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Peroxynitrite-mediated oxidation of albumin to the protein-thiyl free radical

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Abstract

Nitric oxide reacts with superoxide to produce peroxynitrite, which may be an important mediator of oxidant-induced cellular injury. Here we report that peroxynitrite is able to oxidize a protein, bovine serum albumin (BSA), to the corresponding protein-thiyl free radical as demonstrated by electron paramagnetic resonance (EPR)-spin-trapping experiments with both α-phenyl-*N*-*tert*-butyl nitrone (PBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). BSA radical adduct yields increased with pH indicating peroxynitrite anion as its main forming agent. Reaction with peroxynitrite may be another aspect of the antioxidant action of albumin in extracellular fluids.

Key words: Peroxynitrite; Albumin; Thiols; Thiyl radical; Cysteine; Spin-trapping

1. Introduction

A variety of cell types including endothelial cells [1,2], macrophages [3,4], and neurons [5,6] produce superoxide and nitric oxide. These species are free radical intermediates which react with each other in aqueous solution at an almost diffusion-controlled rate ($k = 6.7 \times 10^9$ $M^{-1} \cdot s^{-1}$) [7] to yield the peroxynitrite anion (Eqn. 1). Therefore, peroxynitrite production by cells is expected to occur and this has already been demonstrated in the case of macrophages [8], neutrophils [9] and endothelial cells [10]. Peroxynitrite and its protonated form, peroxynitrous acid (Eqn. 2; $pK_a = 6.8$) [11], are short-lived intermediates at physiological pH ($t_{1/2} < 1$ s) [11,12] and behave as potent oxidants which are able to oxidize biomolecules such as deoxyribose [12], lipids [13], methionine [14] and thiols [11]. As a consequence, peroxynitrite is being increasingly recognized as a reactive intermediate that may participate in many injury processes associated with oxidative biological damage [15,16].

$$O_2^{\bullet} + {}^{\bullet}NO \rightarrow ONOO^- \qquad \text{(Eqn. 1)}$$

$$ONOO^- + H^+ \rightleftarrows ONOOH \qquad \text{(Eqn. 2)}$$

Recently we have demonstrated, by using EPR-spin-trapping techniques, that both peroxynitrite and peroxynitrous acid can trigger free radical production from nearby molecules in a metal-independent pathway [17]. The demonstration that low molecular thiols such as glutathione and cysteine are oxidized to the corresponding thiyl free radical intermediates [17] was particularly important since these compounds are part of one of the most important defense mechanism against biological oxidative damage [18]. Considering that peroxynitrite is also able to oxidize the single cysteine residue of BSA [11], it became important to examine whether this process results in the formation of the corresponding proteinthiyl free radical. We now report that indeed, peroxynitrite-mediated oxidation of BSA leads to the proteincysteinyl radical as ascertained by spin-trapping experiments with both DMPO and PBN. This is the first demonstration that peroxynitrite can oxidize high-molecular-weight biomolecules to free radical intermediates.

2. Materials and methods

2.1. Chemicals and biochemicals

BSA (fraction V), cysteine, DMPO, PBN, Chelex-100, urea, and *N*-ethyl maleimide were from Sigma Chemical Company (St. Louis, MO). DMPO was purified by charcoal filtration [19], and PBN and urea were recrystallized from hexane [20] and ethanol–H_2O (70:1, v/v), respectively. Peroxynitrite was synthesized, purified and kept frozen as previously described [11–13]. Stock solutions of peroxynitrite were prepared in 0.1 M NaOH and the concentrations were determined by absorbance at 302 nm ($\varepsilon = 1670$ $M^{-1} \cdot cm^{-1}$) [11–13]. The sulfhydryl group of BSA was blocked by reaction with *N*-ethyl maleimide as previously described [11,21]. All solutions were prepared using distilled water treated with a Millipore Milli-Q system .The phosphate buffer was pretreated with Chelex-100 to remove transition metal ion contamination.

