophoresis buffer containing 0.04 M Tris, 1 mM EDTA and 5 mM acetate, adjusted to pH 7.8 at 50 V for 3–4 h. The gels were examined and photographed under UV light.

RESULTS

Interaction of DNA with peroxidized proteins and amino acids

Incubation of plasmid DNA with several peroxidized proteins induced band retardation and broadening in agarose gels. We tested some of the 14 proteins currently known to form OOH groups [13]. The results (Figure 1) show that exposure of pBR322

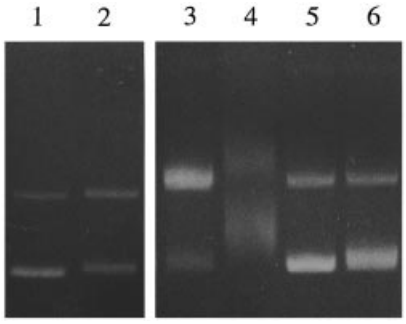

Figure 1 The effect of native and peroxidized proteins on the electrophoretic migration of pBR322 DNA

The plasmid (0.3 μl of aqueous solution containing 0.1 μg of DNA/μl) was incubated for 1 h at 37 °C with native or peroxidized protein (27 μl of aqueous solution containing 2 mg of protein/ml). The proteins were peroxidized by irradiation with ^{60}Co γ rays in the presence of oxygen to a dose of 2000 Gy and the H_2O_2 generated removed with catalase. Formation of protein OOHs under these conditions was confirmed by the iodide assay. Aliquots (32 μl) of the mixture were subjected to 3 h electrophoresis in agarose gel containing ethidium bromide and the bands photographed under UV light. DNA was incubated with (lane 1) native insulin, (lane 2) irradiated insulin, (lane 3) native apotransferrin, (lane 4) irradiated apotransferrin, (lane 5) native α casein and (lane 6) irradiated α casein.

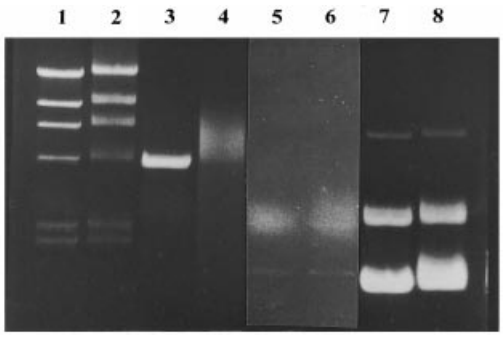

Figure 2 Gel-shift assay of the interaction between BSA-OOH and DNA derived from different sources

The BSA (2 mg/ml) was peroxidized by exposure to γ rays and the concentration of OOHs generated measured by iodide assay, after removal of H_2O_2 with catalase. Solutions of DNA from different sources (6 μl) were incubated for 1 h with 24 μl of the native or peroxidized BSA at 37 °C. The final concentration of BSA-OOH in all samples was 138.4 μM, or 4.6 OOH/BSA. Samples (32 μl) were subjected to 3 h electrophoresis in agarose gels. Lanes 1 and 2, 0.2 μg of λ/*Hin*dIII DNA with native or peroxidized BSA, respectively. Lanes 3 and 4, 0.26 μg of pBR322/*Eco*R1 DNA with native or peroxidized BSA, respectively. Lanes 5 and 6, a small amount of pGEM with native or peroxidized BSA, respectively. Lanes 7 and 8, approx. 0.6 μg of pUC18 DNA with native or peroxidized BSA, respectively.

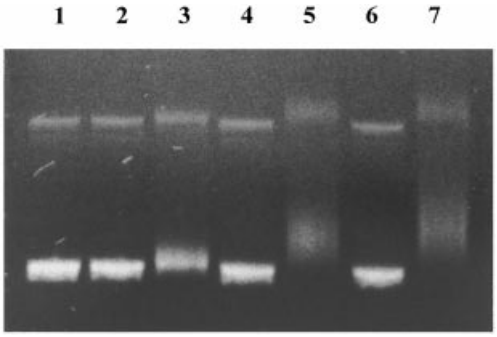

Figure 3 The effect of increasing amounts of peroxidized BSA on the electrophoretic migration of pBR322 DNA

The pBR322 DNA was incubated with native or peroxidized BSA and the mixture subjected to agarose gel electrophoresis for 4 h. The protein (2 mg/ml in water) was irradiated with γ rays under oxygen to a dose of 2000 Gy. The initial BSA-OOH concentration was 151 μM (5 OOH/BSA). Aliquots (27 μl) of the protein solution were incubated with 3 μl of 0.1 μg/μl of the DNA for 30 min at 37 °C. Control mixtures contained identical amounts of native BSA. Lane 1, DNA alone. Lanes 2 and 3, DNA with native or peroxidized BSA, respectively, at a ratio of 60:1 BSA/DNA (w/w). Lanes 4 and 5, as lanes 2 and 3, respectively, but at ratio of 120:1. Lanes 6 and 7, as lanes 2 and 3, respectively, but at ratio of 180:1.

