Nitration of Tyrosine 92 Mediates the Activation of Rat Microsomal Glutathione S-Transferase by Peroxynitrite*

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There is increasing evidence that protein function can be modiﬁed by nitration of tyrosine residue(s), a reaction catalyzed by pro- teins with peroxidase activity, or that occurs by interaction with peroxynitrite, a highly reactive oxidant formed by the reaction of nitric oxide with superoxide. Although there are numerous reports describing loss of function after treatment of proteins with peroxynitrite, we recently demonstrated that the microsomal glutathione S-transferase 1 is activated rather than inactivated by peroxynitrite and suggested that this could be attributed to nitration of tyrosine residues rather than to other effects of peroxynitrite. In this report, the nitrated tyrosine residues of peroxynitrite-treated microsomal glutathione S-transferase 1 were characterized by mass spectrometry and their functional signiﬁcance determined. Of the seven tyrosine residues present in the protein, only those at positions 92 and 153 were nitrated after treatment with peroxynitrite. Three mutants (Y92F, Y153F, and Y92F, Y153F) were created using site-directed mutagenesis and expressed in LLC-PK1 cells. Treatment of the microsomal fractions of these cells with peroxynitrite resulted in an ~2-fold increase in enzyme activity in cells expressing the wild type microsomal glutathione S-transferase 1 or the Y153F mutant, whereas the enzyme activity of Y92F and double site mutant was unaffected. These results indicate that activation of microsomal glutathione S-transferase 1 by peroxynitrite is mediated by nitration of tyrosine residue 92 and represents one of the few examples in which a gain in function has been associated with nitration of a speciﬁc tyrosine residue.

The microsomal glutathione S-transferase 1 (MGST1) is a member of the membrane-associated proteins in eicosanoid and glutathione metabolism superfamily of proteins (1). Although these proteins share common structural characteristics, their biological functions are quite varied, including roles in xenobiotic metabolism, cellular protection, pain, and inﬂammation. Human membrane-associated proteins in eicosanoid and glutathione metabolism includes six proteins: 5-lipoxygenase-activating protein, leukotriene-C4 synthase, MGST1, MGST2, MGST3, and microsomal prostaglandin-E synthase. Rat hepatic

MGST1 exists as a homotrimer of identical 17.3-kDa subunits and has a high degree of amino acid sequence similarity (83%) to human MGST1. In contrast to the cytosolic GSTs, an important characteristic of micro- somal GST1 is that the enzyme can be activated by various treatments, including limited proteolysis, heating, radiation, and exposure to sul- hydryl modifying reagents and reactive oxygen species. In addition, since MGST1 possesses glutathione peroxidase activity, it has been sug- gested that this enzyme may play a protective role under conditions of oxidative stress (2, 3).

Activation of MGST1 also occurs after exposure to reactive nitrogen species (RNS), including nitric oxide (NO) donors and peroxynitrite (ONOO−) (3, 4), in contrast to the cytosolic GSTs, which are inhibited by RNS (5). The ONOO−-induced activation of MGST1 was associated with tyrosine nitration, polymer formation, and protein fragmentation but not protein S-oxidation (3). This increase in enzyme activity by ONOO− is unusual, since tyrosine nitration of proteins is almost always associated with a loss of function. For example, a number of enzymes involved in protection against oxidative stress are inhibited by ONOO−, including superoxide dismutases (6), glutathione reductase (7), cytosolic GSTs (5), catalase (8, 9), and glutathione peroxidase (8, 10). The activation of MGST1 by RNS, together with its GSH peroxidase activity, suggest that this enzyme may play an important role in limiting the extent of oxidative tissue injury under pathophysiological conditions of excessive ONOO− formation, especially if other antioxidant defense mechanisms are compromised.

The tyrosine residues of MGST1 that are nitrated by ONOO− have not been identiﬁed. In the present study, we used two mass spectromet- ric techniques, MALDI-TOF MS and ESI-MS/MS, to identify the tyro- sine residues of puriﬁed rat hepatic MGST1 that are nitrated after ONOO− treatment. Subsequently, we determined the functional con- sequences resulting from nitration of these tyrosine residues using site- directed mutagenesis.

