3-Bromotyrosine and 3,5-Dibromotyrosine Are Major Products of Protein Oxidation by Eosinophil Peroxidase: Potential Markers for Eosinophil-Dependent Tissue Injury in Vivo†

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Received October 8, 1998

ABSTRACT: Detection of specific reaction products is a powerful approach for dissecting out pathways that mediate oxidative damage in vivo. Eosinophil peroxidase (EPO), an abundant protein secreted from activated eosinophils, has been implicated in promoting oxidative tissue injury in conditions such as asthma, allergic inflammatory disorders, cancer, and helminthic infections. This unique heme protein amplifies the oxidizing potential of H_2O_2 by utilizing plasma levels of Br^- as a cosubstrate to form potent brominating agents. Brominated products might thus serve as powerful tools for identifying sites of eosinophil-mediated tissue injury in vivo; however, structural identification and characterization of specific brominated products formed during EPO-catalyzed oxidation have not yet been reported. Here we explore the role of EPO and myeloperoxidase (MPO), a related leukocyte protein, in promoting protein oxidative damage through bromination and demonstrate that protein tyrosine residues serve as endogenous traps of reactive brominating species forming stable ring-brominated adducts. Exposure of the amino acid L-tyrosine to EPO, H_2O_2, and physiological concentrations of halides (100 mM Cl^-, ≤100 μM Br^-) produced two new major products with distinct retention times on reverse phase HPLC. The products were identified as 3-bromotyrosine and 3,5-dibromotyrosine by electrospray ionization mass spectrometry and multinuclear (^1H and ^{15}N) NMR spectroscopy. Formation of the ring-brominated forms of the amino acid occurred readily at neutral pH with the enzymatic system and a variety of reactive brominating species, including HOBr/OBr^-, *N*-bromoamines, and *N*,*N*-dibromoamines. Addition of primary amines (e.g., N^α-acetyllysine and taurine) to L-tyrosine exposed to either HOBr/OBr^- or the EPO−H_2O_2−Br^- system enhanced phenolic ring bromination, suggesting *N*-bromoamines are preferred brominating intermediates in these reactions. Reduction of *N*-bromoamines (e.g., N^α-acetyl,N^ϵ-bromolysine) by L-tyrosine was shown to result in the loss of reactive halogen with a near stoichiometric increase in the level of tyrosine ring bromination (i.e., carbon−bromine bonds). Although both EPO and MPO could use Br^- to halogenate protein tyrosine residues in vitro, only EPO effectively brominated the aromatic amino acid at physiological levels of halides and H_2O_2. Collectively, these results suggest that 3-bromotyrosine and 3,5-dibromotyrosine are attractive candidates for serving as molecular markers for oxidative damage of proteins by reactive brominating species in vivo. They also suggest that in biological mixtures where amine groups are abundant, the trapping of EPO-generated HOBr/OBr^- as *N*-bromoamines will serve to effectively "funnel" reactive brominating equivalents to stable ring-brominated forms of tyrosine.

Reactive oxidant species generated by leukocytes are of central importance in immune surveillance and host defense mechanisms; however, the reactive intermediates formed also have potential to harm normal tissue and contribute to inflammatory injury (*1*−*7*). One pathway for oxidative damage may involve formation of reactive halogenating species by the leukocyte-derived proteins, eosinophil peroxidase (EPO)[1] and myeloperoxidase (MPO) (*8*−*15*). EPO and MPO are abundant leukocyte proteins which are secreted by activated phagocytes (eosinophils and neutrophils or monocytes, respectively) along with other granule contents into the extracellular milieu and phagolysosomal compartments of their respective cells of origin. These heme proteins utilize H_2O_2 generated by the activated cells as a substrate to catalyze the peroxidation of halides (X^-) forming potent halogenating intermediates that possess "active" halogen in the formal +1 oxidation state (in the form of diatomic

† This work was supported by grants from the National Institutes of Health (HL61878) and the American Heart Association.

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[1] Abbreviations: EPO, eosinophil peroxidase; ESI, electrospray ionization; GC, gas chromatography; HOBr, hypobromous acid; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MPO, myeloperoxidase; MS, mass spectrometry; *m*/*z*, mass-to-charge ratio; OBr^-, hypobromite.

10.1021/bi982401l CCC: $18.00
Published on Web 03/05/1999

halogen, X_2; hypohalous acid, HOX; and hypohalite, OX^-) (*3*, *6*, *8*, *9*, *15*). These species are in rapid equilibrium; at neutral pH, the predominant forms are a mixture of the acid and its conjugate base (HOX/OX^-) (*16*−*20*). Peroxidase-generated HOX and OX^- are potent cytotoxic oxidants which possess microbicidal and viricidal activity (*1*−*6*, *21*−*23*). However, the reactive intermediates generated by these peroxidases are also potentially deleterious and can damage host tissues, contributing to inflammatory injury. Halogenated products have the potential to serve as excellent molecular markers to identify sites where EPO and MPO promote oxidative damage because there are no other known pathways in humans which result in covalent incorporation of Br or Cl atoms into biomolecules.