plasmid to α casein, insulin or apotransferrin (group-1 proteins) peroxidized by radiation-generated $HO^{\bullet}$ radicals produced the DNA gel shifts. Similar results were obtained with BSA, and most of the studies reported here were carried out with this protein because its peroxidized derivatives have been the most extensively studied so far [13]. Attempts to test several other proteins were not successful. DNA treated with the basic proteins lysozyme or histone could not be detected on the gel, even before electrophoresis. On the other hand, even native haemoglobin, urease, ribonuclease, turkey egg albumin and β lactoglobulin (group-2 proteins) caused considerable retardation and broadening of DNA bands, so that the effects of their hydroperoxides could not be studied.

The results shown in the Figures are representative of at least three electrophoretic runs for each experiment. The reproducibility of the distance travelled by the plasmid was very close for any given sample of DNA or protein. The extent of the DNA-band retardation and broadening was less reproducible, for reasons which are not clear. However, the overall effects of the interaction of group-1 proteins with DNA were always the same in a qualitative sense: the bands were broad and retarded by comparison with controls, and the unperoxidized proteins never produced such effects.

Tests with DNA from different sources demonstrated the general nature of the DNA–protein hydroperoxide interaction. In experiments shown in Figure 2, each DNA sample was exposed to the same amount of native or peroxidized BSA before electrophoresis. Lanes 1 and 2 contained λ/*Hin*dIII DNA with the native or peroxidized BSA, the DNA in lanes 3 and 4 was pBR322/*Eco*R1, in lanes 5 and 6 was pGEM, and in the last 2 lanes was pUC18. The migration of all DNA samples was retarded and more heterogeneous after treatment with BSA-OOH, with each band in different DNA samples affected to a similar extent. Between samples the degree of gel shift varied, with pBR/*Eco*R1 apparently the most altered and pGEM the least.

The effects of several variables on the interaction of peroxidized BSA with DNA are illustrated in Figures 3–5. The migration pattern of pBR322 plasmid DNA incubated with increasing amounts of native or peroxidized BSA is shown in Figure 3. Lane 1 contained control DNA without protein. Both the relaxed circular (slower) and supercoiled (faster) forms are clearly evident. The following lanes are paired, with lanes 2, 4 and 6 showing controls made up of DNA incubated with increasing amounts of native BSA, and lanes 3, 5 and 7 showing DNA incubated with identical amounts of peroxidized BSA. Since the exact concentration of the protein OOH groups was known from the iodide analysis and the concentration of the BSA from the dissolved weight and value of M_r, the average number of OOH

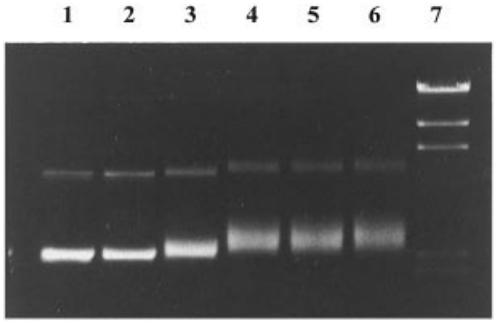

Figure 4 Gel shift of pBR322 DNA incubated with increasing amounts of BSA-OOH at a constant BSA/DNA ratio

Samples of BSA (2 mg/ml in water) were irradiated for various times in a ^{60}Co source at 30 Gy/min under oxygen and treated with catalase. The irradiations were timed so that all were completed simultaneously. The concentrations of BSA-OOH groups in all solutions were measured, with the protein solution irradiated for the longest time having 5 OOH groups per molecule. Thus, each sample contained an identical amount of BSA, but different amounts of BSA-OOH. From each protein sample, 27 μl was added to 3 μl of solution containing 0.1 μg/μl DNA. After incubation at 37 °C for 1 h, the mixtures were run on agarose gels for 4 h. Lane 1, DNA alone. Lanes 2–6, DNA treated with 0, 41, 82, 119 and 161 μM protein peroxide. Lane 7, molecular-mass marker λ/*Hin*dIII (23130, 9416, 6557, 4361, 2322 and 2027 bp).

