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Original Contribution

Production of lysozyme and lysozyme-superoxide dismutase dimers bound by a ditryptophan cross-link in carbonate radical-treated lysozyme

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ABSTRACT

Despite extensive investigation of the irreversible oxidations undergone by proteins in vitro and in vivo, the products formed from the oxidation of Trp residues remain incompletely understood. Recently, we characterized a ditryptophan cross-link produced by the recombination of hSOD1-tryptophanyl radicals generated from attack of the carbonate radical produced during the bicarbonate-dependent peroxidase activity of the enzyme. Here, we examine whether the ditryptophan cross-link is produced by the attack of the carbonate radical on proteins other than hSOD1. To this end, we treated hen egg white lysozyme with photolytically and enzymatically generated carbonate radical. The radical yields were estimated and the lysozyme modifications were analyzed by SDS-PAGE, western blot, enzymatic activity and MS/MS analysis. Lysozyme oxidation by both systems resulted in its inactivation and dimerization. Lysozyme treated with the photolytic system presented monomers oxidized to hydroxy-tryptophan at Trp^{28} and Trp^{123} and *N*-formylkynurenine at Trp^{28}, Trp^{62} and Trp^{123}. Lysozyme treated with the enzymatic system rendered monomers oxidized to *N*-formylkynurenine at Trp^{28}. The dimers were characterized as lysozyme-Trp^{28}-Trp^{28}-lysozyme and lysozyme-Trp^{28}-Trp^{32}-hSOD1. The results further demonstrate that the carbonate radical is prone to causing biomolecule cross-linking and hence, may be a relevant player in pathological mechanisms. The possibility of exploring the formation of ditryptophan cross-links as a carbonate radical biomarker is discussed.

1. Introduction

Oxidative post-translational modification of proteins is being extensively investigated because, among other reasons, proteins are major targets of radicals and oxidants under physiological conditions [1, 2]. The amino acid residues most susceptible to oxidation are the sulfur-containing residues Cys and Met and the aromatic residues His, Phe, Tyr, and Trp. Relevantly, the reversible oxidation of protein-Cys residues is emerging as a fundamental cell regulatory mechanism [3–6]. In contrast, the irreversible oxidation of protein residues can result in loss of protein function, protein fragmentation, protein aggregation and/or altered protein turn-over, leading to cell and tissue dysfunction and to various human pathologies [1, 2, 7–10].

Despite extensive investigation of the irreversible oxidations undergone by proteins in vitro and in vivo, the products formed from the oxidation of Trp residues remain partially characterized [11–17]. Trp residues possess multiple sites of attack by oxidants and radicals, giving rise to many oxidized products, the most frequently characterized of which have been protein-tryptophanyl radicals and protein-bound products, such as tryptophan hydroperoxide, hydroxy-tryptophan, *N*-formylkynurenine and kynurenine [13–15, 18–20] (Fig. 1). Additionally, Trp residues are the strongest UV chromophore in proteins, rendering diverse products through a rich photochemistry involving both protein-tryptophanyl radical- and singlet oxygen-mediated pathways (Fig. 1) [21–23]. Moreover, the low frequency of Trp in proteins (approximately 1%) compared with other aromatic residues, such as Tyr (approximately 3–4%), most likely contributes to the limited

Abbreviations: CoC, carbonatotetrammine cobalt(III) complex; DMPO, 5,5-dimethylpyrroline-N-oxide; DTPA, diethylenetriamine-N,N,N′,N′-pentaacetic acid; hSOD1, human superoxide dismutase 1; Lyso, lysozyme; MNP, 2-methyl-2-nitrosopropane

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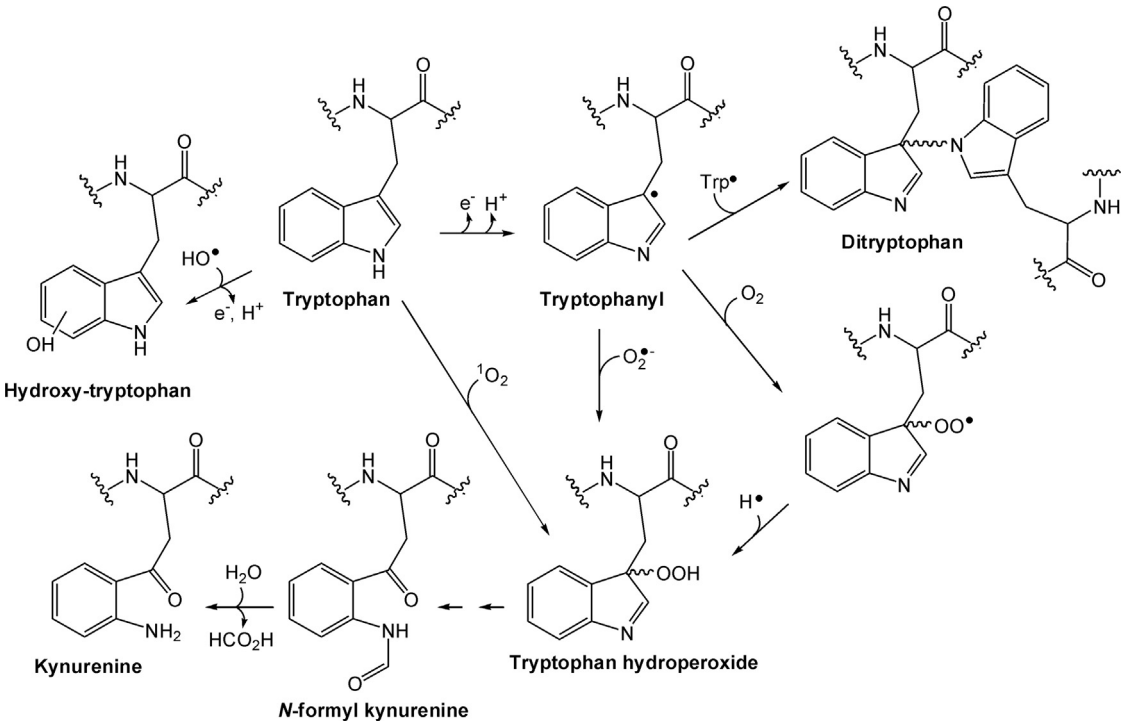