Chloride (Cl^-) is by far the most abundant halide in vivo (e.g., 100 mM in plasma; *24*, *25*); consequently, formation of chlorinating species by leukocyte peroxidases has been widely studied. HOCl/ClO^- is the primary oxidant formed by MPO at plasma levels of halides (*8*, *14*). EPO preferentially oxidizes another halide, bromide (Br^-), and the psuedohalide thiocyanate (SCN^-) as substrates (*8*, *9*, *15*, *26*−*29*); however, the enzyme also is capable of generating reactive chlorinating species, particularly when alternative substrates are limiting (*9*, *11*, *15*). HOCl/ClO^- chlorinates amines to form *N*-chloroamines (RNHCl) and *N*,*N*-dichloroamines ($RNCl_2$), which retain their oxidizing equivalents (*18*, *30*). *N*-Chloroamines are typically long-lived reactive species and can accumulate in media following phagocyte activation; they thus may promote cytotoxic reactions far from their site of origin (*30*−*33*). In contrast, *N*-chloroamines formed following reaction of HOCl/ClO^- with α-amino acids are labile and rapidly decompose to form aldehydes (*34*, *35*). Production of chlorinating species by neutrophils in vitro was first demonstrated by trapping the reactive halogen through reaction with the *β*-amino acid, taurine, forming stable *N*-chloroamine derivatives (*32*, *36*). HOCl/ClO^- production by phagocytes in vitro has also been demonstrated by utilizing aromatic compounds or proteins as trapping agents (*12*, *37*−*42*). Structural identification of endogenous protein traps for reactive chlorinating species has permitted direct demonstration of their formation in vivo, as well as provided a means for studying mechanisms of oxidative injury by inflammatory cells (*12*, *41*, *42*).

Although the biochemical properties and products of leukocyte peroxidase-dependent chlorination reactions have been explored in a wealth of studies, much less work has focused on the biological role(s) of bromide (Br^-) and reactive brominating species in human health and disease. Bromide is found in virtually all animal tissues; its concentration in plasma and extracellular fluids ranges between 20 and 120 μM (*24*, *27*, *43*). Weiss and colleagues were the first to report a role for Br^- in a mammalian system by demonstrating that eosinophils preferentially utilize Br^- at physiological concentrations of halides to form a reactive brominating species which was likely hypobromous acid (HOBr) (*8*). Activation of eosinophils in media containing physiological levels of Cl^- and $^{82}Br^-$ resulted in covalent incorporation of the radioactive isotope into proteins (*8*). Subsequent in vitro studies in which exogenous aromatic trapping agents were employed revealed that eosinophils selectively utilize Br^- present in media, forming a potent brominating agent, even when the Cl^- concentration is more than 20000-fold greater than the Br^- concentration (e.g., 140 mM Cl^- vs 5 μM Br^-) (*9*). Moreover, at least 25−35% of the oxygen consumed during a respiratory burst in stimulated eosinophils could be ascribed to the generation of brominating species (*9*). The selective preference of eosinophils for utilization of physiological concentrations of Br^- mirrors the halide specificity of purified EPO (*9*, *15*).

Eosinophil activation and oxidative damage are thought to play a critical role in tissue injury during asthma, allergic reactions, and host defenses against various malignancies and helminthic infections (*1*−*3*). However, direct demonstration of the oxidation pathways involved has proven to be difficult due to the evanescent nature of reactive oxidant species, and the noninformative products they typically produce. The unique nature of leukocyte-generated reactive brominating species makes brominated oxidation products excellent candidates for serving as molecular markers for this pathway in vivo. Primary amine-containing species (e.g., taurine) have been used as traps to detect and quantify brominating agents formed by isolated eosinophils and purified EPO (*8*, *15*) in vitro. The *N*-bromoamines (RNHBr) and *N*,*N*-dibromoamines ($RNBr_2$) formed, however, are not long-lived (*8*, *44*) and are both reduced by H_2O_2 and rapidly scavenged by thiols forming noninformative products (*15*, *45*, *46*). Reactions of select α-amino acids exposed to a peroxidase−H_2O_2−Br^- system have been examined in several studies; unstable intermediates are formed that decompose into species without oxidizing or brominating activity (*19*, *28*, *44*, *45*). Indirect evidence suggests that aldehydes are produced in these reactions; in the case of L-serine, mass spectrometry has identified glycolaldehdye as a product (*34*). Nitrogen−bromine, carbon−bromine, and sulfur−bromine derivatives of proteins have been inferred in several studies in which proteins were used as a trap of radioactive brominating agents (*8*, *47*−*50*). However, to date, definitive structural identification of stable brominated products generated on proteins exposed to leukocyte peroxidases has not yet been reported.

The aim of this study was to identify stable and specific protein oxidation products which might serve as molecular markers for leukocyte-generated reactive brominating species in vivo. We now report that 3-bromotyrosine and 3,5-dibromotyrosine are readily formed by reaction of free and protein-bound tyrosine residues with either HOBr/OBr^- or EPO in the presence of H_2O_2 and plasma levels of halides. We also demonstrate that *N*-bromoamines are particularly effective brominating agents for the aromatic amino acid and that the trapping of peroxidase-generated HOBr/OBr^- as *N*-bromoamines serve to effectively "funnel" reactive brominating equivalents to stable ring-brominated forms of tyrosine. Collectively, these results suggest that protein tyrosyl residues are endogenous traps of reactive brominating species and that 3-bromotyrosine and 3,5-dibromotyrosine are attractive molecular markers for exploring the role of protein oxidative damage by EPO in vivo.

