THE REACTIVITY OF THE SH GROUP OF BOVINE SERUM ALBUMIN WITH FREE RADICALS

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The reactivity of the SH group of bovine serum albumin (BSA) towards free radicals generated by several different systems including γ-radiolysis and hydrogen peroxide/metal salt mixtures was investigated. On exposure of BSA (1 mg/ml and 5 mg/ml) to HO$\cdot$ radicals generated radiolytically the protein-SH concentration was found to decrease in a dose-dependent manner. At 40 mg/ml albumin no loss of SH was observed. O_2^- and $HO_2\cdot$ radicals were much less aggressive towards the SH group.

The effect of divalent metal salts (copper or iron) plus hydrogen peroxide was studied separately and in combination. H_2O_2 alone caused a decrease in SH group concentration the rate of which was not decreased by the presence of desferrioxamine and so was apparently *not due to reactions catalysed by adventitious* metal ions. Copper alone caused a dose-dependent decrease in SH group concentration and the mixture of the two agents caused a greater loss of SH than each separate component. However, this latter effect was again resistant to the effects of desferrioxamine. The SH group of BSA was only moderately sensitive to the presence of ferrous iron alone and in a system containing both ferrous iron and H_2O_2 rates of SH oxidation were obtained that were identical to those obtained with H_2O_2 alone. Desferrioxamine again did not alter the rate of SH oxidation in these experiments. We suggest that the highly reactive free radical HO$\cdot$ *is not able to reach and to oxidize the SH group of BSA when generated by metal/H_2O_2 mixtures, in* contrast to HO$\cdot$ generated radiolytically. Less reactive radicals and non-radical species such as H_2O_2 have more potential for doing so.

KEY WORDS: SH groups, bovine serum albumin, free radicals.

INTRODUCTION

The reaction of free radicals with proteins resulting in damage has long been recognised to occur and in the past few years there has been an upsurge of interest in this topic, mostly directed towards the investigation of how radical-induced modifications of proteins lead to increased rates of proteolysis.[1-6] This particular aspect is clearly an example of free radical damage to protein. The reaction of proteins with free radicals can, in certain circumstances, be seen as a form of antioxidant action, protecting some other more critical target. As discussed by Halliwell,[7] albumin may have such a secondary role as an antioxidant in the blood by virtue of its high concentration (40 mg/ml) and its high turnover rate (half-life = 20 days). In fact, albumin could act as an antioxidant in two ways: by a non-specific 'mopping-up' of reactive free radicals

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and also by specifically binding copper ions that might otherwise catalyse the production of free radicals. It is the former, radical-scavenging property of albumin with which this paper is mostly concerned.

Albumin reacts with and is damaged by oxidizing free radicals as shown by numerous studies.[2,3 8-11] It is well established that certain amino acid residues in proteins are particularly susceptible to free radical attack and one such is cysteine, the thiol group of which is prone to oxidation. Albumin contains only one thiol group per molecule, but this is the major source of protein thiol in the plasma and may be of particular significance to the putative free radical scavenging role of albumin. The thiol group of bovine serum albumin (BSA) has been shown to be accessible to electrophilic agents such as bromosulphopthalein and ethacrynic acid[12] and also peroxides including t-butyl hydroperoxide and benzoyl peroxide.[13] Wayner et al.[14] have demonstrated the oxidation of plasma protein thiol groups in systems generating peroxyl free radicals. The aim of the present study was to assess the potential of the thiol group of BSA to react with free radicals. We have used several different free radical generating systems including gamma radiolysis and hydrogen peroxide/metal mixtures and we have determined the rate of oxidation of the BSA thiol group under these conditions.

MATERIALS AND METHODS

Chemicals

BSA Fraction V (SIGMA) was employed. Other samples of BSA were used (Merck) which gave essentially the same results; the only difference consisted of small differences in the content of SH. All other reagents were of analytical grade and Millipore-filtered de-ionised water was used throughout.

