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Bilirubin attenuates radical-mediated damage to serum albumin

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Oxidative damage to biological macromolecules has been implicated in a number of diseases. Much interest has focused on how non-proteinaceous, low-molecular weight antioxidants prevent oxidative damage to lipids, while comparatively little is known about protein antioxidation. Here we show that bilirubin (BR), the end-product of heme catabolism, when bound to bovine serum albumin (BSA), is oxidised by hydroxyl ($^{\bullet}OH$), hydroperoxyl ($HO_2^{\bullet}$), and superoxide anion ($O_2^{-\bullet}$) radicals to so far mostly uncharacterised products. The initial oxidation rates of BSA-bound BR decreased in the order $^{\bullet}OH > HO_2^{\bullet} > O_2^{-\bullet}$. BR protected its carrier protein from oxidative damage inflicted by $^{\bullet}OH$ radicals. This protective action included a reduction in the $^{\bullet}OH$-mediated cleavage of BSA, conversion of Trp into kynurenine and formation of 'bityrosine-specific' fluorescence. BR also strongly inhibited $^{\bullet}OH$-mediated formation of protein carbonyls, whereas ascorbate and Trolox (a water-soluble analogue of vitamin E) were much less effective. These results support an antioxidant-protective function of BR and point towards significant differences in the efficacies of various antioxidants in the prevention of oxidative damage to lipids and proteins.

Protein oxidation; Antioxidant; Irradiation; Ascorbate; Vitamin E; Kynurenine

1. INTRODUCTION

Bilirubin (BR), the end product of heme degradation, can be toxic for humans (when accumulating at high concentrations) and has long been regarded as a waste product. Formation of BR from its precursor, biliverdin (BV), is catalysed by biliverdin reductase, an enzyme found in various isoforms and with no other known function [1]. In birds and reptiles that lack BV reductase, the non-toxic BV is excreted directly. This prompts the question of why during evolution this additional, energy-requiring step has been introduced [2].

It has been suggested that BR is an antioxidant of physiological significance [3–6]. In the circulation, most of the pigment is present as a tight complex with serum albumin at levels of 5–17 μM [7]. BR efficiently protects albumin-bound fatty acids from peroxyl radical-mediated oxidations in vitro [3] and, when incorporated into liposomes, acts as a chain-breaking antioxidant at least as efficient as α-tocopherol [4]. Similarly, conjugated BR (the water-soluble form of BR present in bile) is also an efficient scavenger of peroxyl radicals [8] and can synergise with membrane-bound α-tocopherol to prevent lipid peroxidation [9]. Albumin-bound BR has been shown to protect human ventricular myocytes against reactive oxygen species (ROS)-induced damage [10], and an earlier report indicated a protective activity for BR against photo-oxidative damage to albumin's amino acids [11]. We have recently observed that $^{\bullet}OH$-mediated oxidation of BSA and other proteins can proceed via a chain reaction, as assessed by the loss of amino acid residues, and that this chain reaction was inhibited substantially by BR or Trolox [12]. In this communication we describe additional protective activities of BR on oxidative damage to BSA.

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Abbreviations: BR, bilirubin; BV, biliverdin; BSA, bovine serum albumin; $HO_2^{\bullet}$, hydroperoxyl radical; $^{\bullet}OH$, hydroxyl radical; $O_2^{-\bullet}$, superoxide anion radical; Kyn, kynurenine; ROS, reactive oxygen species; Trp, tryptophan.

2. MATERIALS AND METHODS

BSA (fatty acid free), uric acid, ascorbic acid, glutathione, dinitrophenyl hydrazine and BR were purchased from Sigma; Chelex-100 from Bio-Rad; Trolox from Aldrich; and Trolox acetate was prepared from Trolox by its O-acylation using acetic anhydride by Dr. C. Suarna (The Heart Research Institute). Other chemicals were of the highest purity available, and solvents of HPLC grade.