EXPERIMENTAL PROCEDURES

Materials. Organic solvents and H_2O_2 were obtained from Fisher Scientific Co. (Pittsburgh, PA). Methanesulfonic acid and bromine were purchased from Fluka Chemical Co. (Ronkonkoma, NY). D_2O and L-[^{15}N]tyrosine were purchased from Cambridge Isotopes, Inc. (Andover, MA). All other

reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Isolation and Characterization of EPO and MPO. EPO was isolated from porcine whole blood according to the method of Jorg (*11*) employing guaiacol oxidation as the assay (*22*). The purity of EPO preparations was assured before use by demonstrating an RZ of >0.9 (A_{415}/A_{280}), SDS−PAGE analysis with Coomassie Blue straining, and in-gel tetramethylbenzidine peroxidase staining to confirm no contaminating MPO activity (*51*). MPO was initially purified from detergent extracts of human leukocytes by sequential lectin affinity and gel filtration chromatography as described previously (*52*). Trace levels of contaminating EPO were then removed by passage over a sulfopropyl Sephadex column (*53*). MPO preparations were concentrated, dialyzed against water, and stored in 50% glycerol at −20 °C. The purity of isolated MPO was established by demonstrating an RZ of 0.87 (A_{430}/A_{280}), SDS−PAGE analysis with Coomassie Blue staining, and in-gel tetramethylbenzidine peroxidase staining to confirm no contaminating EPO activity (*51*). Enzyme concentrations were determined spectrophotometrically utilizing extinction coefficients of 89 000 and 112 000 M^{-1} cm^{-1}/heme of MPO (*10*) and EPO (*54*, *55*), respectively. The concentration of the MPO dimer was calculated as half of the indicated concentration of heme-like chromophore.

General Procedures. Protein content was measured by the Markwell-modified Lowry assay with BSA as the standard (*56*). Amino acid analysis was performed at the Cleveland Clinic Foundation Research Institute Protein Chemistry Core Laboratory. HOBr free of Br^- and bromate was prepared from liquid bromine as described previously (*44*); on the day of use HOBr was quantified spectrophotometrically (ϵ_{331} = 315 M^{-1} cm^{-1}) as its conjugate base, OBr^- (*19*). Methionine sulfoxide was quantified by reverse phase HPLC and fluorescence detection following precolumn derivitization with *o*-phthalic aldehyde mercaptoethanol reagent as described previously (*57*). The concentrations of H_2O_2 (ϵ_{240} = 39.4 M^{-1} cm^{-1}) (*58*), *N*-bromoamine (ϵ_{288} = 430 M^{-1} cm^{-1}) (*15*), and *N*,*N*-dibromoamine (ϵ_{336} = 371 M^{-1} cm^{-1}) (*15*) were determined spectrophotometrically.

Oxidation of Free L*-Tyrosine and Protein-Bound Tyrosyl Residues*. Reactions were initiated by addition of oxidant (H_2O_2, HOBr, or *N*-bromo- or *N*,*N*-dibromoamine) and performed in sodium phosphate buffer (pH 7.0) at 37 °C for 60 min under the conditions described in the figure legends and table footnote. The pH dependence of 3-bromotyrosine and 3,5-dibromotyrosine formation from L-tyrosine and *N*-bromotaurine was performed in phosphate buffer (50 mM final) composed of mixtures of phosphoric acid and monobasic and dibasic sodium phosphate. The pH of each reaction mixture was determined at the end of the incubation period and did not change by more than 0.1 pH unit over the course of the reaction. Proteins oxidized in vitro were prepared for analysis by first precipitating and desalting them in a single-phase extraction mixture comprised of H_2O/methanol/H_2O-saturated diethyl ether (1:3:7, v:v:v) (*40*), followed by acid hydrolysis in methanesulfonic acid (below).

Protein Hydrolysis. Preliminary experiments demonstrated the nonenzymatic formation of 3-bromotyrosine and 3,5-dibromotyrosine in proteins (e.g., BSA or ribonuclease A) subjected to hydrolysis with either HBr or HCl (presumably due to trace contaminants of HBr). Proteins were subsequently hydrolyzed under halide-free conditions by incubating the desalted protein pellet with 4 N methanesulfonic acid (0.5 mL) supplemented with 1% phenol for 24 h at 100 °C. Prior to the initiation of hydrolysis, acid mixtures were degassed under vacuum and then sealed under a blanket of argon. Control experiments demonstrated that under these conditions, no detectable (detection limit of <1 fmol on-column for GC/MS analysis) intrapreparative formation of oxidation products occurred. Complete protein hydrolysis under these conditions was also confirmed in independent experiments by comparing the recovery of L-tyrosine from BSA subjected to hydrolysis with 4 N methanesulfonic acid or conventional HCl hydrolysis conditions (6 N HCl for 24 h at 110 °C; *40*).

