Peroxynitrite-induced nitration of tyrosine-34 does not inhibit *Escherichia coli* iron superoxide dismutase

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The peroxynitrite anion is a potent oxidizing agent, formed by the diffusion-limited combination of nitric oxide and superoxide, and its production under physiological conditions is associated with the pathologies of a number of inflammatory and neurodegenerative diseases. Nitration of *Escherichia coli* iron superoxide dismutase (Fe-SOD) by peroxynitrite was investigated, and demonstrated by spectral changes and electrospray mass spectroscopic analysis. HPLC and mass studies of the tryptic digests of the mono-nitrated Fe-SOD indicated that tyrosine-34 was the residue most susceptible to nitration by peroxynitrite. Exclusive nitration of this residue occurred when Fe-SOD was exposed to a cumulative dose of 0.4 mM peroxynitrite. Unlike

with human Mn-SOD, this single modification did not inactivate *E. coli* Fe-SOD at pH 7.4. When Fe-SOD was exposed to higher concentrations of peroxynitrite (7 mM), eight tyrosine residues per subunit of the protein, of the nine available, were nitrated without loss of catalytic activity of the enzyme. The pK_a of nitrated tyrosine-34 was determined to be 7.95 ± 0.15 , indicating that the peroxynitrite-modified enzyme appreciably maintains its protonation state under physiological conditions.

Key words: electrospray mass spectrometry, 3-nitrotyrosine, pK_a , protein digestion.

INTRODUCTION

The superoxide dismutases (SODs) are key enzymes in the metabolism of oxygen free radicals, whose primary activity is the dismutation of the superoxide radical anion ($O_2^{\cdot-}$) into hydrogen peroxide and molecular oxygen [1]. The substrate of SODs, $O_2^{\cdot-}$, reacts rapidly with $NO^{\cdot}$ at almost diffusion-limited rates to form the peroxynitrite anion $ONOO^-$. Peroxynitrite is a powerful oxidant that reacts with a variety of biomolecules, and is capable of nitrating tyrosine or tryptophan residues in proteins and oxidizing metalloenzymes, DNA or lipids [2]. Tyrosine nitration induced by peroxynitrite to yield 3-nitrotyrosine is a well established reaction, and has been reported to occur in some proteins, including Mn-SOD [3,4] and Cu,Zn-SOD [5], cytochrome P450 [6], cytochrome *c* [7] and ribonucleotide reductase [8]. The mechanism by which these reactions occur involves the homolysis of peroxynitrous acid, leading to the formation of nitrogen dioxide and hydroxyl radicals [9,10].

In some inflammatory conditions where a large amount of nitric oxide is generated from the increased expression and activity of NO synthases, the rate of formation of peroxynitrite from $NO^{\cdot}$ and $O_2^{\cdot-}$ can outcompete the rate of dismutation of $O_2^{\cdot-}$ by SODs. Consequently, the peroxynitrite-induced nitration of tyrosine residues of proteins, including SODs, represents a marker of peroxynitrite-mediated tissue injury in inflammatory conditions associated with the pathology of a wide range of disorders, such as neurodegenerative diseases and bacterial or viral infections. It has been reported that bovine erythrocyte Cu/Zn-SOD was nitrated by peroxynitrite without significant loss of enzymic activity [5,11]. The only apparent modification was the nitration of tyrosine-108. Under the same conditions,

Escherichia coli Mn-SOD and Fe-SOD were shown to be much more sensitive to peroxynitrite-induced nitration [5].

MacMillan-Crow et al. [3] reported that peroxynitrite inactivated human Mn-SOD by inducing the formation of both nitrotyrosine and dityrosine. Analysis of the proteolysed protein revealed that three residues (Tyr-45, Tyr-193 and Tyr-34) of the total of nine tyrosine residues in each subunit were nitrated, with Tyr-34 being the most susceptible residue. On the other hand, Yamakura et al. [4] showed that the single modification in the peroxynitrite-inactivated human Mn-SOD is the nitration of Tyr-34. The present study was undertaken to examine further the modification of Fe-SOD by peroxynitrite. UV spectroscopy, electrospray ionization MS (ESI-MS) and peptide analysis, after tryptic digestion, were used to analyse the peroxynitrite-induced nitration of *E. coli* Fe-SOD. We report that Tyr-34 of *E. coli* Fe-SOD is the residue that is most susceptible to nitration by peroxynitrite, and that exclusive nitration of this residue does not inactivate the enzyme.