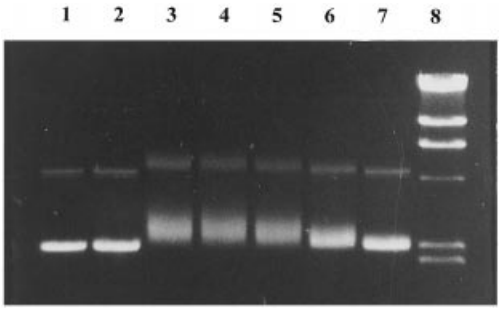

Figure 5 The effect of the length of incubation of pBR322 with BSA-OOH

The protein (2 mg/ml in water) was oxidized by exposure to 2000 Gy of γ rays under oxygen and 27 μl incubated with 3 μl of DNA solution containing 0.1 μg DNA/μl for various times. The final protein peroxide concentration in the mixture was 59.4 μM (2 OOH/BSA). Lane 1, DNA alone. Lane 2, DNA with native BSA. Lanes 3–7, DNA incubated with BSA-OOH for 120, 90, 60, 30 and 5 min, respectively. Lane 8, molecular-mass markers.

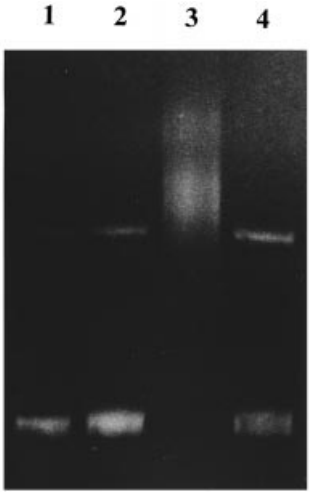

Figure 6 Reversal of the mobility shift of DNA by proteolysis

BSA (2 mg/ml) was peroxidized by irradiation and the BSA-OOH concentration measured. The pBR322 DNA (0.5 μg) was incubated for 90 min at 37 °C with the native or peroxidized BSA at a protein/DNA ratio of 180:1 (w/w). Lane 1, DNA alone. Lane 2, DNA with native BSA. Lane 3, DNA with peroxidized BSA. Lane 4, same sample as in lane 3, but after additional treatment with 1 μg of proteinase K per 50 μl of sample for 1 h before electrophoresis.

groups per protein molecule could be calculated by simple division. In this experiment, the initial number of the OOH groups was 5 per BSA molecule. If the size of the pBR322 plasmid is taken as 8.64 kb, the M_r is 2.88×10^6, giving 1.81×10^{15} bases/μg of DNA. Thus the sample migrating in lane 3 contained initially 13800 BSA-OOH groups for each DNA molecule, or 1.6 per base. This was increased to 3.2 in lane 5 and to 4.8 in lane 7. It should be noted that the actual number of protein OOH groups able to react with DNA is likely to be much lower than the total present, because of spontaneous decay of these peroxides [8] and the large sizes of the molecules, resulting in unfavourable steric factors for the interaction. However, the results demonstrate that native BSA at all the concentrations used had no effect on the electrophoretic migration of DNA and that the peroxidized BSA retarded both its relaxed and supercoiled forms. It is also clear that the extent of retardation and band broadening increased with the ratio of BSA-OOH to DNA.

Results shown in Figure 4 demonstrate that the gel retardation and spreading of DNA treated with peroxidized BSA was a function of the amount of protein OOHs in the mixture. In this experiment the BSA-to-DNA mass ratio was constant at 180:1 (w/w), or 7490 molecules of BSA per DNA. Samples of BSA containing different amounts of OOHs were prepared by exposing a 2 mg/ml protein solution to different doses of γ rays, with timing of the irradiation arranged so that incubation of all DNA samples with the irradiated protein began simultaneously and was terminated after 1 h. The final BSA-OOH concentrations ranged from 0 (lane 2) to 161 μM (lane 6). Lane 1 shows the bands of DNA in the absence of BSA. Even the lowest concentration of BSA hydroperoxides used (41 μM, or an average of 1.4 OOH groups per BSA molecule) produced a visible gel shift in the DNA.

In practice, detection of the binding required a mass ratio of 60:1, or roughly 2500 BSA molecules for every DNA (Figure 3), much higher than the protein/DNA ratios typical of chromatin. This requirement was imposed by the limitations of the assay, relying on a significant modification of the mass of migrating DNA. However, as the gel shift was caused by the crosslinking between DNA and proteins dependent on the presence of OOH groups on the latter, it seems likely that one OOH per protein is sufficient for the binding. Qualitatively, the maximum mobility change resulted from incubation of the DNA with BSA having 2.8 OOH groups per molecule (lane 4), with treatment with higher BSA-OOH concentrations producing little further gel shift. In this experiment, there were initially 2.3 protein OOH groups per DNA base. We did not test the possibility that longer exposures of DNA to higher hydroperoxide levels might have led to greater gel shifts, but the results summarized in Figure 5 show that this is likely. Here the pBR322 DNA was incubated for increasing periods with BSA-OOH at concentrations resulting in 3.3 OOH groups per base at the start of incubation. Lane 1 contained only plasmid DNA and lane 2 DNA treated at 37 °C for 120 min with 162 times its mass of native BSA. The samples in lanes 3–7 had the same amounts of BSA and DNA as lane 2, but with the BSA peroxidized by radiation. The DNA + BSA-OOH mixtures were incubated for 120, 90, 60, 30 and 5 min.