EXPERIMENTAL PROCEDURES

Materials—Hydroxyapatite, CM-Sepharose, GSH, 1-chloro-2,4-di- nitrobenzoate, Triton X-100, manganese (IV) dioxide, Dulbecco’s mod- ified Eagle’s medium, and nutrient mixture F-12 were purchased from Sigma. Sequence-grade modiﬁed trypsin was from Promega (Madison, WI). Monoclonal anti-nitrotyrosine antibody was from Cayman Chemical Co. (Ann Arbor, MI) and horseradish peroxidase-linked goat anti- mouse IgG was obtained from Bio-Rad (Mississauga, Ontario, Canada). Chemiluminescence reagents were from Kirkegaard and Perry (Gaithersburg, MA). All other chemicals were of reagent grade and were obtained from common commercial sources.

Enzyme Purification and Enzyme Activity Assay—Rat hepatic MGST1 was puriﬁed from male Sprague-Dawley rats (250–300 g) by hydroxyapatite and CM-Sepharose chromatography as described (3). The purity of the enzyme preparation was assessed by SDS-PAGE on
Tyrosine Nitration and Microsomal Glutathione S-Transferase 1

15% gels under reducing conditions. The protein migrated as a single band at about 17 kDa (4). Prior to experiments, GSH was removed from the preparation by dialysis against buffer containing 10 mM potassium phosphate, pH 7.0, 0.1 mM EDTA, 1% Triton X-100, and 20% glycerol using a System 500 Microdialyzer (Pierce). Enzyme activity was determined by the spectrophotometric method of Habig et al. (11). Samples (1.0 ml) contained 100 mM potassium phosphate, pH 6.5, 0.5% Triton X-100, 1.0 mM GSH, and 1.0 mM 1-chloro-2,4-dinitrobenzene at 25 °C.

Preparation of Peroxynitrite (ONOO−) and Treatment of MGST1—ONOO− was synthesized from acidified nitrite and H2O2 as described (12). Prior to each experiment, excess H2O2 in ONOO− solutions was removed by MnO2 chromatography (3). Purified enzyme (20 μg/ml) or microsomal fractions (200–400 μg/ml protein) in 100 mM potassium phosphate, pH 7.0, containing 100 μM diethylenetriaminepentaacetic acid (DTPA), were exposed to the indicated concentrations of ONOO− at room temperature for 10 s. To keep the pH value of the reaction unchanged, ONOO− was added to the protein or microsomal solution as a small volume during vigorous mixing, and the reaction was terminated by dilution of the sample into the assay buffer for the determination of enzyme activity or into the sample buffer for SDS-PAGE.

For immunoblot analysis, samples treated with ONOO− were resolved on a 15% SDS-PAGE gel under non-reducing conditions. Proteins were transferred to polyvinylidene difluoride membranes and incubated with a 3-nitrotyrosine-specific antibody. The immunoreactive protein bands were visualized by enhanced chemiluminescence.

Identification of Nitration Sites by MALDI-TOF MS—Protein bands corresponding to the MGST1 monomer (17.3 kDa) with and without ONOO− treatment were excised from Coomassie Blue-stained, 15% reducing SDS-PAGE gels (approximately 2 μg of purified protein/band). Gel slices were washed three times with 50% ACN, 25 mM ammonium bicarbonate, pH 8.0 (15 min each time under gentle agitation) to remove the Coomassie Blue and then were incubated with 100% ACN for 5 min and vacuum-dried. To reduce and alkylate proteins, dried gel slices were incubated with 10 mM DTT in 100 mM ammonium bicarbonate at 55 °C for 45 min followed by incubation with 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 30 min at room temperature in the dark. Gel slices were then washed with 25 mM ammonium bicarbonate and dried with 100% ACN as described above.

In-gel digestion was performed by swelling the dried gel slices with 10 μg/ml trypsin in 25 mM ammonium bicarbonate, pH 8.0, for 30 min on ice. Excess trypsin solution was removed and digestion continued at 37 °C for 16 h. Tryptic peptides were extracted with 50% ACN/5% trifluoroacetic acid, dried under vacuum, and reconstituted in 3 μl of 70% ACN, 0.1% trifluoroacetic acid. Reconstituted extract (0.5 μl) was mixed with 0.5 μl of matrix (10 mg/ml α-cyano-4-hydroxy-trans-cinnamic acid in 70% ACN, 0.1% trifluoroacetic acid), spotted onto a stainless steel 100-well MS plate, and air-dried. Samples were analyzed using a Voyager DE-Pro MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA) operated in the delayed extraction/reflector mode with an accelerating voltage of 20 kV, grid voltage setting of 72%, and a 50-ns delay. Three spectra (100 laser shots/spectrum) were obtained for each sample. External calibration was performed using a Sequazyme Peptide Mass Standard kit (Perseptive Biosystems, Framingham, MA) containing the following standards: des-Arg-bradykinin, angiotensin-1, and Glu-fibrinopeptide B. Peptide mass fingerprinting was conducted with the data base search tool MS-Fit using Protein Prospector (version 3.2.1), available at prospector.ucsf.edu/. The modifications due to nitration were included in the search for both control and ONOO−-treated samples. The criteria for identification of the protein by MALDI-TOF MS were: a match of at least four peptides from the protein compared with the data base of rat proteins with similar to observed molecular mass and with an error of less than 50 ppm for all peptides, including modified ones.