Reverse Phase HPLC Quantification of L*-Tyrosine Oxidation Products.* Quantitative determination of 3-bromotyrosine and 3,5-dibromotyrosine production from free L-tyrosine was performed utilizing reverse phase HPLC with a C18 column (Beckman Ultrasphere, 5 μm resin, 4.6 mm × 250 mm) equilibrated with solvent A [0.1% trifluoroacetic acid (pH 2.5)]. L-Tyrosine and its oxidation products were eluted at a flow rate of 1 mL/min with a linear gradient generated with solvent B [0.1% trifluoroacetic acid in methanol (pH 2.5)] as follows: 0% solvent B for 5 min, 0 to 100% solvent B over the course of 30 min, and 100% solvent B for 10 min. 3-Bromotyrosine and 3,5-dibromotyrosine were monitored on a diode array detector and quantified at 280 nm by employing a standard curve constructed with authentic synthetic standards prepared with HOBr and purified by reverse phase HPLC as shown in Figure 1.

3-Bromotyrosine and 3,5-dibromotyrosine in protein hydrolysates were quantified by reverse phase HPLC with electrochemical (coulometric) detection on an ESA (Cambridge, MA) CoulArray HPLC instrument equipped with four electrochemical cells (channels) utilizing porous-carbon graphite electrodes arranged in series and set to increasing specified potentials: channel 1, 320 mV; channel 2, 440 mV; channel 3, 540 mV; and channel 4, 620 mV. Amino acid hydrolysates (50 μL) were injected onto a Progel TSK ODS-80 TM column (5 μm, 4.6 mm × 250 mm) equilibrated with mobile phase A [15 mM lithium phosphate and 3 mg/L lithium dodecyl sulfate (pH 3.2)]. Products were eluted at a flow rate of 1 mL/min with a nonlinear gradient generated with mobile phase B [50% methanol, 15 mM lithium phosphate, and 3 mg/L lithium dodecyl sulfate (pH 3.2)] as follows: isocratic elution at 0% mobile phase B for 10 min, 0 to 15% mobile phase B over the course of 10 min, isocratic elution at 15% mobile phase B for 10 min, 15 to 20% mobile phase B over the course of 10 min, isocratic elution at 20% mobile phase B for 10 min, 20 to 100% mobile phase B over the course of 20 min, and isocratic elution at 100% mobile phase B for 20 min. Peak identity was established by demonstrating the appropriate retention time, redox potential, and ratio of integrated currents in adjacent channels, and by co-injection of an authentic standard for each analyte. L-Tyrosine, 3-bromotyrosine, and 3,5-dibromotyrosine standards (1−100 pmol each on-column) were also dissolved together and used to generate an external calibration curve.

Instrumentation. Electrospray ionization mass spectrometry (ESI/MS) was performed on a Quatro II triple-quadruple mass spectrometer (Micromass, Inc.) interfaced with an HP

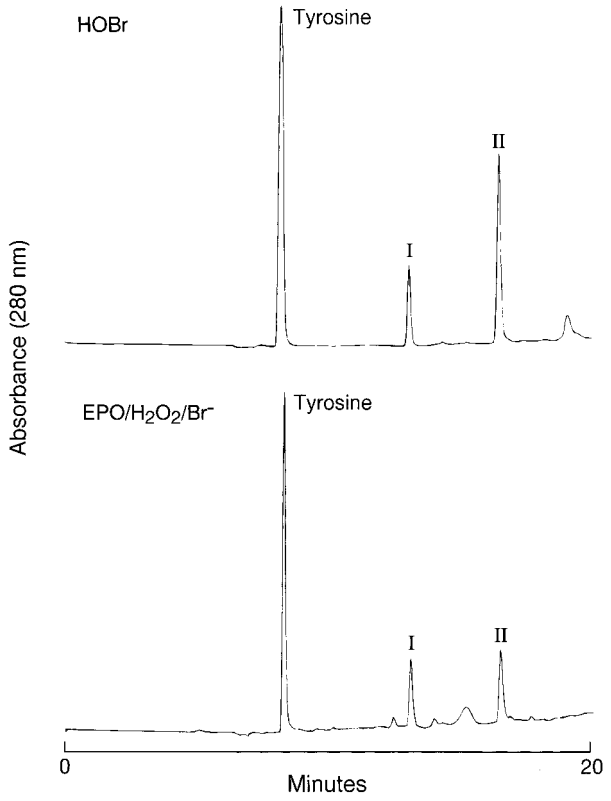

FIGURE 1: Reverse phase HPLC separation of L-tyrosine oxidation products generated by eosinophil peroxidase. (Top panel) L-Tyrosine (2 mM) was incubated with HOBr (2 mM) in sodium phosphate buffer (50 mM, pH 7.4) at 37 °C for 60 min. Products were subsequently analyzed by reverse phase HPLC as described in Experimental Procedures. (Bottom panel) L-Tyrosine (100 μM) was incubated with eosinophil peroxidase (57 nM), H_2O_2 (100 μM), NaCl (100 mM), and NaBr (1 mM) in sodium phosphate buffer (20 mM, pH 7.4) at 37 °C for 60 min. Products were then analyzed by reverse phase HPLC as described in Experimental Procedures. The major tyrosine oxidation products generated by EPO (peaks I and II, lower panel) were subjected to the structural studies described below (Figures 2 and 3).