Enzymic digestion of the protein component in the DNA–BSA-OOH complex proved that the DNA band broadening and retardation were due to attachment of the peroxidized protein to DNA (Figure 6). The extent of this reversal was surprising, especially in the case of supercoiled DNA. It is also evident that the crosslinking did not produce any detectable strand breaks in the DNA. Incubation of DNA with 10 mM of irradiated glutamic acid, lysine, proline, valine, leucine or isoleucine, all of which are known to form appreciable amounts of hydroperoxides [8], did not lead to any gel shift.

Factors affecting the DNA–protein hydroperoxide interaction

Results summarized above are consistent with crosslinking of DNA and protein molecules, apparently dependent on the presence of protein OOHs. This dependence was confirmed in several ways. In the first series of tests, we attempted to remove the OOHs from the oxidized proteins before incubation with the DNA. Protein hydroperoxides are readily reduced by several reagents [8]. Of these, Fe^{2+} and Fe^{2+}EDTA cause rapid decomposition of most of the OOHs [11,12], but they induced significant mobility retardation and band broadening in pBR322 DNA directly. Even the presence of the native proteins did not protect the DNA. Other peroxide-reducing agents tested were $NaBH_4$ and ascorbate. After complete reduction of the OOH

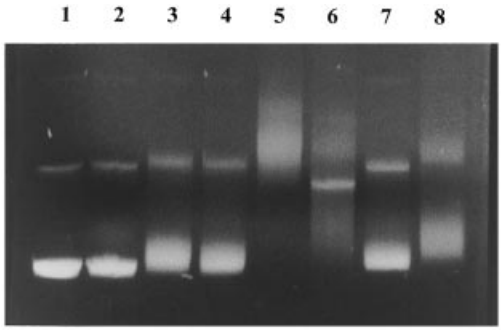

Figure 7 The effect of reduction of the BSA-OOH groups on the migration of the DNA–protein complex

BSA solutions (native or irradiated with 2000 Gy of γ rays) were treated with catalase and then incubated for 2 h with 100 mM $NaBH_4$, or allowed to stand for 48 h at 20 °C. Samples treated with $NaBH_4$ were dialysed against four changes of H_2O for 43 h. A 26 μl aliquot of each sample was then incubated for 1 h at 37 °C with 4 μl of pBR322 solution containing 0.1 μg DNA/μl. Other samples were treated with 5 mM ascorbate. The samples were photographed after 4h electrophoresis in agarose gels. Lane 1, DNA alone. Lane 2, with native BSA. Lane 3, with freshly peroxidized BSA. Lane 4, with 2-day-old peroxidized BSA. Lanes 5 and 6, with native or peroxidized BSA, respectively, treated with $NaBH_4$. Lanes 7 and 8, with native or peroxidized BSA, respectively, in the presence of ascorbate.

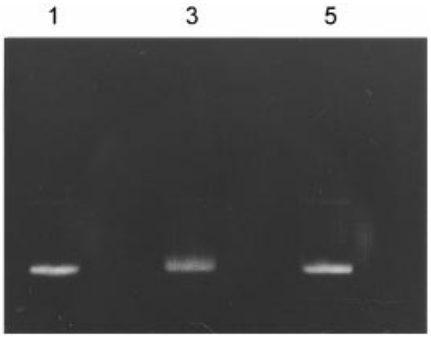

Figure 8 Reversal of $NaBH_4$-induced DNA gel shift by *N*-ethylmaleimide

Results of agarose gel electrophoresis of pBR322 DNA incubated with BSA treated with $NaBH_4$. Lane 1, DNA alone. Lane 3, DNA incubated with native BSA treated with $NaBH_4$ as in the experiments described in Figure 7. The borohydride was removed by triple filtration through Centricon 30 membranes and the reduced protein treated for 15 min with 2 mM *N*-ethylmaleimide before incubation with the DNA. Lane 5, as lane 3, but with BSA peroxidized by 2000 Gy of radiation.

groups from radiation-peroxidized BSA, as determined by the iodide assay, both agents were removed by dialysis and the protein was incubated with pBR322. Native BSA subjected to identical treatment was used as control. The results of subsequent electrophoresis (Figure 7) show that treatment of both the native and irradiated BSA with the reducing agents caused extensive retardation and broadening of DNA bands (lanes 5–8). In the case of $NaBH_4$, the gel shift was caused by new SH groups generated from some of the 17 disulphide bonds of BSA [34]; when the peroxidized BSA was reduced by 100 mM $NaBH_4$, the borohydride filtered off and the BSA treated with 2 mM N-ethylmaleimide, the protein did not induce any gel shift in pBR322 DNA (Figure 8). The mechanism of the gel shift induced by ascorbate was not studied.