γ-Radiolysis experiments were carried out using the ^{60}Co-source facility at the School of Biological Sciences of the Macquarie University, Sydney. All irradiations were carried out in closed glass vials with the samples being bubbled constantly with the appropriate gas. Most samples were dissolved in 0.1 M phosphate buffer, pH 7.4, and gassed with either O_2 (to give equimolar amounts of $^{\bullet}OH$ and $O_2^{-\bullet}$ at 12.4 μM/min each) or N_2 (to give $^{\bullet}OH$ at 12.4 μM/min $^{\bullet}OH$). Some samples were dissolved in the same phosphate buffer supplemented with either 10 mM sodium formate (final pH about 7.4) and gassed with O_2 (to give $O_2^{-\bullet}$ radicals at 14.7 μM/min), or 0.1 M formic acid (final pH about 4) and gassed with O_2 (to give $HO_2^{\bullet}$ radicals at 14.7 μM/min). In the case of N_2 gassing, samples were pre-gassed with N_2 for 15 min prior to irradiation. Some samples were dissolved in the above phosphate buffer and irradiated under a constant gassing with an N_2O:O_2 (4:1, v/v) mixture leading to the formation of $^{\bullet}OH$ radicals (at the rate of 24.8 μM/min). In contrast to the N_2-gassed samples, the O_2 present in these samples would allow addition reactions of oxygen to the carbon-centered radicals generated by the initial H-abstraction of BSA's amino acids by $^{\bullet}OH$ radicals. Individual samples were irradiated for the indicated time period, an aliquot withdrawn, and placed in dry ice and kept frozen until analysed.

BSA-bound BR was prepared by addition of freshly dissolved BR (in 50 mM NaOH) to BSA (in 0.1 M phosphate buffer, pH 7.4). This results in binding of BR that is representative of the in vivo form of the pigment bound to the primary binding site on albumin (see [4]). The control (no BR) BSA samples were supplemented with the same volume of 50 mM NaOH. The final pH of the solutions was 7.4. All buffers and solutions were treated thoroughly with Chelex-100 prior to use to remove contaminating transition metals. Successful elimination of catalytically significant level of transition metals was verified by the ascorbate autoxidation method [13].

BR was determined in the supernatants resulting from the EtOH precipitation of control and irradiated BSA samples, using an HPLC method described elsewhere [14].

For carbonyl and 'bityrosine' determinations, the thawed control and irradiated samples (100 μl) were treated with EtOH (1:4, v/v), the precipitated protein pelleted, washed with EtOH and resuspended in 100 μl 6 M guanidine hydrochloride. For measurement of bityrosine, the resuspended pellet was diluted appropriately with phosphate buffer and its fluorescence intensity determined (λ_{ex} = 325 nm, λ μl of this was mixed with 0.02% dinitrophenyl hydrazine (in 1 M HCl), incubated at 50°C for 30 min, made 1 ml with 10% NaOH, and the absorbance was measured at 450 nm. A molar extinction coefficient of 2.2×10^4 M · cm^{-1} [16] was used to quantitate protein carbonyls. Tryptophan (Trp) and kynurenine (Kyn) were determined after acid hydrolysis of the BSA samples by HPLC as detailed elsewhere [12,17].

Protein concentration was determined using the bicinchoninic acid assay supplied by Sigma. SDS-electrophoresis was carried out on 15% polyacrylamide gels using the reducing Laemmli system [18]; the gels were stained with Coomassie brilliant blue R-250, and the Bio-Rad molecular weight markers were used to estimate the size of the bands of the irradiated protein.