EXPERIMENTAL

Materials

Peroxynitrite was synthesized in a quenched-flow reactor from sodium nitrite and acidified hydrogen peroxide as described previously, and was used without further purification [12]. Solutions of sodium peroxynitrite were freshly prepared before each experiment. Concentrations of these solutions (typically 80–120 mM in 1.5 M NaOH) were determined at 302 nm using the molar absorption coefficient $\epsilon_{302} = 1670 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [13]. *E. coli* Fe-SOD, bovine Tos-Phe-CH₂Cl ('TPCK')-treated trypsin, authentic 3-nitrotyrosine and all other common chemicals were

Abbreviations used: SOD, superoxide dismutase; NBT, Nitro Blue Tetrazolium; PMS, phenazine methosulphate; ESI-MS, electrospray ionization mass spectrometry.

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purchased from Sigma–Aldrich Co. Water was from a Milli-Q reagent water system (Millipore). UV spectra were recorded on a Cary Varian 1E spectrophotometer. ESI-MS was performed on an LC/MS/MS API 365 mass spectrometer (PerkinElmer SCIEX) equipped with series 200 pumps (PerkinElmer) and a 785A UV/visible detector (PerkinElmer).

Graphics were generated in Microcal Origin[®] 5.0. The molecular masses of proteins and peptides were calculated using the Biopolymer Calculator program (<http://paris.chem.yale.edu/>). Structural comparisons of Mn-SOD and Fe-SOD were performed using the Swiss-PdbViewer 3.7 program (Glaxo Wellcome Experimental Research; <http://www.expasy.ch/spdbv/>).

Peroxynitrite treatment of *E. coli* Fe-SOD

Nitration was performed by the bolus addition (1 or 2 μ l) of a solution of peroxynitrite (115 mM) to a solution of Fe-SOD (15 μ M) in 50 mM sodium phosphate buffer (pH 7.4) containing 1 mM EDTA at room temperature. After each addition of peroxynitrite, the solution was incubated for 1 min and the spectral modifications were monitored spectrophotometrically. The pH of all assays was verified at the end of experiments to ensure that conditions were not changed significantly by additions of alkaline peroxynitrite solution.

ESI-MS analysis

Both native and peroxynitrite-modified Fe-SOD were dialysed extensively against water before MS studies. Analytical samples were prepared by mixing native (30 μ M) or modified (15 μ M) SOD in water (25 μ l) with a mixture of 0.1% formic acid in water/acetonitrile (1:1, v/v; 25 μ l).

Trypsin digestion and peptide analysis

Native Fe-SOD (30 μ M) or peroxynitrite-treated Fe-SOD (15 μ M) was incubated with trypsin (1:50, w/w) for 16 h at 37 °C in 100 mM ammonium carbonate buffer (pH 8.0). Peptides were analysed by using an LC/MS/MS system on an octadecyl silica gel reverse-phase column (Hypersil 250 mm $\times$ 3 mm; 5 μ m pore size PEP100) with a linear gradient from 100% A to 100% B over 60 min (A: 20% acetonitrile, 80% water and 0.1% TFA; B: 80% acetonitrile, 20% water and 0.1% TFA).

Determination of the pK_a of mono-nitrated Fe-SOD

After exposure to peroxynitrite, mono-nitrated Fe-SOD (30 μ M) was dialysed against 1 mM NaOH. To this solution, sequential volumes (approx. 10 μ l) of 0.01 M HCl were added. After each addition, the pH of the mixture was measured using a micro-electrode, and the spectral modifications at 355 nm and 430 nm were monitored.