RADICAL GENERATION

Gamma-radiolysis

BSA was dissolved in phosphate buffer 10 mM, pH 7.4, or in formate buffer 10 mM, pH 4.0 or 7.2. Then samples were exposed to a 2000 Ci cobalt source at a dose rate of 0.78 Krads/min to generate defined free radicals by gamma-radiolysis of water, according to techniques previously described.[15] For the specific generation of $HO\cdot$ radicals, BSA solutions in phosphate buffer were bubbled with a N_2O/O_2 gas mixture prior to irradiation. For the generation of O_2^- and $HOO\cdot$ radicals the samples contained 10 mM formate buffer at pH 7.2 and pH 4.0, respectively.

Radical Generation with H_2O_2 and Divalent Metal

H_2O_2, $FeSO_4$, $CuSO_4$ and BSA stock solutions were freshly prepared in water. In all the experiments the SH groups of BSA were measured at the specified times after the addition of H_2O_2

SH DETERMINATION

SH group determination was carried out using Ellman's reagent as described previously.[12] Aliquots of BSA solutions (0.1–3.6 ml according to the BSA concentration) were added to phosphate buffer (0.1 M, pH 7.4) in a cuvette (final volume 1.1 ml, or 4 ml) and then 0.1 ml of 5, 5′ dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent) in the same buffer was quickly added to a final concentration of 1–2 mM and quickly mixed with a plastic rod. The change of absorbance at 410 nm was recorded with time; the end of reaction occurred within 3–8 min. Other details are reported in the legends to the Figures.

TRYPTOPHAN FLUORESCENCE

Before and after irradiation aliquots of BSA were taken and diluted to 0.05 mg/ml in water for measurement of tryptophan-dependent fluorescence using 290 nm excitation and 350 nm emission.[16]

RESULTS AND DISCUSSION

Gamma-Radiolysis

Three concentrations of BSA (1, 5 and 40 mg/ml) were exposed to increasing doses of radiation (Figures 1 and 2). The SH groups of 1 and 5 mg/ml BSA were oxidized by exposure to HO· radicals in a dose-dependent fashion. On the other hand, HO· radicals were unable to produce an effect when a concentration of 40 mg/ml of BSA was employed (not shown). HO_2· and O_2^- radicals produced a smaller decrease in SH concentration than HO· radicals; this effect was observed with 1 mg/ml of BSA but no change in SH concentration at all was seen with higher concentrations (5 mg/ml, Figure 2 and 40 mg/ml, not shown). Figure 1b shows the fluorescence variation of BSA (1 mg/ml) after gamma-irradiation. The SH group concentration and fluorescence decreased in parallel with increasing exposure to radicals.