Further evidence for the role of the protein OOH groups in the interaction with DNA was obtained by allowing them to decay

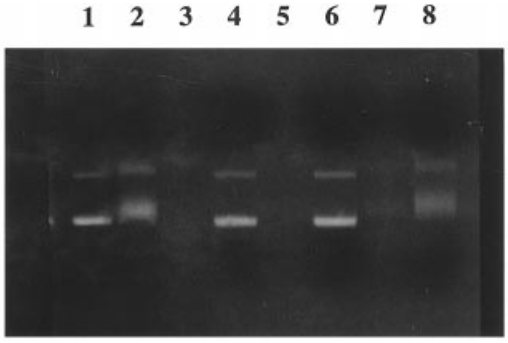

Figure 9 The effect of salt on the crosslinking of BSA-OOH with DNA

The protein (2 mg/ml), oxidized by irradiation, contained 193 μM BSA-OOH (average of 6.4 OOH/BSA). After treatment with catalase, 27 μl samples were mixed with 3.3 μl of water or 1.5 M NaCl and then incubated with 3 μl of pBR322 DNA (0.1 μg/μl) for 30 min at 37 °C. Controls were incubated with native BSA. The solutions were subjected to agarose gel electrophoresis for 2 h. Lane 1, DNA with native BSA. Lane 2, DNA with irradiated BSA. Lanes 3, 5 and 7 contained no samples to avoid the effect of diffusion of the salt present in adjacent lanes. Lane 4, DNA incubated with native BSA before electrophoresis in the presence of 150 mM NaCl. Lane 6, peroxidized BSA incubated with DNA in the presence of 150 mM NaCl before electrophoresis. Lane 8, peroxidized BSA incubated with DNA, with NaCl (150 mM final concentration) added after the incubation but before electrophoresis.

spontaneously at 20 °C for 43 h. This reduced the extent of the gel shift induced by freshly peroxidized protein (Figure 7, lanes 3 and 4). The effect was much more pronounced when most of the OOH groups were allowed to decay by standing for 15 days (results not shown). However, the most direct evidence that protein OOHs are necessary for the crosslinking with DNA was provided by irradiation under conditions precluding their formation. This was achieved by exposure of 2 mg/ml BSA solutions to γ radiation under N_2O, with the absence of peroxides confirmed by the iodide assay. Subsequent electrophoresis of pBR322 DNA incubated with the irradiated BSA for 90 min at 37 °C showed that it migrated like pure DNA or DNA treated with native BSA. These experiments also eliminated the possibility that the DNA–protein crosslinking was due to modifications of the protein by radiation, independent of the presence of OOH groups. Such modifications would include changes in side chains of amino acids, general denaturation, aggregation or relocation of bound metals.

The nature and mechanism of formation of the DNA–protein crosslinks

This was investigated in several experiments. Electrostatic bonds should be disrupted in high ionic strength solutions, whereas weak interactions are susceptible to detergents and mechanical forces. The effect of ionic strength was studied by incubating BSA-OOH with pBR322 DNA in the presence of 0.15 M NaCl. The results (Figure 9) show that the salt prevented the crosslinking when present during incubation of the DNA with peroxidized protein, but was unable to dissociate the already crosslinked macromolecules. Inclusion of 0.06 % (v/v) Nonidet or Triton X-100 during treatment of the DNA with peroxidized BSA had no effect on the gel shift (results not shown). Similarly, the crosslinks proved resistant to vigorous vortexing and filtration through membranes.

In a preliminary study of the mechanism of the crosslinking we tested the effects of anti-oxidants, metal chelators and free-radical scavengers. BSA peroxidized by radiation was mixed with the reagent under test and then incubated with pBR322

Table 1 The effect of radical scavengers and chelating agents on the migration of DNA incubated with peroxidized BSA

pBR322 DNA (3 μl, 0.1 μg/μl) was incubated for 2 h at 37 °C with the indicated reagents and subjected to agarose-gel electrophoresis in the presence of ethidium bromide. The resultant bands were examined under UV light. BSA was oxidized by irradiation in a ^{60}Co source to a dose of 2000 Gy under an oxygen atmosphere. The concentration of BSA-OOH groups was measured by the iodide assay (see Materials and methods section). The final concentrations were: BSA, 54 μg; BSA-OOH, 135 μM; chelating agents, 10–50 μM; other reagents, as shown.