SOD assay

Before each assay, peroxynitrite-modified SODs were dialysed extensively for 2 days against water, and the concentrations of native and peroxynitrite-modified SODs were verified by the Bradford method [13a]. Activities were determined by measuring the superoxide-mediated reduction of Nitro Blue Tetrazolium (NBT) by an NADH/phenazine methosulphate (PMS) system [14] in the presence of EDTA in sodium phosphate buffers at two different pH values. Conditions were as follows: 50 μ M NBT, 100 μ M NADH, 5 μ M PMS and 1 mM EDTA in a 50 mM phosphate buffer at pH 7.4 or in a 100 mM phosphate buffer at pH 9.0, in a final volume of 500 μ l at 25 °C. In a typical assay,

the rate of NBT reduction was inhibited by 80% at a SOD concentration of 150 nM at pH 7.4, and by 75% at a SOD concentration of 300 nM at pH 9.0. The relative activity (RA) of SOD was calculated as:

$$RA (\%) = \{1 - [(r_{me} - r_{ne}) / (r_o - r_{ne})]\} \times 100$$

where r_{me} , r_{ne} and r_o are the initial rates of NBT reduction with modified SOD, native SOD and in the absence of SOD respectively. Initial rates correspond to the slope of absorbance (at 620 nm) against time from 0 to 2 min.

RESULTS

Spectroscopic and MS analysis

Successive additions of a peroxynitrite solution (approx. 100 mM) to a solution of *E. coli* Fe-SOD in phosphate buffer at pH 7.4 resulted in the formation of nitrotyrosine, as demonstrated by a spectral change in the visible region with the appearance of a band at 420 nm (Figure 1). When exposed to a cumulative dose of 0.4 mM peroxynitrite, only one tyrosine residue per subunit of enzyme was nitrated, as indicated by the value of the absorbance at 420 nm ($\epsilon = 3110 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 7.4). This was confirmed by ESI-MS, with a deconvoluted mass of 21179 Da (native protein 21134 Da) corresponding to the addition of one nitro group per protein subunit (Figure 2). Both unmodified SOD and SOD with an increase in molecular mass of 90 Da (21224 Da), consistent with the addition of two nitro groups per subunit, were also present as a small fraction of the peroxynitrite-exposed protein (Figure 2B). The nitration yield reached a maximum when the cumulative concentration of peroxynitrite was greater than 7 mM, corresponding to the nitration of eight of the nine tyrosines available per subunit (Figure 1). The spectral data show that peroxynitrite caused a concentration-dependent increase in tyrosine nitration without time-dependence, with an apparent linear relationship with the cumulative concentration of peroxynitrite up to 4 mM

Figure 1 Spectral study of the nitration of tyrosine residues in *E. coli* Fe-SOD

Shown is cumulative peroxynitrite concentration against the number of nitrotyrosine residues per enzyme subunit. A solution of peroxynitrite (115 mM) was added sequentially to a solution of Fe-SOD (15 μ M) in phosphate buffer (pH 7.4) at 25 °C, and the absorbance spectrum was recorded. Total nitrotyrosine content per enzyme subunit was quantified by absorbance at 420 nm using $\epsilon = 3110 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (measured using authentic 3-nitrotyrosine), and the molar concentration of the enzyme was determined using the Bradford assay [13a]. The absorbance spectra after each addition of peroxynitrite are represented in the inset.

Figure 2 ESI-MS study of tyrosine nitration in *E. coli* Fe-SOD

(A) Native enzyme; (B) enzyme exposed to a cumulative dose of 0.4 mM peroxynitrite. The deconvoluted mass spectra obtained from the original spectra are displayed in the inset. Samples were prepared and analysed as described in the Experimental section.

(Figure 1). The last tyrosine residue to be nitrated required a cumulative dose of approx. 2.5 mM peroxynitrite (from 4 to 6.5 mM; Figure 1), suggesting that this eighth residue presents a lower susceptibility to nitration.