1100 HPLC system (Hewlett-Packard). L-Tyrosine oxidation products were resolved on an Ultrasphere C18 column (Beckman, 5 μm, 4.6 mm × 250 mm) at a flow rate of 1 mL/min and a linear gradient between H_2O (with 0.3% formic acid) and methanol (with 0.3% formic acid) over the course of 30 min. The column eluent was split (970 μL/min to the UV detector and 30 μL/min to the mass detector) and analyzed by the mass spectrometer in the positive ion mode with a cone potential of 20 eV. Gas chromatography−mass spectrometry (GC−MS) analysis of L-tyrosine oxidation products was performed following derivatization to their *n*-propyl per HFB derivatives (*12*) on a Perkin-Elmer TurboMass mass spectrometer equipped with a chemical ionization probe. Chromatographic separations were performed on a 20 m PE-5MS column (0.18 mm inside diameter, 0.18 μm film; Perkin-Elmer), and mass spectra were acquired in the negative ion mode.

NMR studies were performed at 25 °C in D_2O/H_2O (1:9 v:v) with a Varian Unity-Plus 500 spectrometer (499.843 MHz for ^{1}H). ^{1}H chemical shifts were referenced to external sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 in D_2O. A Nalorac indirect detection probe was employed for ^{1}H and ^{1}H, ^{15}N two-dimensional NMR studies. The intense HOD signal was attenuated by transmitter preirradiation, and digital signal processing was employed to suppress phase distortions for ^{1}H spectra. ^{15}N NMR chemical shifts of L-tyrosine and its oxidation products were established through heteronuclear multiple bond correlation spectroscopy experiments as previously described (*34*).

RESULTS

Reverse Phase HPLC Identification of Major L*-Tyrosine Oxidation Products of Eosinophil Peroxidase*. Preliminary experiments of analysis of protein (e.g., BSA or ribonuclease A) exposed to HOBr, the major oxidant generated by EPO at plasma levels of halides (*8*, *9*, *15*), demonstrated that protein tyrosine residues were major targets for oxidation. For example, incubation of BSA (1 mg/mL) with HOBr (100 nmol) at neutral pH resulted in a loss of approximately 50 nmol of tyrosine residues as assessed by amino acid composition analysis. To explore the potential products generated during tyrosine oxidation by HOBr, we incubated free L-tyrosine with either HOBr or isolated EPO, H_2O_2, and Br^- (in the presence of plasma levels of Cl^-) and then analyzed the reaction products by reverse phase HPLC (Figure 1). Both HOBr and the enzymatic system generated two new major oxidation products with distinct retention times (designated peaks I and II). Use of heat-killed eosinophil peroxidase or omission of either cosubstrate (H_2O_2 or Br^-) resulted in no detectable production of peaks I and II by the enzymatic system. The reaction products (peaks I and II) formed were stable to treatment with acid (4 N HCl at 100 °C for 24 h), prolonged (1 week) incubation at 37 °C with H_2O_2 [in phosphate buffer (pH 7.0) supplemented with DTPA], and addition of a 100-fold molar excess of either reducing agents or nucleophilic scavengers (e.g., $NaCNBH_3$, sodium hydrosulfite, methionine, β-mercaptoethanol, taurine, or ammonium acetate). These results strongly suggested that the products in peaks I and II were not *N*-bromoamines (*8*, *15*, *44*−*46*).

Structural Identification of 3-Bromotyrosine and 3,5-Dibromotyrosine as Major Products of L*-Tyrosine Oxidation by Eosinophil Peroxidase-Generated Reactive Brominating Species*. To characterize the structures of the L-tyrosine oxidation products generated by the EPO−H_2O_2−Br^- system, LC−MS analysis was performed. The positive ion mass spectrum of peak I was consistent with a monobrominated derivative of tyrosine (Figure 2, upper panel) and contained a single major compound possessing a molecular ion with a mass-to-charge ratio (*m*/*z*) of 260 [$(M + H)^+$]. The mass spectrum also demonstrated the isotopic cluster expected for a monobrominated compound (1:1 M:M + 2), with ions at *m*/*z* 260 ([M + H]$^+$ for the ^{79}Br-containing isotopomer) and 262 ([M + H]$^+$ for the ^{81}Br-containing isotopomer). These results, combined with the chemical stability of the product to acid, peroxide, reductants, and nucleophilc compounds, suggested that peak I was the stable ring-brominated product, 3-bromotyrosine (Figure 2, upper panel inset). The positive ion mass spectrum of material in peak II (Figure 2, lower panel) was consistent with a dibrominated derivative of L-tyrosine and contained a single major component with an ion at *m*/*z* 338 [$(M + H)^+$]. The mass spectrum demonstrated the isotopic cluster (1:2:1 M:M + 2:M + 4) expected for a dibrominated derivative of tyrosine, with ions at *m*/*z* 338 ([M + H]$^+$ for the $^{79}Br_2$-containing isotopomer), 340 ([M + H]$^+$ for the ^{79}Br^{81}Br-containing isotopomer), and 342 ([M