2.2. Electron paramagnetic resonance spectroscopy

EPR spectra were recorded at room temperature on a Bruker ER 200 D-SRC spectrometer. The reaction mixtures (500 μl, final volume) at room temperature were transferred to flat cells immediately after peroxynitrite addition and the spectra usually recorded after 1 min incuba-

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Abbreviations: BSA, bovine serum albumin; EPR, electron paramagnetic resonance; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; PBN, α-phenyl-*N*-*tert*-butyl nitrone.

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tion. Due to the short half-life of peroxynitrite at pH 7.5 [11,12] the reactions were completed in less than 10 s. The scanning time was not critical, however, because the EPR signal intensities were maximum immediately after peroxynitrite addition and did not decay significantly over 1 h time frame.

3. Results

Reaction of BSA (1.0 mM) with peroxynitrite (1.0 mM) in the presence of the spin-trap PBN (50 mM) in phosphate buffer (100 mM), pH 7.5, led to the detection of an EPR spectrum characteristic of a strongly immobilized nitroxide (Fig. 1A), indicating that the trapped radical is constrained within the protein. Indeed, the detected anisotropic spectrum (a^N_{ZZ} = 3.05 mT) (Fig. 1A) is identical to the one obtained during the oxidation of BSA by Ce(IV) which was ascribed to the PBN-protein-cysteinyl radical adduct [21]; it is also very similar to other PBN-protein thiyl adduct spectra reported in the literature such as those derived from myosin (a^N_{ZZ} = 3.25 mT) [21] and hemoglobin (a^N_{ZZ} = 3.08 mT) [22]. Further evidence that peroxynitrite was oxidizing the single cysteine residue in BSA to the corresponding thiyl radical was obtained first by reacting peroxynitrite with BSA whose thiol residue was blocked by pre-treatment with *N*-ethyl-maleimide. Under these conditions, the formation of the PBN-protein-cysteinyl radical adduct was almost completely inhibited and no other EPR signals were evident (Fig. 1B). Next, another spin-trap, DMPO, was used to trap the radical formed during peroxynitrite-mediated oxidation of BSA. Again, the EPR spectrum obtained has the dominant triplet pattern expected for a slowly tumbling nitroxide (Fig. 2). In this case, however, the a^H_β splitting can be seen, particularly in the central region of the spectrum since the a^H_β doublet splitting value for DMPO adducts is typically much higher than those of PBN adducts [22,23]. The easily measured EPR parameter of the DMPO radical adduct obtained (a^H_β = 1.6 mT) (Fig. 2) is also consistent with the trapping of the BSA-cysteinyl radical [24]. Furthermore, the DMPO radical adduct yield was strongly inhibited in experiments using sulfhydryl-blocked BSA (Fig. 2B).

The yield of the immobilized PBN-protein-cysteinyl radical adduct obtained during peroxynitrite-mediated oxidation of BSA was dependent on the pH of the incu-