3. RESULTS AND DISCUSSION

Fig. 1 shows the time-dependent disappearance of BSA-bound BR under different irradiation conditions. Exposing the BSA-bound pigment to either $^{\bullet}OH/O_2^{\bullet-}$, $HO_2^{\bullet}$ or $O_2^{\bullet-}$ resulted in rates of BR oxidation of 8.2 ± 1.5 μM · min^{-1}, 4.5 ± 1.2 μM · min^{-1} or 3.2 ± 0.7 μM · min^{-1}, respectively. BR oxidation by $^{\bullet}OH/O_2^{\bullet-}$ was primarily due to $^{\bullet}OH$ as irradiation of an N_2-saturated solution of BSA–BR in phosphate buffer under anaerobic conditions (yielding $^{\bullet}OH$ only) caused disappearance of albumin-bound BR at the initial rate of 10.5 μM · min^{-1}, i.e. similar to that observed with $^{\bullet}OH/O_2^{\bullet-}$. Also, irradiation of a solution of BSA–BR under N_2O:O_2 (4:1, v/v), where $^{\bullet}OH$ were formed almost exclusively, resulted in an initial rate of BR oxidation of about 8 μM · min^{-1}. Furthermore, under the irradiation conditions used for $O_2^{\bullet-}$ generation, some 0.5% of the radical are present as $HO_2^{\bullet}$, which may be contributing to the observed loss of BR. Indeed, the reaction of BR with $O_2^{\bullet-}$ has been reported to be rather slow [19].

Since oxidation of BSA–BR with peroxyl radicals results in formation of biliverdin (BV) [4], we tested irradiated samples for the formation of the latter pigment. However, only small amounts (<1% yields) of BV were formed with oxygen-centered primary radicals. The majority of BR was converted to colourless products (not shown), indicative of cleavage of the tetrapyrrole moiety of BR.

The concentration of BSA used in our experiments

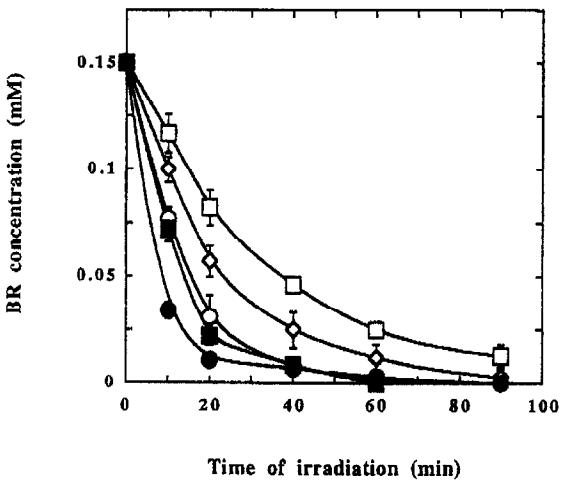

Fig. 1. γ-Radiation of BSA-bound BR. BSA–BR (75 μM BSA, 150 μM BR) dissolved in 0.1 M phosphate buffer (pH 7.4) was exposed to either $^{\bullet}OH$ in N_2^- (●) or N_2O:O_2 (4:1, v/v)-saturated solutions (○), or $^{\bullet}OH/O_2^{\bullet-}$ (■), $HO_2^{\bullet}$ (◇ or $O_2^{\bullet-}$ (□). Formation of the various radicals was achieved as described in section 2. At the indicated time points, aliquots were withdrawn and analysed for BR by HPLC (see section 2). The results shown represent mean values of 3 identical experiments with variation of ±10%.

was 75 μM (5 mg · ml^{-1}), which represented 'saturating' conditions [12,20], i.e. when all of the generated radicals reacted with the protein rather than with each other or possible impurities present in the samples. This enabled us to carry out a material balance [21]. The concentration of the pigment used was 150 μM; in the case of the highly reactive, and hence more or less indiscriminative $^{\bullet}OH$, this represented, on a molar basis, only some 0.5% of total targets, as each BSA molecule comprises 525 amino acid residues. Due to its low selectivity [22], we assume that a large proportion of $^{\bullet}OH$ formed reacted with the protein by H-abstraction, forming protein carbon-centered radicals which, in the presence of oxygen, will be converted rapidly to protein peroxyl radicals. We have recently presented evidence that irradiation of a 'saturated' BSA solution under conditions where $^{\bullet}OH$ are formed, results in a destructive oxidation chain reaction, and that reaction of BR with protein radicals rather than direct scavenging of the chain-initiating radicals may explain the rapid disappearance of the pigment [12]. The fact that BR also disappeared rapidly under anaerobic irradiation suggests that the pigment can efficiently react with carbon-centered radicals. BR consumption under conditions where $O_2^{\bullet-}$ or $HO_2^{\bullet}$ only were formed is consistent with the known higher reactivity of the pigment towards these radicals than BSA's amino acid residues [22].