Analysis of Trypsin Cleavage Products by ESI-MS/MS—Samples were analyzed with an ESI/MS/MS system composed of Waters Capillary LC (Waters Corp., Milford, MA) coupled to a Finnigan LCQ Deca iontrap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a nanospray ionization source. Separation was performed using a picofrit column (PFC7515–PP18–5–3PK, New Objective, Woburn, MA) with a 75 μm inner diameter and a 15 μm tip. The stationary phase was 5 μm ProteoSep C18 matrix packed in a column of 5 cm length. The mobile phase was ACN, 0.1% trifluoroacetic acid. Separation of peptides was achieved using a gradient of ACN from 5 to 65% in 45 min, followed by a wash with 95% ACN. MS/MS spectra were analyzed using SEQUEST® (ThermoFinnigan) software. For the ESI-LC MS/MS spectra, a correlation score above 2 was considered significant for peptides with a charge of 2 to accept the deduced sequence, whereas the sequence of peptides with a lower score was deemed manually.

Site-directed Mutagenesis—Plasmid (pCMV5) containing rat liver MGST1 cDNA was a generous gift from Dr. Ralf Morgenstern (Institute of Environmental Medicine, Karolinska Institute, Stockholm, Sweden). The plasmid was digested with EcoRI (Promega), and the MGST1 cDNA was inserted into pcDNA3.1(−). All mutations were generated using the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA). pcDNA3.1-MGST1 was used as template for generating mutants Y92F and Y153F, and subsequently pcDNA3.1-MGST1 (Y153F) was used as the template for generation of double mutants (Y92F and Y153F). Oligonucleotides bearing mismatched bases at the residues to be mutated (underlined) were synthesized by ACGT Corp. (Toronto, Ontario, Canada) as follows: Y92F (5′-C GGC CTC TGC TAC GCC GCG-3′) and Y153F (5′-G CTC AGG AGC AGA CTG TAC TTG TAA AGA GAT TTG GCA CC-3′). All mutations were confirmed by DNA sequencing.

Cell Culture and Gene Transfection—LLC-PK1 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco’s modified Eagle’s medium/F-12 medium (1:1) supplemented with 10% fetal bovine serum, 5 μg/ml insulin, 2 mM glutamine, 10 mM HEPES, pH 6.9, 50 units/ml penicillin, and 50 μg/ml streptomycin. For transient transfections, LLC-PK1 cells were seeded in 150-mm cell culture plates, and the expression plasmids were introduced using FuGENE 6 (Roche Applied Science), according to the manufacturer’s instructions. After 24-h incubation with the transfection reagents, cells were harvested, lysed by sonication in 100 mM potassium phosphate, pH 7.4, and microsomal fractions (100,000 × g pellet) prepared by ultracentrifugation. MGST1 activity in both ONOO−-treated and non-treated microsomal fractions was assessed as described above. MGST1 activity determinations were performed in triplicate, and data are presented as the mean ± S.D. Data were analyzed by the appropriate statistical test, as indicated. p values <0.05 were considered statistically significant.

RESULTS

Tyrosine Nitration and Activation of MGST1 by ONOO−—Exposure of purified MGST1 to 2.0 mM ONOO− resulted in a 4.3-fold increase in enzymatic activity (p < 0.001) (Fig. 1). However, decayed ONOO− or 2.0 mM H2O2 did not affect enzyme activity (Fig. 1). The presence of 3-nitrotyrosine in the activated protein was demonstrated using SDS-PAGE (under non-reducing conditions) and immunoblot analysis employing a monoclonal anti-nitrotyrosine antibody. Immunoreactive staining of the 17.3-kDa MGST1 monomer increased in intensity after exposure to increasing concentration of ONOO− (Fig. 2). In addition, there was
staining evident in proteins bands of about 34 and 50 kDa, as observed previously (3), indicating the formation of tyrosine-nitrated dimers and trimers.