Since at the same radical concentrations O_2^- and HO_2· did not have as great an effect as did HO·, it would seem that these radicals are, as may be expected, less active in attacking and oxidising the sulphydryl group. Differences in the reactivity of oxygen radicals towards proteins has previously been described by other authors showing that hydroperoxyl radicals and superoxide radical anions are less reactive than hydroxyl radicals.[1-5] The greater effect of HO· radical was also evident as fluorescence changes (Figure 1b); confirming the observations of Dean et al.[1] Interestingly, at the highest radiation doses, O_2^- and HO_2· radicals were more aggressive than HO· in terms of producing changes in the apparent protein SH concentration but in these cases the changes observed were in the opposite direction i.e. *increases* in the apparent SH concentration. In fact a white precipitate was observed in samples exposed to the highest concentrations of O_2^- and HO_2· radicals (100 and 200 Krads); in these samples very high levels of SH group concentration were observed (5–10 fold the control value). Albumin is very rich in disulphide bridges and their reductive cleavage must explain the high increase in SH concentration in these cases. A similar result is implied in the paper of Davies et al.[4] The S-S bridge cleavage could be due to a direct action towards disulphide bridges, of which there are 16 in BSA, or may
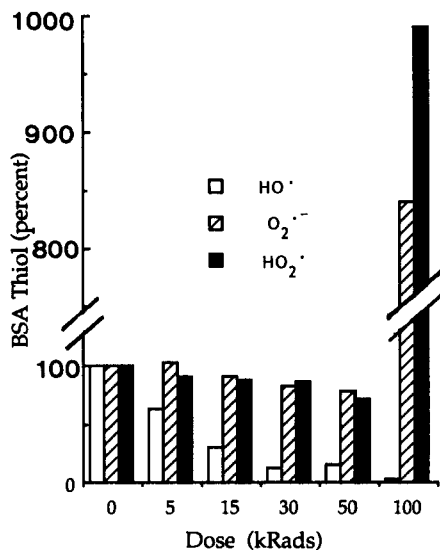
FIGURE 1a   Changes in the amount of SH in BSA (1 mg/ml) after different exposures to radiation-generated free radicals. Control values for SH: for HO· experiments, $5.68 \pm 0.58 \mu M$, for HO_2· experiments $5.56 \pm 0.61 \mu M$, for O_2^- experiments $6.11 \pm 0.70 \mu M$ (Means + S.D)
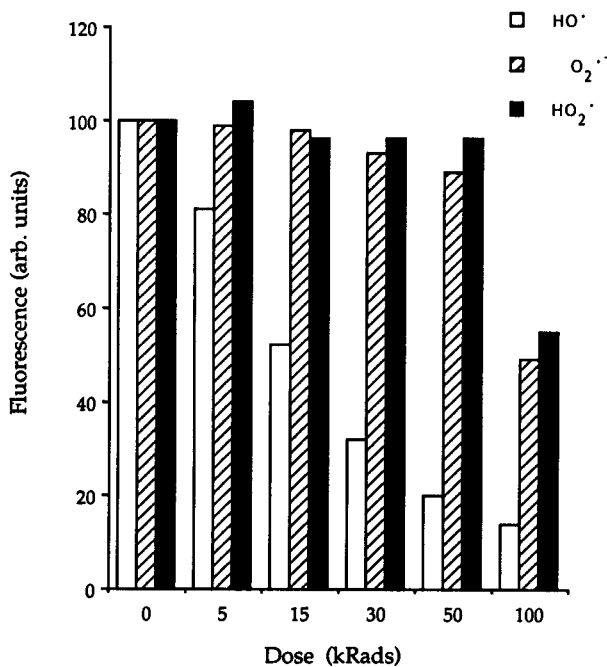

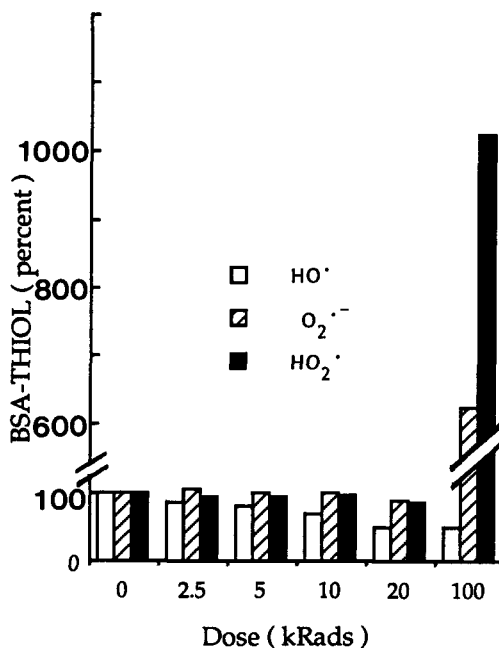FIGURE 1b   Changes in tryptophan fluorescence in BSA (1 mg/ml) after exposure to radiation-generated free radicals.

FIGURE 2   Changes in the amount of SH in BSA (5 mg/ml) after exposure to radiation-generated free radicals. Control values: 29.1 $\pm$ 0.6 μM for HO$\cdot$ experiments, 27.5 $\pm$ 0.6 μM for HO$_2\cdot$ experiments, 27.0 $\pm$ 0.5 μM for O$_2^-$ experiments.

be a secondary effect after the attack of other protein residues, as described by Dean et al.,[11] facilitating the exposure of the S-S bridges. At these high doses however, oxygen will be limiting and the observed effect is most likely due to $CO_2^{\cdot-}$ radicals rather than $O_2^{\cdot-}$.

H_2O_2 and Divalent Metals

HO$\cdot$ radicals are known to be generated by reaction of H_2O_2 with a divalent ion such as Cu^{++} or Fe^{++}. However, as H_2O_2 (see Pirisino et al.,[13]) or divalent ions may potentially react independently with sulphydryl groups, it is important to determine the contribution of each independent reaction.