Identification of the nitrated tyrosine residue

To identify the nitration site, *E. coli* Fe-SOD was incubated with 0.4 mM peroxynitrite and analysed by ESI-MS to confirm the mono-nitration. The peroxynitrite-exposed protein was then digested with trypsin, which cleaves after lysine and arginine residues. Tryptic digests were analysed by liquid chromatography/MS using absorbance at 278 nm to monitor the eluted peptide fragments (Table 1). With our chromatographic conditions, only 11 peptides of the 13 expected (A–M; Table 1) re eluted for both native and modified SOD. Of the 11 peptides eluted for the native protein (peptides A–E, G–K and M; Table 1), only peptide C (fragment His³⁰–Lys⁴³; MH⁺ 1695.4 Da) was nitrated, being converted into peptide C' (MH⁺ 1741.5 Da) in the peroxynitrite-modified SOD with an increase in molecular mass corresponding to an additional nitro group (Figure 3). The sequence of this peptide C (His³⁰–His–Gln–Thr–Tyr–Val–Thr–Asn–Leu–Asn–Asn–Leu–Ile–Lys⁴³) included only one tyrosine residue, i.e. Tyr-34. The retention time of fragment C' (approx. 21 min) was greater than that of peptide C (17 min) on reverse-phase HPLC, consistent with the higher hydrophobicity of C' due to the introduction of a nitro group. All other eluted peptides (A, B, D, E, G–K and M) were recovered in the modified SOD with similar retention times and molecular masses as in the native protein (Table 1). Peptides F (3624.9 Da) and L (2547.9 Da) were not eluted by the experimental chromatographic conditions, but were detected without chromatographic separation by direct-infusion ESI-MS analysis of the mixtures of the tryptic digests of native or modified SOD. In this case, the spectra of the doubly charged ions of F (1813.0 Da) and L (1274.8 Da) were obtained (Table 1). These data clearly indicate that nitration of Tyr-34 is the only modification when *E. coli* Fe-SOD is exposed to 0.4 mM peroxynitrite.

Activity of nitrated SOD

The catalytic properties of native and nitrated SOD were evaluated by a standard SOD assay based on the superoxide-induced reduction of NBT by a mixture of NADH and PMS [14].

Table 1 Identification of the structural modification induced by peroxynitrite in *E. coli* Fe-SOD using ESI-MS

MW is the calculated molecular mass of the peptide fragments. R_T is the HPLC retention time on a reverse-phase column of trypsin-digested peptide fragments from native and peroxynitrite-modified SODs. m/z values are given for the singly ($z = 1$), doubly ($z = 2$) and triply ($z = 3$) charged molecular ions of peptide fragments, determined by ESI-MS. In peptide C', Y* denotes 3-nitrotyrosine. ne, not eluted.