DNA with ...	Appearance of DNA bands
No addition	2, Narrow
BSA	2, Narrow
BSA-OOH	Retarded, diffuse
BSA-OOH + 26 mM formate	2, Narrow
BSA-OOH + 26 mM DMSO	Retarded, diffuse
BSA-OOH + 26 mM mannitol	Retarded, diffuse
BSA-OOH + 13 mM *t*-butanol	Retarded, diffuse
BSA + 1 mM trolox	Retarded, diffuse
BSA-OOH + 1 mM trolox	Retarded, diffuse
BSA-OOH + deferrioxamine	Less retarded and diffuse
BSA-OOH + neocuproine	Less retarded and diffuse
BSA-OOH + both chelators	Less retarded and diffuse

DNA (Table 1). None of the compounds used were able to inhibit the crosslinking by a mechanism related to their antioxidant or scavenging properties. The action of formate was probably due to an increase in ionic strength, having a similar effect to NaCl (Figure 9). The reduction of gel shift and band broadening by chelators suggest a role for metals in the crosslinking process, even though none were added deliberately. There appear to be two possible sources of such metals. One is inadvertent contamination of the macromolecules by traces of redox-active transition metals, and the other is mobilization and activation by radiation of metal ions already bound to the protein. We tested the first possibility by overnight treatment of DNA or peroxidized BSA with Desferal, followed by co-incubation of the macromolecules. This reduced the DNA gel shift relative to the untreated molecules. Treatment of DNA was more effective, suggesting that it was the major carrier of the metals mediating the crosslinking. The effect of any bound metals that might be released from the protein by the irradiation was studied by gel electrophoresis of pBR322 DNA incubated with increasing concentrations of Fe^{2+}EDTA. Up to 10 μM of the chelated iron was without effect, whereas addition of between 50 and 300 μM Fe-EDTA induced extensive retardation and broadening of the DNA bands; this was found to be due to the HCl used to stabilize the iron. It appears therefore that metal ions already bound to DNA assisted the crosslinking, whereas any present in solution at likely concentrations had no effect.

DISCUSSION

Results illustrated in Figures 1–8 show that both the faster-migrating supercoiled and slower open-circle forms of DNA were present in the pBR322 samples and that they did not have any detectable double-strand breaks. The mobility shift and band broadening observed after incubation with proteins containing OOH groups can be due to a decrease in the negative charge of DNA, a change in its shape or molecular mass, or a combination of these. All our findings are consistent with the conclusion that the peroxidized proteins reacted with DNA by forming strong intermolecular crosslinks. Several lines of evidence show that the formation of these links is a general phenomenon, dependent on the presence of intact protein OOH groups.

All of the DNA samples tested showed the gel shift, with its extent largely determined by the protein/DNA ratio (Figure 2). Of the several proteins used, those that did not affect the migration of DNA in their native state (group 1) caused a clear gel shift when peroxidized (Figure 1). Unfortunately, the rapid and sensitive agarose gel electrophoresis assay of crosslinking limits the range of proteins that can be studied. Proteins containing high proportions of lysine or arginine, such as histones or lysozyme, evidently prevented the uptake of ethidium bromide when bound to DNA, whereas some others induced DNA gel shifts by an unknown mechanism, even in their native state.

In studies of the effects of variables such as the presence of OOH groups, time of interaction with DNA, the number of protein OOH groups per DNA molecule, and others, we used BSA as a model protein. BSA is typical of many intra- and extracellular soluble proteins, is readily available in sufficiently pure state and gives high yields of OOHs on exposure to a range of ROS [13]. An added advantage is that, of all proteins, the formation and properties of BSA hydroperoxides have been studied extensively for the last 10 years. Potential interference by molecules and ions known to bind to BSA proved to be unimportant, as the peroxidizability of protein samples from several suppliers and research laboratories was not affected by any trace contaminants that may have been present.

The DNA mobility shift was observed only when the group-1 proteins were peroxidized by irradiation (Figure 1). Whereas the use of a relatively large excess of peroxidized protein was necessary for visualization of its effect on the mobility of DNA, we have no evidence that the interaction requires a high ratio of protein to DNA. In fact, the actual number of protein OOH groups in these experiments was only in the order of 2 per DNA base (Figures 3 and 4). Since the peroxidized protein had only about 1.5–5 such groups per molecule and it is unlikely that, for steric reasons, more than 1 or 2 could react, the crosslinking process was surprisingly efficient.