Figure 3 shows the time-course of changes in the SH concentration of BSA in the presence of increasing concentrations of H_2O_2; the experiment was carried out with 40 mg/ml BSA in 10 or 100 mM phosphate buffer pH 7.4. A dose-related decrease in SH concentration was observed in both cases. This effect was apparently not due to metal-dependent free radical production, due to traces of metals (such as Fe^{++}) present as contaminants in reagents, since in the presence of 0.1 mM desferrioxamine, a chelator of ferric ions which quenches metal-dependent free radical formation, we observed kinetic profiles overlapping with those of H_2O_2 alone (Figure 3).

Interestingly, we observed a kinetic profile with 10 mM phosphate buffer different to that seen with 100 mM phosphate buffer (Figure 3). The reaction rate of BSA thiol groups with hydrogen peroxide was initially (in the first minute) the same; however, in 10 mM phosphate an increasingly slower reaction rate than in 100 mM phosphate
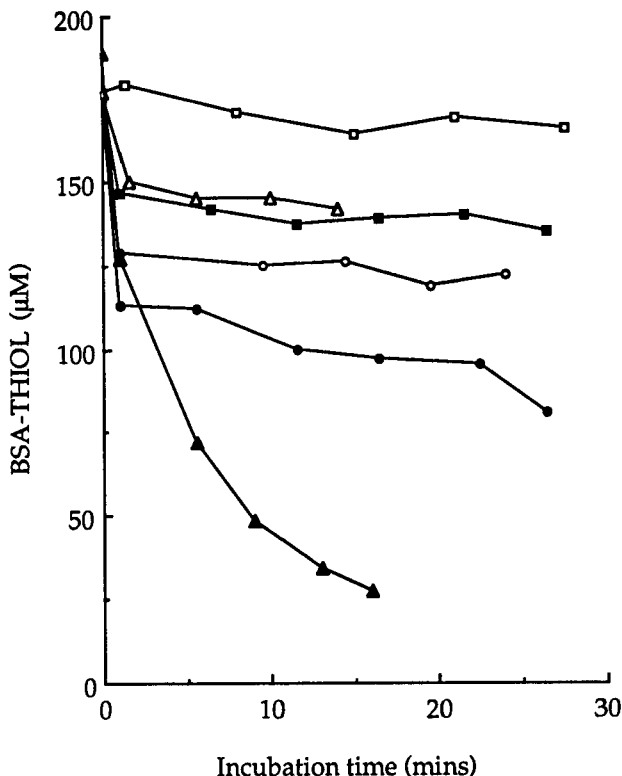
FIGURE 3   Time-course of SH oxidation of BSA (40 mg/ml) with increasing concentrations of hydrogen peroxide in 10 mM phosphate buffer. □, 0.55 mM; ■, 4.4 mM; ○, 8.8 mM; ●, 17.6 mM; △, 4.4 mM plus 0.1 mM desferrioxamine. ▲, 8.8 mM H_2O_2 in 100 mM phosphate buffer.

was seen. For example, approximately all of the total available BSA SH groups were attacked by 8.8 mM H_2O_2 within 20 min at 100 mM phosphate, whereas only a 30% decrease in SH content was observed within the same time in 10 mM phosphate buffer.

Cu^{++} can react with the SH group of BSA. The SH concentration decreased in the presence of copper in a dose-related manner (experimental range: 0.1–0.8 mM; Figure 4 shows the effect of 0.1 mM Cu^{++}) but the effect of copper was not influenced by phosphate concentration, as it was in the previous case of hydrogen peroxide. Other heavy metal ions, such as Ni^{++} and Hg^{++} (not shown), behaved in the same manner as Cu^{++}, exhibiting kinetic profiles similar to those obtained with 10 mM phosphate (an immediate drop, increasing with the dose, in the SH content, followed by constant values for 15–20 min).

Figure 4 shows the time-course of changes in the SH concentration (BSA 4 mg/ml) in a system that generates free radicals (2.5 mM H_2O_2; 0.1 mM Cu^{++}) in the presence of 10 mM or 100 mM phosphate buffer.