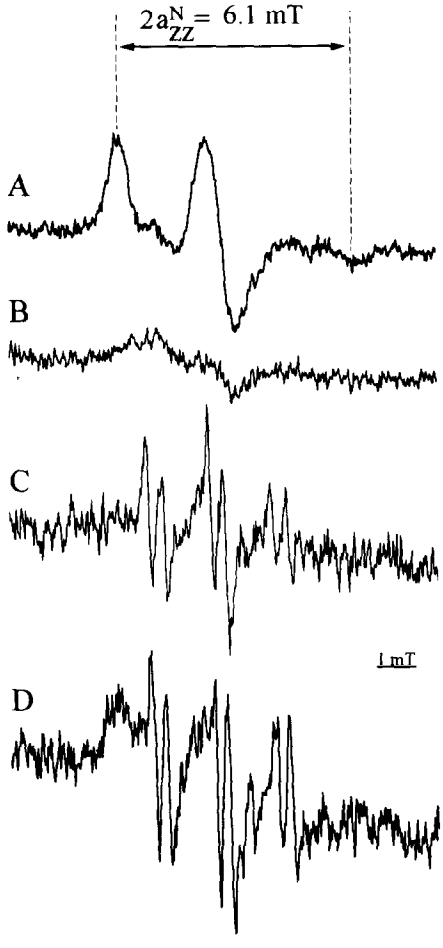

Fig. 1. EPR spectra of PBN radical adducts obtained during peroxynitrite-mediated oxidation of BSA. The spectra were obtained after 1 min of incubation at room temperature of 1.0 mM BSA, 50 mM PBN, and 1.0 mM peroxynitrite in 0.1 M phosphate buffer. (A) Native BSA at pH 7.5. (B) BSA pretreated with *N*-ethylmaleimide at pH 7.5. (C) Untreated BSA at pH 11.6. (D) The spectrum resulting from the addition of urea (5 M, final concentration) to the mixture in (A). Instrumental conditions: microwave power, 20 mW; modulation amplitude: 0.25 mT (A,B,D), 0.1 mT (C); time constant, 0.5 s; rate 0.04 mT/s; gain: 10×10^5 (A), 1.25×10^6 (B), 2.5×10^6 (C), 3.2×10^6 (D).

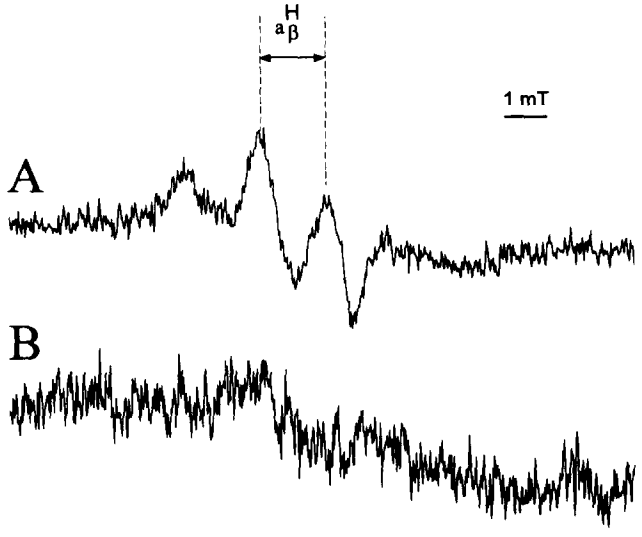

Fig. 2. EPR spectra of DMPO radical adducts obtained during peroxynitrite-mediated oxidation of BSA. The spectra were obtained after 1 min of incubation at room temperature of 1.0 mM BSA, 80 mM DMPO, and 0.8 mM peroxynitrite in 0.1 M phosphate buffer, pH 7.5. (A) Native BSA. (B) BSA pretreated with *N*-ethylmaleimide. Instrumental conditions: 30 mW; modulation amplitude: 0.25 mT; time constant: 0.5 s; rate: 0.04 mT/s; gain: 1.25×10^6 (A), $3.2\ 10^6$ (B).

bation mixture (Fig. 3). At pH 6.0 the adduct was barely detectable, but its yield increased as the pH was increased up to 9.0, at which value protein denaturation becomes important [25] leading to an apparent decrease in the adduct concentration (Fig. 3). This occurs because BSA denaturation led to changes in the EPR spectrum which became a composite of two adducts, the immobilized adduct (Fig. 1A) and the almost freely rotating PBN adduct whose spectrum is shown in Fig. 1C. This species, which became the dominant species at pH 11.5, appears to be the denatured PBN-albumin cysteinyl radical adduct since it presents some degree of immobilization and has hyperfine splitting constants (a_{ZZ}^{N} = 1.6 mT; a_{β}^{H} = 0.36 mT) (Fig. 1C) which are consistent with a PBN-thiyl radical adduct [26]. In agreement, the almost isotropic signal was also obtained by denaturation of the immobilized adduct resulting from the addition of either NaOH to reach pH 11.5 (not shown) or 5 M urea (Fig. 1D) to the incubation mixture shown in Fig. 1A.