**Identification of Sites of Tyrosine Nitration in MGST1**—Peroxynitrite-treated and non-treated MGST1 were separated by 15% SDS-PAGE, and the monomeric MGST1 (17.3 kDa) underwent in-gel digestion with trypsin. Peptide mass fingerprinting by MALDI-TOF MS confirmed the identification of the 17.3-kDa band as rat MGST1 (SwissProt accession number P08011, M_ 17,472, pI 9.6) (~57% protein coverage for both control (non-treated) and ONOO⁻-treated samples). There are 7 tyrosine residues in MGST1 (Table 1). Tryptic peptides containing these tyrosine residues were observed by either MALDI-TOF MS and/or ESI-MS/MS with the exception of the tryptic peptide containing Tyr₁⁴₅. Most likely this was not observed due to poor ionization of the peptide fragment containing Tyr₁⁴₅. Based on the sequence of MGST1, digestion using either Asp-N or Glu-C would not have been suitable for identification by MALDI-TOF MS.

The tryptic peptide containing Tyr₉² (a.a. 74–107) was observed in both untreated and ONOO⁻-treated MGST1 by ESI-MS/MS, whereas the nitrated form of the Tyr₉² peptide was detected only in the ONOO⁻-treated sample. Tyrosines 115 and 120 present within the same peptide (1897.03 m/z) (aa 114–129) were observed using both types of MS, but there was no evidence for nitration of either of these tyrosine residues in ONOO⁻-treated samples. Similarly, Tyr₁³⁷ and Tyr₁⁵₃ lie within the tryptic peptide comprising residues 131–147 (1851.95 m/z). Nitration of these two tyrosine residues also was not detected. However, in all cases, the methionine residue at position 143 was always found in the oxidized form, and this modification could have prevented the identification of other potential modifications within the same peptide. The tyrosine residue located near the C terminus, Tyr₁⁵₃, was found in a short 3-amino acid peptide (aa 152–154) by ESI-MS/MS or as a peptide that resulted from a single missed cleavage comprising the penultimate 5 amino acids (aa 150–154) by MALDI-TOF MS. Nitration at Tyr₁⁵₃ in ONOO⁻-treated samples was observed in both peptides. The MS data are summarized in Table 1. The Sequest summary for parental and nitrated peptides observed in ONOO⁻-treated samples and a schematic representation of the MS/MS sequence for peptides containing nitrated Tyr₁⁵₃ are shown in Fig. 3.

**Cellular Functional Analysis**—The functional consequences of nitration of tyrosine residues Tyr₉² and Tyr₁⁵₃ of MGST1 were determined by comparing the wild type protein to several MGST1 mutants, in which these tyrosine residues were substituted with phenylalanine, alone or in combination (Y₉²F, Y₁⁵₃F, and a Y₉²F,Y₁⁵₃F double mutant). These mutations would prevent any potential nitration at the mutated amino acid site while maintaining similar overall chemistry. Expression vectors containing the wild type or mutated MGST1 (both single and double sites) were transiently transfected in LLC-PK₁ cells and the MGST1 activity in the microsomal fractions assessed. Immunoreactive MGST1 protein was readily detected in transfected cells but not in non-transfected cells or cells transfected with empty vector (Fig. 4A), consistent with a 2–3-fold increase in basal MGST1 activity in MGST1-transfected cells (Fig. 4B). As expected, the basal MGST1 activity was similar in microsomes containing either the wild type or mutated proteins. However, after treatment with 2.0 mM ONOO⁻ for 10 s, there was a significant increase in MGST1 activity only in microsomal fractions from cells transfected with wild type MGST1 or with the Y₁⁵₃F mutant (Fig. 4B). In contrast, MGST1 activity in the Y₉²F mutant or the double mutant was unaltered by ONOO⁻, suggesting that nitration of Tyr₉² is required for the peroxynitrite-induced activation of MGST1.