The decrease in SH group concentration was greater and faster when H_2O_2 and Cu^{++} were together than when hydrogen peroxide was alone. This was in accordance with a presumed additive action above that of hydrogen peroxide alone due to the generation of oxidative free radicals. However, when desferrioxamine was also added
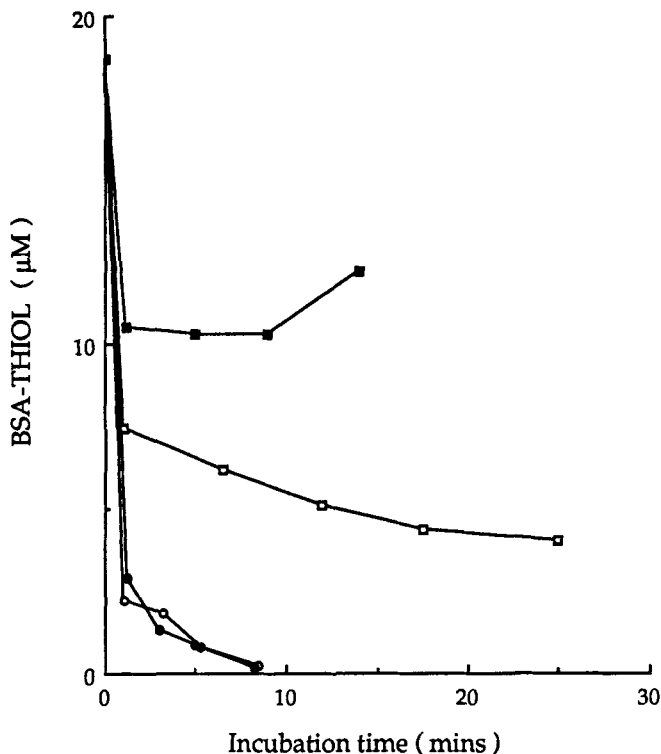
FIGURE 4   Time-course of SH oxidation of BSA (4 mg/ml) with copper (0.1 mM) and hydrogen peroxide (2.5 mM) in 10 mM phosphate buffer. ■, copper alone; □, H_2O_2 alone; ○, copper/H_2O_2; ●, copper/H_2O_2/ desferrioxamine (0.1 mM).

it had no effect (Figure 4). A further experiment, carried out with 40 mg/ml BSA and 5.5 mM H_2O_2 and 0.1 mM Cu^{++}, confirmed the lack of effect of 0.1 mM desferrioxamine (data not shown). Desferrioxamine thus seems incapable of binding copper and preventing copper-mediated loss of protein thiol in this system.

These results did not explain why the system containing copper plus peroxide produced a greater decrease in SH groups in comparison with hydrogen peroxide alone. The same experiments were subsequently repeated, using Fe^{++} instead of Cu^{++}, at concentrations of reagents which usually generate HO· radicals[2] and with various concentrations of BSA (1, 4, 40 mg/ml) in the presence of 10 mM phosphate buffer (Figure 5). Experiments were also carried out in 100 mM phosphate buffer but the results are not shown as they were essentially similar to those carried out in 10 mM buffer. In all cases except one (40 mg/ml BSA, 10 mM phosphate; data not shown), the combination of iron plus hydrogen peroxide gave a rate of oxidation the same as that found with hydrogen peroxide alone. This finding, which was in contrast with the previous experiment which utilized copper, seems to suggest that HO· radicals are not attacking the SH- group in this system. It is possible that they are produced at a site too distant from the SH group and react with other residues before reaching it. In support of the idea that free radicals produced by the metal-catalysed breakdown of hydrogen peroxide are not responsible for oxidation of the SH-group, in all examined
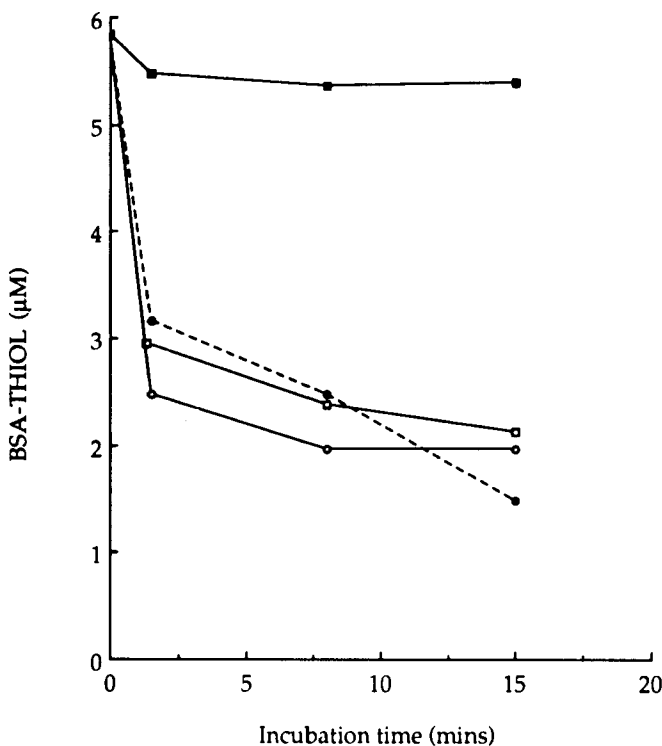
FIGURE 5   Time-course of SH-oxidation of BSA (1 mg/ml) with ferrous iron (0.1 mM) and hydrogen peroxide (2.5 mM) in 10 mM phosphate buffer. ■, iron alone; □, H_2O_2 alone; ○, iron/H_2O_2; ●, iron/H_2O_2/ desferrioxamine (0.1 mM).