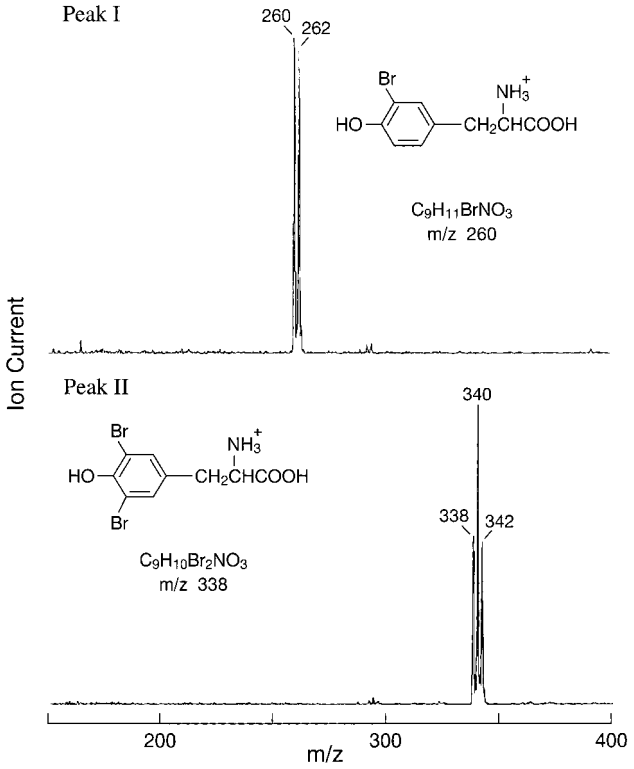

FIGURE 2: Positive ion electrospray mass spectra of 3-bromotyrosine and 3,5-dibromotyrosine generated by the EPO−H_2O_2−Br^- system. L-Tyrosine oxidation products generated by the EPO−H_2O_2−Br^- system were analyzed by LC−MS as described in Experimental Procedures. The positive ion ESI mass spectra of the L-tyrosine oxidation products constituting peak I (top panel) and peak II (bottom panel) are shown. The predicted *m/z* values of protonated 3-bromotyrosine and 3,5-dibromotyrosine are 260 and 338, respectively (insets). Note that the mass spectrum for peak I demonstrates the isotopic cluster expected for a monobrominated tyrosine adduct, with ions of relative intensity of 1:1 M:M + 2. Likewise, note that the mass spectrum for peak II demonstrates the isotopic cluster expected for a dibrominated tyrosine adduct, with ions of relative intensity of 1:2:1 M:M + 2:M + 4. LC−MS analysis of peaks I and II generated by addition of HOBr to L-tyrosine (Figure 1, top panel) yielded similar results (data not shown).

+ H]$^+$ for the $^{81}Br_2$-containing isotopomer). These results, combined with the chemical stability of the compound, suggested that peak II was the stable ring-brominated product, 3,5-dibromotyrosine.

GC−MS analysis of the L-tyrosine oxidation products generated by the EPO−H_2O_2−Br^- system (peaks I and II) was also consistent with their structural assignment as 3-bromotyrosine and 3,5-dibromotyrosine, respectively. The negative ion mass spectrum of the *n*-propyl per heptafluorobutyryl derivative of the product in peak I demonstrated the anticipated molecular anion $(M)^{\bullet -}$ at *m/z* 593. Major ions observed in the mass spectrum which also demonstrated the isotopic pattern of a monobrominated species were observed at *m/z* 573 [$(M - HF)^-$], 553 [$(M - 2HF)^-$], and 445 [$(M - CF_3CF_2CHO)^-$] (data not shown). Likewise, the negative ion mass spectrum of the *n*-propyl per heptafluorobutyryl derivative of peak II was consistent with the structural assignment as 3,5-dibromotyrosine. Major ions observed in the mass spectrum which demonstrated the isotopic pattern of a dibrominated species included ions at *m/z* 671 [$(M)^{\bullet -}$], 651 [$(M - HF)^-$], 524 [$(M - CF_3CF_2CO)^-$], and 509 [$(M - CF_3CF_2CONH)^-$].