A number of oxidative modifications are known to occur on proteins exposed to highly reactive oxidants [23–26]. Among them, we examined protein fragmentation, Trp degradation, and formation of kynurenine (Kyn), bityrosine and carbonyls, using aerobic irradia-

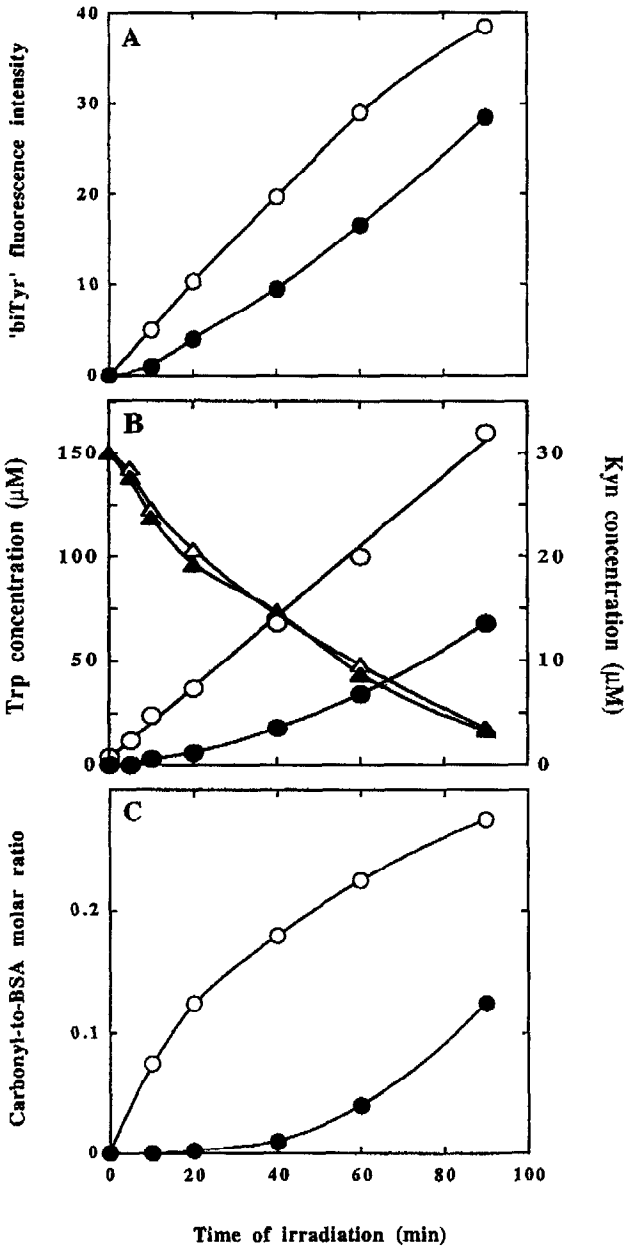

Fig. 2. γ-Irradiation-induced oxidative damage of BSA. BSA was exposed for various periods of time to $^{\cdot}OH/O_2^{\cdot-}$ in an O_2-saturated system in the absence (open symbols) and presence of BR (filled symbols). At the time points indicated, aliquots were withdrawn and analysed for selected markers of oxidative damage as described in section 2. (A) Formation of 'bityrosine'. (B) Degradation of BSA's Trp residues (circles) and the formation of Kyn (triangle). (C) Formation of protein carbonyls. The carbonyl values of all samples were corrected for the carbonyl content determined in the control, non-irradiated BSA.

tion conditions that resulted in the generation of equimolar amounts of $^{\cdot}OH$ and $O_2^{\cdot-}$.

As reported previously by others [23,24,26], irradiation of BSA with large doses of radicals resulted in cleavage of the 69 kDa polypeptide chain, as assessed by the appearance of more or less discrete bands of lower molecular weight on polyacrylamide gels (not shown). This polypeptide cleavage is believed to be initiated by α-hydrogen abstraction [23]. In the presence of BR at twice the molar concentration of BSA, irradiation-induced cleavage of the polypeptide chain was partially inhibited as indicated by the slower rates of disappearance of the 69 kDa band and appearance of the low-molecular weight bands. However, the observed protection was rather small and protein cleavage did not appear to represent an early event in protein oxidation and was difficult to quantitate.