Peptide	Sequence	MW (Da)	R_T (min)		m/z					
			Native	Nitrated	$z = 1$		$z = 2$		$z = 3$	
					Native	Nitrated	Native	Nitrated	Native	Nitrated
A	S ¹ FELPALPYAK ¹¹	1235.5	23.73	23.66	1236.2	1236.2	618.8	618.8	—	—
B	D ¹² ALAPHISAETIEYHYGK ²⁹	2015.2	14.52	14.59	2016.1	2016.6	1008.5	1008.5	672.9	673.4
C	H ³⁰ HQTYVTNLNNLIK ⁴³	1694.9	17.03	—	1695.4	—	848.5	—	565.8	—
C'	H ³⁰ HQTY*VTNLNNLIK ⁴³	1739.9	—	20.97	—	1741.5	—	871.0	—	581.3
D	G ⁴⁴ TAFFGK ⁵⁰	708.8	4.16	4.24	709.9	709.9	—	—	—	—
E	S ⁵¹ LEEIR ⁵⁷	859.0	14.52	14.59	860.0	860.0	430.7	430.2	—	—
F	S ⁵⁸ SEGGVFNNAAQVWNHTFYWNCLAPNAGGPTGK ⁹¹	3624.9	ne	ne	—	—	1813.0	1813.0	—	—
G	V ⁹² AEAIASFGSADF ¹⁰⁷	1630.8	26.61	26.31	1631.4	1631.4	816.4	816.4	544.8	544.8
H	A ¹⁰⁸ QFTDAIK ¹¹⁶	964.1	6.15	6.41	965.0	965.0	483.3	483.3	—	—
I	N ¹¹⁷ FGSGWTWLVK ¹²⁷	1294.5	30.62	30.37	1295.2	1295.2	648.4	648.4	—	—
J	N ¹²⁸ SDGK ¹³²	519.5	2.73	2.73	520.3	520.3	—	—	—	—
K	L ¹³³ AIIVSTSNAGTPLTTDPTLLTVDVWEHAYYIDYR ¹⁶⁷	3868.3	41.38	41.72	—	—	1935.1	1935.1	1290.7	1290.7
L	N ¹⁶⁸ ARPGYLEHFWALVNWFEVAK ¹⁸⁸	2547.9	ne	ne	—	—	1274.8	1274.8	—	—
M	N ¹⁸⁹ LAA ¹⁹²	387.4	4.50	4.46	388.2	388.2	—	—	—	—

Figure 3 ESI-MS spectra of peptides **C** and **C'**

The sequence of peptide **C** is His³⁰-His-Gln-Thr-Tyr-Val-Thr-Asn-Leu-Asn-Asn-Leu-Ile-Lys⁴³ (calculated mass: 1694.9), and that of peptide **C'** is His³⁰-His-Gln-Thr-[3-nitroTyr]-Val-Thr-Asn-Leu-Asn-Asn-Leu-Ile-Lys⁴³ (calculated mass 1739.9). Masses 848.5 and 871.0 correspond to the doubly-charged molecular ions of **C** and **C'** respectively, and 565.8 and 581.3 correspond to the triply-charged peptides.

Figure 4 Determination of the pK_a of peroxynitrite-modified *E. coli* Fe-SOD

pK_a was estimated from changes in the molar absorption coefficient (ϵ) at 355 nm (■) and 430 nm (●) of a solution of Fe-SOD (30 μ M) exposed to 0.4 mM peroxynitrite as a function of pH.

This method has the advantage of measuring SOD activities at a range of pH values (> 7), as the rate constant for the reaction between superoxide and NBT is little affected by increasing pH [15]. The activity of the nitrated SOD was found to be pH-dependent. At pH 7.4, the enzymic activity of Fe-SOD was unaffected by treatment with up to 7 mM peroxynitrite and subsequent dialysis. At the same concentration of enzyme (verified by the Bradford assay [13a]), when the pH of the assay was changed from 7.4 to 9.0, the peroxynitrite-treated SOD was inactivated by 60% compared with the activity of the native enzyme.

Spectroscopic pK_a determination

From changes in absorbance at 355 and 430 nm as a function of pH, the apparent acid dissociation constant (pK_a) of authentic 3-nitrotyrosine was determined to be 7.15 ± 0.10 in aqueous solution (results not shown). Likewise, absorbances at these same wavelengths of the mono-nitrated Fe-SOD were found to be highly pH-dependent, consistent with the presence of a nitrotyrosine residue (Figure 4). The pH profile for the mono-nitrated Fe-SOD indicated a pK_a of 7.95 ± 0.15 corresponding to nitrated Tyr-34 (i.e. approx. 2 units lower than for the phenolic group of a tyrosine residue).

DISCUSSION

A number of previous studies have shown that protein modifications associated with nitrative stress, such as tyrosine nitration, are often associated with the loss of catalytic activity [3,4,6–8]. Some results indicate that nitration of a single tyrosine residue is sufficient to induce changes in the catalytic properties of proteins. In addition to nitrating tyrosine residues in proteins, peroxynitrite is also known to be a powerful thiol oxidant [16–18] and to induce dityrosine formation [19]. The data presented here indicate that Fe-SOD can undergo peroxynitrite-induced nitration of Tyr-34, and that this single modification does not affect the catalytic properties of the enzyme. Moreover, even when a maximum of eight tyrosine residues per SOD monomer were nitrated at a cumulative peroxynitrite concentration of 7 mM, the activity of the enzyme was not significantly affected at pH 7.4.