Spreading of the DNA bands during electrophoresis after incubation with peroxidized proteins indicates that the interaction does not conform to any particular stoichiometry. At a 60:1 (w/w) ratio, there were 2507 BSA molecules for every DNA, but the numbers actually crosslinked with any particular DNA molecule must have varied considerably. This suggests that the crosslinking is not dependent on the binding of the peroxidized protein to a unique site on DNA, because otherwise the migration of DNA in the gel would be retarded, but the bands would remain narrow. In fact, there are probably many sites on DNA able to form the crosslinks. Such sites would need to be accessible on both the supercoiled and relaxed DNA, as both forms were affected (Figure 3).

The crosslinking was a function of the number of protein OOHs present and the duration of the interaction with DNA. The first point is illustrated in Figure 4, where the mobility shift increased with the number of protein OOH groups. Study of the rate of formation of the DNA–BSA-OOH complex (Figure 5) shows that the crosslinking process is relatively slow and progressive. This probably reflects the binding of an increasing number of protein molecules to DNA, with the number of OOH groups and access to DNA limiting the final extent of crosslinking. Under the conditions employed, maximum crosslinking was achieved in about 90 min. There was no indication that the process might be co-operative, with binding of some BSA molecules altering the conformation of DNA and assisting the attachment of more protein. In fact, the extent of reversal of the gel shift by proteolysis (Figure 6) shows that the crosslinking

caused little conformational alteration to the DNA. Rather, the results suggest that DNA may have sites with high and low reactivity for the OOH groups, the former reacting within a few minutes, and with the latter requiring longer incubations. Alternatively, the kinetics of the crosslinking may depend mainly on the rates of formation of free radicals from the hydroperoxides [12]. These will vary with the location of OOH groups, presence of metals, temperature and other factors.

The findings that both the supercoiled and linear forms of DNA crosslinked and that the process was not confined to a specific peroxidized protein confirms the essentially random nature of the interaction. The source of DNA also seemed to have little influence on the crosslinking (Figure 2). All samples tested crosslinked to peroxidized BSA and each DNA fragment was able to form such crosslinks. Apparent differences in the extent of crosslinking shown by various DNA samples were probably due to differences in the amounts of protein and DNA in individual bands. For example, even if the efficiency of crosslinking was similar for the λ/HindIII and pBR322 DNAs, partitioning of the BSA-OOH between the several bands of the former would result in a small gel shift in comparison with pBR322. The pUC18 samples had a higher DNA/protein ratio, with consequent smaller increase in effective mass of the crosslinked molecules and a less pronounced gel shift.

The role of transition metals in the generation of crosslinks between peroxidized proteins and DNA needs further study. We can exclude the possibility that the irradiated proteins acted simply as chelators for metals causing crosslinking or damage to the DNA, independently of the presence of OOHs. First, the reduction in gel shift by chelating agents was incomplete. Second, BSA irradiated under N_2O did not crosslink with DNA, even though it should have had an unaltered amount of bound metal ions. The partial inhibition of crosslinking by chelators was probably due to a decrease in the amounts of free radicals involved in the crosslinking, generated catalytically from protein hydroperoxides by metals attached to the DNA [12].

The reversal of the DNA mobility shift by proteolysis (Figure 6) indicates that attachment of intact protein molecules or large peptides is responsible for the effect. Protein fragments can be produced by aerobic irradiation, although their yields are low [35] and evidently any BSA fragments left attached to the DNA after proteinase-K treatment were too small to affect its migration. However, the enzyme would not remove any covalently bonded amino acids or small peptides. In the absence of effective repair mechanisms, such adducts might still affect the biological activity of DNA, especially during replication and gene transcription. Studies of the effects of NaCl (Figure 9) and of the actions of detergents and mechanical forces suggested that the DNA–protein crosslinking was probably covalent.

The exact location or the mechanism of the formation of the crosslinks are not known at present. The requirement for intact OOHs shows that the macromolecules are linked by direct reaction of protein OOH groups with DNA, resulting in covalent bonding, or that the OOH groups can give rise to reactive intermediates able to induce the crosslinking. Of these alternatives, the direct crosslinking is less likely. The chemistry of peroxides is dominated by homolytic scission of the O–O or O–H bonds, with subsequent reactions of the free-radical intermediates giving products determined by the composition of the system [36,37]. This knowledge is of limited help in the derivation of the crosslinking mechanism. The most relevant past experiments have dealt with the action of peroxides on DNA. Unfortunately, most of them employed H_2O_2, often in the presence of metals, as the oxidizing agent [38–42], when the HO$^\bullet$ free radicals generated produced DNA damage analogous to that induced by ionizing radiations, except for some site specificity due to the localized binding of the catalytic metal [42]. However, the interaction of DNA with free radicals derived from the protein hydroperoxides will give different results because they produce insignificant amounts of HO$^\bullet$ [12].