4. Discussion

We have previously reported that peroxynitrite oxidizes BSA [11] and herein we demonstrate that this process is mediated by one-electron transfer with formation of a protein-cysteinyl radical (Figs. 1, 2) and probably nitrogen dioxide [17]. The latter species can potentially oxidize the sulfhydryl group of another BSA molecule [17,27,28] in which case, it would be partially responsible for the protein-thiyl radical formation. This radical was detected and identified by spin-trapping experiments in the presence of either PBN (Fig. 1) or DMPO (Fig. 2). In both instances, the EPR parameters of the trapped radical were in agreement those reported for BSA-cysteinyl radical adducts (see section 3). Also, no radical adducts could be detected in experiments using BSA whose thiol residue was blocked (Figs. 1B, 2B). In the absence of a free sulfhydryl group to react with peroxynitrite, we would expect its protonation to peroxynitrous acid and the consequent hydroxyl radical-like reactivity [15,17] leading to a protein-derived peroxyl radical, as previously reported during the oxidation of blocked BSA by iron(II)-EDTA-hydrogen peroxide [24]. Excess peroxynitrite, however, leads to decomposition of DMPO spin adducts to EPR-silent products [17], a process that could explain the non-detection of radical adducts in the reaction of blocked BSA with peroxynitrite (Figs. 1B, 2B).

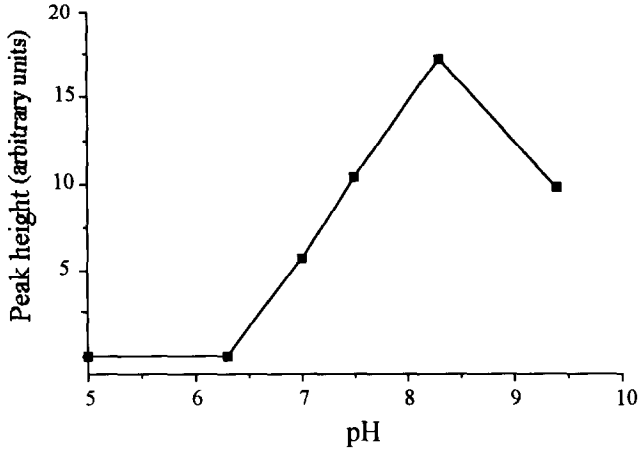

Fig. 3. Effect of pH on the yield of PBN-BSA-cysteinyl radical adduct produced during peroxynitrite-mediated oxidation of BSA. The spectra were obtained after 1 min. of incubation at room temperature of 1.0 mM BSA, 50 mM PBN, and 1.0 mM peroxynitrite in 0.1 M phosphate buffer, whose pH was varied. The instrumental conditions were the same as those in Fig. 1A except for the gain which was changed depending on radical adduct yield. The measured peak heights are the mean values obtained from two experiments, and correspond to the central peak of the PBN-BSA-cysteinyl radical adduct.

The yield of the immobilized PBN-BSA radical adduct increased with the pH to the value at which protein denaturation becomes a relevant process (Figs. 1, 3). This result is in agreement with our previous studies demonstrating that the main intermediate responsible for BSA oxidation is the peroxynitrite anion [11] rather than peroxynitrous acid or its energized intermediate with hydroxyl-like reactivity [15].

Peroxynitrite-mediated oxidation of both low- [17] and high-molecular-weight thiols to thiyl radical intermediates (this report) may have several biological consequences since these radicals are reactive species which can initiate free radical chain reactions [29]. In the case of low-molecular-weight thiols such as glutathione it has been proposed that the thiyl radical can be detoxified, to some extent, by superoxide dismutase through the formation of oxidized glutathionyl radical anion which reacts with oxygen producing superoxide anion [30,31]. Another protective mechanism may involve the recently characterized thiol-specific antioxidant enzyme [32,33]. The biological fates of protein-thiyl radicals have been less analyzed in the literature. Under low oxygen tensions, these radicals could lead to protein aggregation through the formation of inter and/or intramolecular disulfide bonds [34,35]. On the other hand, further oxidation of thiyl radicals to sulfinic or sulfonic acids can lead to partial or total loss of the biological activity of proteins [11,36,37]. Albumin oxidation by peroxynitrite may prevent attack of more critical plasma proteins such as enzymes and antibodies. In this sense, our results support and further expand the concept of albumin being an important antioxidant in plasma and extravascular space [38].

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