Fig. 1. Some of the characterized products of the oxidation of protein-tryptophan residues [13–15, 18–20].

characterization of the oxidation products of protein-Trp residues.

Nevertheless, protein-Trp residues have a unique potential to interact with other proteins and cellular structures [16, 24, 25], and their oxidation may have profound physiological consequences. For instance, we recently characterized a novel ditryptophan cross-link caused by the recombination of human SOD1-tryptophanyl radicals, which are produced by attack of the carbonate radical generated during the bicarbonate-dependent peroxidase activity of the enzyme (Fig. 1) [13]. In addition, we demonstrated that this ditryptophan cross-link contributes to triggering the non-amyloid aggregation of human SOD1 (hSOD1), a process that may be involved in the pathogenic mechanism of amyotrophic lateral sclerosis [26].

In this context, we considered it relevant to examine whether this ditryptophan cross-link is produced by the attack of the carbonate radical on proteins other than hSOD1. Among the radicals produced in vivo, the carbonate radical ($E^{o\prime}=1.78$ V, pH 7.0) is only less oxidizing than the hydroxyl radical ($E^{o\prime}=2.3$ V, pH 7.0) [27]. In contrast with the latter, the carbonate radical does not add to double bonds, acting by both electron transfer and hydrogen abstraction mechanisms to produce radicals from the oxidized targets. The inability of the carbonate radical to produce stable adducts has hampered its detection under physiological conditions [28]. Here, we treated hen egg white lysozyme with photolytically and enzymatically generated carbonate radical and characterized the major formed products. Lysozyme was selected as the target of the radical because it is a small (129 amino residues), highly stable enzyme with a well-characterized three-dimensional structure [29]. In addition, lysozyme possesses 6 Trp residues (Trp^{28}, Trp^{62}, Trp^{63}, Trp^{108}, Trp^{111}, Trp^{123}) and 3 Tyr residues (Tyr^{20}, Tyr^{23}, Tyr^{53}). Our results show that lysozyme-Trp^{28}-Trp^{28}-lysozyme and lysozyme-Trp^{28}-Trp^{32}-hSOD1 dimers are produced in lysozyme treated with photolytically and enzymatically generated carbonate radicals. The possibility of exploring ditryptophan cross-links as biomarkers of the carbonate radical is discussed.

2. Experimental procedures

2.1. Materials

All chemicals were purchased from Sigma-Aldrich, Merck or Fisher and were analytical grade or better. The perchlorate salt of carbonatotetrammine cobalt(III) complex ($[Co(NH_3)_4CO_3]ClO_4$) [18] was a generous gift from Dr. Vitor F. Ferreira (Universidade Federal Fluminense). Bovine superoxide dismutase (bSOD1), sheep anti-SOD1 (574597), rabbit anti-lysozyme (AB391), goat anti-rabbit (AO545) and goat anti-sheep (402100) antibodies were purchased from Calbiochem. Mass spectrometry grade trypsin gold was purchased from Promega (Madison, WI, USA). Nano HPLC Column ZORBAX 300SB-C18, lock mass Calibrant external and Tune mix calibrant low concentration were all obtained from Agilent Technologies. Filters (0.22 μm) were obtained from Millipore. The chemiluminescent substrate for western blot analysis was obtained from Thermo Scientific. Desalting columns (PD-10) were acquired from GE Healthcare. Bovine liver catalase was purchased from Boehringer and its activity was determined according to Sigma Aldrich's instructions. All solutions and buffers were prepared using distilled water purified in a Millipore Milli-Q system and treated with Chelex-100® resin (Sigma-Aldrich) to remove trace amounts of metal ion contaminants before use.

2.2. Expression and purification of hSOD1

Plasmids (pET-3d) encoding the enzyme $hSOD1^{WT}$ were kindly provided by Dr. J. S. Beckman from the Linus Pauling Institute. The plasmids were expressed in Escherichia coli strain BL21 (DE3) pLysS, and the enzyme was purified and analyzed as previously described [30]. Typically, recombinant hSOD1 contained approximately 0.7 copper and 0.7 zinc ions per monomer and a specific dismutase activity of 3900 ± 400 U/mg (mg of protein normalized by the copper content). Here, the concentrations of hSOD1 are always expressed as the dimer.