The factors that may influence the selectivity of protein tyrosine nitration have been investigated in studies of the nitration of model proteins [20]: exposure to solvent, the local environment and the presence of neighbouring negatively charged residues may be critical factors in nitration. Although partially exposed to solvent, Tyr-34 in Fe- or Mn-SOD does not fulfil these criteria, because it is located on an α -helical structure and is not in close proximity to a glutamate or aspartate residue. It has been hypothesized that the negatively charged peroxynitrite ion is attracted to the metal centre of SOD by the same electrostatic field responsible for the attraction of the natural substrate, superoxide anion [21–25]. This hypothesis of peroxynitrite attraction has been proposed by Beckman and colleagues [5] for Cu,Zn-SOD, and could explain the high sensitivity of Tyr-34 to peroxynitrite-induced nitration in both Mn- and Fe-SODs.

Tyr-34 is a highly conserved residue in all Mn- and Fe-SODs, being located at the vertex of the funnel conducting to the metal ion, and plays a major role by participating in a hydrogen-bonded network. Nitration of this residue may lead to major modifications near the active site of the protein that could explain the inactivation of human Mn-SOD. But, in view of the structural analogies between Fe-SOD and Mn-SOD, why then does nitration of Tyr-34 not inhibit the iron-dependent enzyme? An analysis of the structural identity of Mn- and Fe-SODs, based on known structures and sequences, shows that their secondary and tertiary structures are very similar [26]. One of the major differences between these two classes of SODs relates to the location of the residues that interact with the solvent molecule co-ordinated to the metal and Tyr-34. In human Mn-SOD, this residue (Gln-143) is located in the connecting loop between the third β -strand and an α -helix, whereas in *E. coli* Fe-SOD the interacting residue, Gln-69, is located on the second α -helix. Introduction of an additional nitro group into Tyr-34 may disrupt the interaction with this essential residue, which could provoke subtle changes in the local structural environment of the metal ion in the Mn-dependent enzyme.

A second possibility is that mono-nitrated Fe- and Mn-SODs differ in their intrinsic ionization state. The pK_a of mono-nitrated Fe-SOD has been determined to be 7.95, implying that the phenolic group of nitrated Tyr-34 keeps its protonation state at pH 7.4. When the pH of the SOD assay was changed from 7.4 to 9.0, i.e. a pH value higher than the pK_a , the activity of the modified enzyme decreased markedly, probably due to the presence of a negative charge that could repel the substrate (anion) and disrupt the hydrogen-bonding network.

Our results demonstrating that nitration of Tyr-34 does not affect the activity of *E. coli* Fe-SOD are consistent with previous studies on the role of this residue, showing that replacement of

Tyr-34 with Phe in *E. coli* Fe-SOD produced a mutant that retained nearly 40% of the activity of the wild type [27,28]. Surprisingly, a similar result was obtained with human Mn-SOD, for which both the wild type and a Y34F mutant had a k_{cat}/K_m value of approx. $10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, close to being diffusion-controlled [29]. The same mutation (Y34F) in *E. coli* Mn-SOD decreased the dismutase activity of the enzyme only slightly, implying that Tyr-34 is not essential in catalysis [30].

In conclusion, this work shows that, despite the similarities between Fe- and Mn-SODs, the catalytic properties of *E. coli* Fe-SOD are not affected when it is exposed to high concentrations of peroxynitrite under physiological conditions. The biological relevance of this observation has to be considered when evaluating the protective effect during some pathological conditions of SODs, which prevent the production of peroxynitrite by scavenging of superoxide before it reacts with nitric oxide.

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