There are few studies of the more relevant action of low-molecular-mass organic peroxides on DNA. Treatment of rat hepatocytes with *t*-butyl hydroperoxide (*t*-butyl-OOH) produced DNA single-strand breaks [43] but, not surprisingly, not the 8-hydroxy-deoxyguanine characteristic of the action of HO$^\bullet$ radicals [44]. The authors reported that the damage was mediated by intracellular iron but did not explain it in terms of the chemistry of *t*-butyl-O$^\bullet$ free radicals.

The studies most relevant to the mechanism of covalent linking of DNA with peroxidized proteins were carried out by Davies and his co-workers [12]. Using EPR and a range of spin traps, they identified the free radicals generated in metal-induced decomposition of amino acid and protein peroxides. Peroxides of the six susceptible amino acids and of BSA, lysozyme and insulin produced R$^\bullet$, ROO$^\bullet$, $O_2^{-\bullet}$ and $CO^{-\bullet}$ free radicals. Another study [45] showed that alkoxyl radicals derived from *t*-butyl-OOH formed covalent adducts at the C_5 and C_6 of pyrimidines and their nucleosides and nucleotides. The much weaker signals from adducts with purines, polynucleotides, DNA and RNA did not allow exact identification of the site of interaction, but the authors concluded that radical addition to these compounds also occurred mainly at the bases.

Extrapolation of these results to our findings suggests that the crosslinking of DNA with protein (Pr) peroxides may be explained by a similar mechanism. This would require the initial formation of alkoxyl radicals in a reaction mediated by a DNA-bound metal (M) [46]:

$$\text{DNA-M}^{n+} + \text{Pr-OOH} \rightarrow \text{DNA-M}^{(n+1)+} + \text{Pr-O}^\bullet + \text{OH}^-$$

followed by:

$$\text{Pr-O}^\bullet + \text{DNA} \rightarrow \text{Pr-O–DNA}^\bullet$$

to give a covalent crosslink. Any further rearrangements or reactions of the DNA radical apparently do not lead to shifts in DNA gel mobility. The major difficulty in using the *t*-butyl-OOH model lies in the sizes of the hydroperoxides. A protein would experience considerable steric constraints in its reaction with DNA, especially in accessing the bases in supercoiled DNA that was not immune to the crosslinking. The Pr-O$^\bullet$ did not react with sugar groups, as DNA chain breaks were a very minor feature of the crosslinking. At present, Davies and co-workers' mechanism is the only one able to account for the crosslinking and only future detailed characterization of the DNA–protein bonds will show if it needs to be modified.

Direct attempts to discover whether the presence of metals can assist the formation of crosslinks were only partly successful. The partial inhibition by chelators suggests that enough metal was present in the system to assist in crosslinking by the reactions shown above. Failure of free-radical scavengers to inhibit the crosslinking (Table 1) does not necessarily indicate lack of involvement of radicals; if the radicals were generated from protein OOH groups by metals bound to DNA, their reactions with nearest DNA strands could not be intercepted by achievable concentrations of the scavengers.

The results of this study support the need to re-examine the view that proteins have a largely protective role in living systems exposed to ROS [1,5,6]. Whereas there is little doubt that many proteins contribute to the anti-oxidant potential of living organisms by possession of reducing groups or ability to bind metals [6], the generation of OOH groups by ROS converts them

into effective second messengers of oxidative stress. The potential physiological relevance of our findings lies in increasing evidence that the generation of protein hydroperoxides in biological systems is highly probable [13,14]. In a cell, the diffusion radius of peroxidized proteins will be much greater than that of most ROS. Thus, protein hydroperoxides can trap some of the chemical potential of the ROS and release it at sites normally protected by distance, or by scavenging with easily replaced molecules. Our study demonstrates for the first time that common proteins oxidized by ROS can crosslink with DNA long after the primary oxidizing radical has decayed. Further, the proteins do not have to be bound to the DNA during the exposure to the oxidant, with damage to DNA involving a two-step reaction sequence:

$$\text{Protein} + \text{ROS} \rightarrow \text{Protein-OOH}$$

$$\text{Protein-OOH} + \text{DNA} \rightarrow \text{DNA–protein}$$

Any protein peroxidized by ROS in a cell can potentially form such crosslinks if it comes into close proximity to DNA by diffusion or during functional association.

The design of strategies aimed at the protection of living organisms from damage by ROS needs to be based on the knowledge of the mechanisms of their reactions, since it is not possible, or desirable, to prevent the formation of some of the ROS. It is also impossible to introduce sufficient amounts of antioxidants or other molecules to scavenge the potentially most dangerous ROS *in vivo* before they can damage vital molecules [47]. Our results suggest that attempts to minimize DNA damage by ROS should concentrate on the prevention of formation and on benign removal of the protein OOH groups.

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