**DISCUSSION**

MGST1 is a homotrimeric membrane-bound protein, with each subunit containing seven tyrosine residues (at positions 18, 92, 115, 120,
in addition to a single cysteine residue (Cys49). Six of the seven tyrosine residues are located in the C-terminal half of the protein. Electron crystallography of two-dimensional crystals of MGST1 has been used to construct a three-dimensional model of the enzyme. In this model, the trimeric enzyme exhibits three repeats of a left-handed four-helix bundle motif (13). The catalytic center of the enzyme is thought to be contained in the first cytosolic loop of the protein between peripheral transmembrane helices designated D and A. Only two of the tyrosine residues (Tyr120 and Tyr153) are located in putative non-membrane domains, the former in the second cytosolic loop and the latter at the intra-lumen C-terminal; the other five are located in one of the four putative transmembrane domains (13). The first cytosolic loop does not contain any tyrosine residues (13) but does contain Cys49, the alkylation or oxidative modification of which results in increased catalytic activity and is thought to act as a sensor of chemical or oxidative stress (14). However, oxidation of Cys49 by reactive oxygen species does not appear to cause enzyme activation, since we (Ref. 3 and Fig. 1) and others (15) could not demonstrate activation of the enzyme by H2O2 or by superoxide generating systems. In studies that have demonstrated activation of the enzyme by H2O2 (16, 17), incubations with H2O2 were performed in the presence of GSH, and the increase in enzyme activity was attributed to S-glutathiolation or polymer formation, rather than oxidation of the Cys49 per se. In our studies, incubations with H2O2 were performed in the absence of GSH, and therefore S-glutathiolation of Cys49 and subsequent activation of the enzyme would not be expected.

To identify sites of tyrosine nitration of MGST1, we utilized mass spectrometric techniques using both MALDI and ESI to maximize cov-
Tyrosine Nitration and Microsomal Glutathione S-Transferase 1

The active site of MGST1 is localized on the cytosolic side of endoplasm reticulum (23). The MGST1 trimer binds one molecule of GSH, and subunit communication is thought to play a role in the activation of the enzyme (24, 25). Thiolate formation follows a biphasic kinetic pattern, with rapid binding of GSH followed by slower formation of thiolate (26). The presumed conformational changes brought about by modification of Cys49 by N-ethylmaleimide markedly increases the rate of thiolate formation and increases kcat. Although nitration of Tyr153 was shown to occur, this residue is the penultimate C-terminal residue, far removed from the catalytic site of the enzyme, and thus might not be expected to influence enzyme conformation or activity. This is supported by the site-directed mutagenesis data (Fig. 4), in which microsomes from cells expressing the Y153F mutant exhibited significant increases in MGST1 activity after exposure to ONOO-. On the other hand, Tyr92 is located in the transmembrane domain on the C-terminal side of the cytosolic loop containing the catalytic domain. Considering the location of Tyr92, it seems reasonable to suppose that in a manner analogous to that occurring after structural modification of Cys49, a conformational change brought about by nitration of this tyrosine residue might increase the rate of thiolate formation and increase catalytic activity. Therefore, in simile with the role of Cys49 as a sensor of chemical and oxidative stress, the nitration of Tyr92 could function as a sensor of nitrosative stress.

Data from this and other studies have shown that activation of MGST1 by RNS occurs by two distinct mechanisms: S-nitrosylation of Cys49 and nitration of Tyr92, and the question arises as to the relative importance of these two mechanisms in vivo. Using the purified enzyme, the degree of activation by ONOO− is about 2.5-fold greater than that by GSNO (3, 4). Using hepatic microsomes, enzyme activity is increased about 3-fold by ONOO− and is not increased by GSNO. Thus in purified enzyme or microsomal preparations, ONOO− is a better activator of the enzyme. The lack of effect of GSNO could be due to contamination of the microsomal fraction by cytosolic GSTs, whose activity is inhibited by GSNO, with a net effect of no change in activity. However, in a recent report in which S-nitrosylation of MGST1 was assessed using biotin switch methodology, no evidence was found for S-nitrosylation of the enzyme after incubation of hepatic microsomes with GSNO or in microsomes from rats treated with lipopolysaccharide to induce nitrosative stress (27). We would therefore speculate that in vivo, under pathological situations associated with increased ONOO− formation, that tyrosine nitration would be the more relevant modification of MGST1.

In a previous study we found that addition of ONOO−-treated MGST1 to microsomal membrane preparations caused a marked reduction in iron-induced lipid peroxidation, consistent with the finding that the GSH peroxidase activity of MGST1 is increased after treatment with ONOO− and suggesting a protective function for the nitrated enzyme (3). Under conditions of oxidative/nitrosative stress, most antioxidant enzymes are inactivated by S-oxidation and/or tyrosine nitration by ONOO−. The data obtained in the present study suggest that nitration of Tyr92 and subsequent activation of MGST1 after exposure to ONOO− may be an important mechanism for cellular protection against nitrosative stress under circumstances in which other antioxidant defense mechanisms are compromised.

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