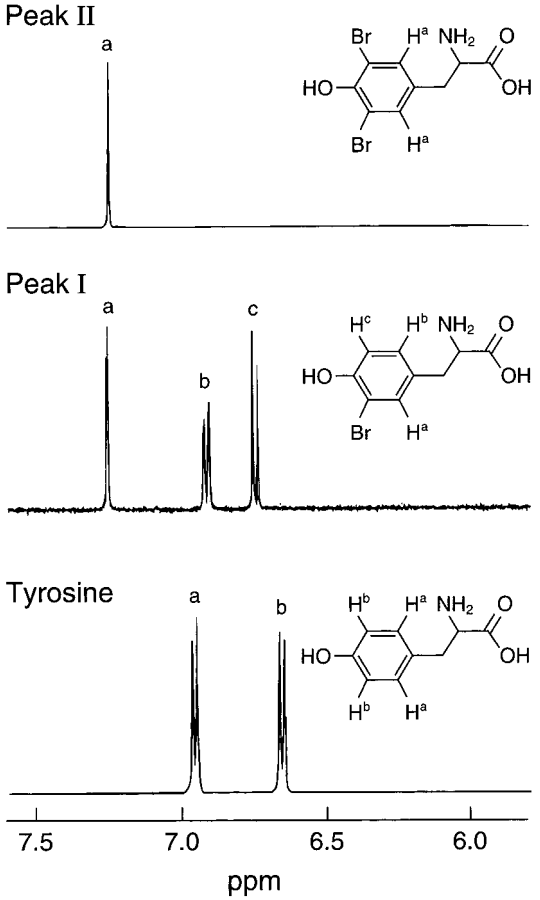

FIGURE 3: Aromatic region of the ^1H NMR spectra of L-tyrosine, 3-bromotyrosine, and 3,5-dibromotyrosine. L-Tyrosine (bottom panel) and the major EPO oxidation products (peaks I and II in the middle and top panels, respectively) were analyzed by ^1H NMR as described in Experimental Procedures. Peak assignments, coupling constants, and relative integrated areas are consistent with the proposed structures (insets) shown.

ESI-MS and GC−MS analysis of the major L-tyrosine oxidation products generated by the EPO system were consistent with formation of compounds having the same mass as 3-bromotyrosine and 3,5-dibromotyrosine. These methods, however, do not unequivocally establish the location of bromine attachment to the amino acid. We therefore utilized multinuclear (^1H and ^{15}N) NMR spectroscopy to identify the precise location of Br attachment to the aromatic amino acid. ^{15}N-labeled L-tyrosine was employed as a starting material for oxidation by the EPO system since the ^{15}N resonance could serve as a nonperturbing and sensitive probe of the immediate chemical environment at the α-amino nitrogen atom (where *N*-bromoamines might be formed), and concomitant examination of the aromatic region of the ^1H NMR spectra to identify any stable ring-brominated adducts would also be feasible. ^1H and ^{15}N NMR analysis of L-[^{15}N]-tyrosine and its EPO−H_2O_2−Br^- oxidation products (peaks I and II) confirmed the identity of these products as 3-bromotyrosine and 3,5-dibromotyrosine, respectively. The chemical shifts, integrated areas, and coupling constants of the resonances in the ^1H NMR spectra were all consistent with formation of the stable ring-brominated adducts (Figure 3). Proton assignments on the aromatic ring were further confirmed by observing allylic coupling between the benzylic protons and the ortho aromatic protons (positions 2 and 6) in both brominated species. Moreover, analysis of L-[^{15}N]-

Table 1[a]

oxidant	3-bromo-tyrosine (nmol)	3,5-dibromo-tyrosine (nmol)	total C−Br bonds (nmol)
(A) HOBr/OBr$^-$	19.6 ± 0.8	7.7 ± 1.1	35.1 ± 1.5
(B) *N*-bromotaurine	31.2 ± 0.4	32.2 ± 0.3	95.6 ± 0.3
(C) *N*,*N*-dibromotaurine	31.2 ± 0.1	27.4 ± 0.5	85.9 ± 1.1
(D) HOBr/OBr$^-$ and Br$^-$ (Br_2)	9.9 ± 0.3	4.3 ± 0.3	18.5 ± 0.3

[a] NaOBr (100 nmol in 2 μL) was added to 100 μL of sodium phosphate buffer (100 mM, pH 7.4) alone (A), in a buffer containing a 200-fold molar excess of taurine-forming *N*-bromotaurine (B), or in a buffer containing 50 nmol of taurine-forming *N*,*N*-dibromotaurine (C). Each of the indicated oxidants was then rapidly mixed with L-tyrosine (400 nmol in 100 μL) and incubated at 37 °C for 60 min. Ring-brominated products of the amino acid were subsequently quantified by reverse phase HPLC as described in Experimental Procedures. In experiment D, the equilibrium content of Br_2 in the final reaction mixture was increased by addition of NaBr (200 mM) to the L-tyrosine solution prior to addition of HOBr/OBr$^-$ as for experiment A. Reactions were performed in capped microcuvettes, and the concentrations of HOBr, *N*-bromotaurine, and *N*,*N*-dibromotaurine stocks were confirmed spectrophotometrically prior to use as described previously (*15*). The total yield of C−Br bonds was calculated as the sum of the number of moles of 3-bromotyrosine + 2 × the number of moles of 3,5-dibromotyrosine formed. Data represent the mean ± standard deviation of triplicate determinations. Similar results were observed in three independent experiments.

tyrosine and the EPO oxidation products by heteronuclear ($^1H-^{15}N$) multiple-bond correlation spectroscopy demonstrated nearly identical chemical shifts of the ^{15}N resonances of the parent and oxidized amino acids, confirming that they did not represent *N*-bromo- and *N*,*N*-dibromoamines (data not shown). Taken together, these results unambiguously establish that the major tyrosine oxidation products generated by the EPO−H_2O_2−Br$^-$ system are 3-bromotyrosine and 3,5-dibromotyrosine.