We have recently observed that exposure of BSA to $^{\cdot}OH/O_2^{\cdot-}$ resulted in the immediate and time-dependent loss of all amino acid residues and that, with the exception of Trp, BR substantially protected all amino acids from such destruction [12]. The results in Fig. 2A confirm the latter observation and they show that $^{\cdot}OH$-mediated conversion of Trp to Kyn is, however, inhibited strongly by BR. At present we do not know whether the presence of BR also inhibits the formation of *N*-formyl kynurenine, which is converted to Kyn during the sample work-up procedure [27]. Since Trp degradation is likely to involve initial hydroxyl radical addition to the aromatic structure, our results suggest that, while BR does not interfere with this step, it efficiently intercepts at the level of cleavage of the indolyl moiety of Trp.

The presence of BR also inhibited the formation of bityrosine fluorescence (Fig. 2B), an assay commonly used to assess protein cross-linking arising from their oxidation by ROS (e.g. [26]). Despite its name bityrosine-specific fluorescence is, however, relatively non-specific and its validity has recently been questioned [28]. The degree of inhibition of Kyn formation in the presence of BR resembled that of bityrosine fluorescence (cf. Fig. 2A and B). Since the fluorescence properties of *N*-formyl kynurenine are very similar to those of bityrosine it cannot be excluded that the observed bityrosine-specific fluorescence may be partially ascribed to *N*-formyl kynurenine formation.

Table I

Inhibition of irradiation-mediated carbonyl formation on BSA by various low-molecular weight compounds

Additive	% inhibition
None	0
Trolox	6
Trolox acetate	2.5
Uric acid	94.1
Ascorbate	37
Reduced glutathione	65.4
BR	96

BSA (final concentration of 75 μM) was exposed to 20-min irradiation under oxygen (corresponding to radical dose of 0.48 mM), either alone or in the presence of additives (final concentration of 150 μM). Following irradiation, samples were analysed for carbonyls as described in section 2.

Fig. 2C shows the extent of carbonyl formation observed during BSA irradiation under O_2 and in the absence of BR, which corresponds well with the published values [26]. Carbonyl formation was almost completely inhibited as long as BR was present, but proceeded at a similar rate as in the control sample following BR consumption (cf. Figs. 1 and 2C). Carbonyl formation is perhaps the most commonly used, single assay to assess protein oxidation. Since formation of protein carbonyls was most strongly inhibited by BR, we compared its efficacy with that of some other small molecular antioxidants used at equimolar concentration. As shown in Table I, only uric acid was as efficient as albumin-bound BR, while glutathione and ascorbate were less protective and both Trolox (a water-soluble analogue of α-tocopherol) and Trolox acetate (a derivative of Trolox lacking antioxidant activity) showed no protection. These results are consistent with a recent report [29] showing virtually no protective activity of ascorbate against the formation of carbonyls on both human serum albumin and human plasma proteins exposed to cigarette smoke (rich in free radicals), while glutathione provided about 50% protection.

The mechanism(s) of protection of BSA's oxidative modification by BR and other antioxidants is not known at present. Our results indicate that BR may act as a chain-breaking antioxidant for both lipids [3] and proteins [12]. The possibility that the pigment may also repair oxidised amino acids, as was shown for other reductants [30], cannot be excluded, however. While our results show that BR is a protective agent for its carrier protein, and thereby further support a role for this pigment as a physiological antioxidant [3–6], they indicate differences in the antioxidation in proteins and lipids. In the latter case, α-tocopherol, together with reductants that reduce the α-tocopheroxyl radical (e.g. ascorbate, BR), appears to represent a major mechanism of antioxidant defence [31–33]. In the absence of α-tocopherol, protein antioxidation must rely on different mechanism(s) that require investigation.

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