cases the addition of desferrioxamine to the system did not change the rate of oxidation.

The results obtained with iron (Figure 5) may in part explain the increased effect found with the copper-plus-hydrogen peroxide system in comparison with that using hydrogen peroxide alone (Figure 4). In fact iron alone (experimental range: 0.1–0.8 mM) produced a lower effect in comparison with copper on the SH group concentration and very small decreases were mostly observed. The more rapid rate of SH loss in the system containing both hydrogen peroxide and copper may therefore be caused by an additive effect of the agents towards the thiol group; this additive action was not readily apparent when iron substituted copper.

An alternative explanation is possible. It is feasible that the albumin contains endogenous copper and that the result obtained by adding H_2O_2 alone is actually due to production of OH· catalysed by protein-bound copper. However, since OH· radicals generated by the Fe/H_2O_2 mixture seem incapable of reaching the SH group, then OH· radicals produced by reaction of H_2O_2 with protein-bound copper should also not reach the SH group *unless* the copper is located in the proximity of the thiol group such that OH· radicals produced by the latter reaction do not have to diffuse

very far. This explanation would also depend on the desferrioxamine being incapable of removing copper from the albumin.

Our observations seem to suggest that highly oxidising free radicals have little opportunity in comparison with other less reactive agents (such as H_2O_2) to reach the BSA thiol group. Using gamma radiolysis the HO$\cdot$ radicals may be generated in the immediate vicinity of the SH group. In gamma-radiolysis experiments at a BSA concentration of 40 mg/ml, no effect of the OH$\cdot$ radical towards SH groups was observed up to an exposure of 200 kRads (equivalent to 1.08 mM OH$\cdot$ concentration). However, this may represent the problem of measuring a small decrease in the concentration of a component having a large initial concentration. According to Wayner et al.[14] and Frei et al.,[17] during ABAP-initiated peroxidation of plasma lipids the plasma protein SH groups, that essentially belong to albumin, are decreased in concentration. The ABAP-peroxyl radical is intermediate in reactivity between the HO$\cdot$ radical and H_2O_2 and may represent the most reactive type of radical to be able to reach and to oxidise the BSA thiol group without reacting indiscriminately with other moieties.

In conclusion it would seem that highly reactive free radicals are not readily capable of reacting with the SH group of BSA, unless generated in its immediate vicinity, because the thiol group is not sufficiently accessible to those agents. More diffusible compounds such as less reactive radicals, hydrogen peroxide or organic substances with electrophilic properties[12] can however be rapidly scavenged by BSA by means of its sulphydryl group. As other authors[14] have indicated that plasma SH groups represent a great and immediate reservoir against the attack of free radicals, further comparative studies are required to clarify differences in reactivity between protein thiols in plasma and purified albumin.

Acknowledgements

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