N-Bromoamines Mediate L*-Tyrosine Ring Bromination at Physiological pH.* The facile formation of ring-brominated L-tyrosine species, and the lack of any detectable *N*-bromoamines in the ^{15}N NMR analysis of tyrosine/HOBr and tyrosine/EPO/H_2O_2/Br$^-$ mixtures, was somewhat unexpected. It is well-known that incubation of HOBr/OBr$^-$ with molecules containing primary amines results in rapid formation of *N*-bromoamines (*19*, *44*). Indeed, inclusion of a large excess of amine-containing compounds in a peroxidase/H_2O_2/halide reaction mixture has been utilized as a method for trapping and quantifying (as an *N*-halo amine) active halogen formation (*8*, *15*). This, and previous reports of the labile nature of *N*-bromoamines (*8*, *28*, *44*, *45*), led us to investigate the possibility that *N*-bromoamines were not observed because they are formed and then rapidly reduced during subsequent phenolic ring bromination at neutral pH. To test this hypothesis, L-tyrosine was first incubated with either HOBr/OBr$^-$, *N*-bromotaurine, *N*,*N*-dibromotaurine, or HOBr/OBr$^-$ in the presence of excess Br$^-$ (to enhance the equilibrium content of Br_2 in the mixture; *20*), and the levels of 3-bromotyrosine and 3,5-dibromotyrosine formed were determined (Table 1). Ring-brominated tyrosine species were readily produced following addition of all reactive brominating species. In particular, *N*-bromoamines formed the stable brominated adducts in almost quantitative yield (96%) when comparing the number of moles of reactive bromine to the number of moles of C−Br bonds formed (Table 1).

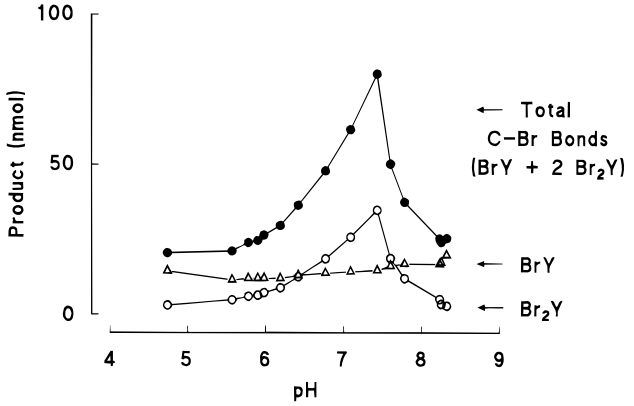

FIGURE 4: *N*-Bromoamines mediate L-tyrosine ring bromination in high yield at physiological pH. *N*-Bromotaurine (100 nmol) was added to L-tyrosine (1 μmol) in sodium phosphate buffer (50 mM) at various pHs at 37 °C as described in Experimental Procedures. Following incubation for 1 h, the contents of 3-bromotyrosine and 3,5-dibromotyrosine were determined by reverse phase HPLC as described in Experimental Procedures. Results represents the mean of duplicate determinations from a typical experiment which was performed in triplicate.

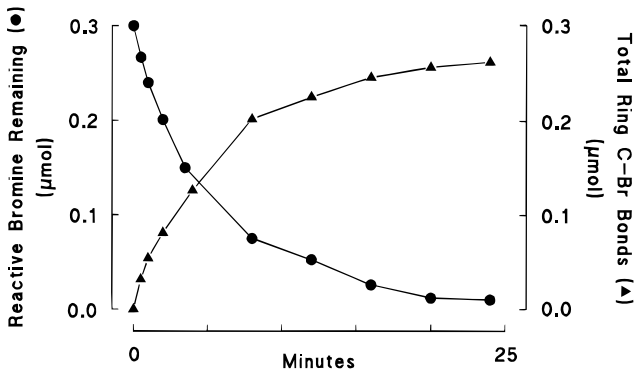

FIGURE 5: Stoichiometric reduction of *N*-bromoamines by L-tyrosine. N^{α}-Acetyl-N^{ϵ}-bromolysine was first prepared by addition of HOBr/OBr$^-$ to a 100-fold molar excess of N^{α}-acetyllysine in sodium phosphate buffer (50 mM, pH 7.4). N^{α}-Acetyl-N^{ϵ}-bromolysine (0.3 μmol) was then mixed with L-tyrosine (1 μmol) in sodium phosphate buffer (50 mM, pH 7.4) at 37 °C. At the indicated times, excess methionine (100 μmol) was added, and the contents of methionine sulfoxide, 3-bromotyrosine, and 3,5-dibromotyrosine present were determined as described in Experimental Procedures. The number of moles of reactive bromine remaining was calculated assuming that 1 mol of *N*-haloamine oxidizes 1 mol of methionine to form 1 mol of methionine sulfoxide (*30*). The total number of moles of ring C−Br bonds was calculated as the sum of the number of moles of 3-bromotyrosine + 2 × the number of moles of 3,5-dibromotyrosine produced. Results represent the mean of duplicate determinations for a typical experiment performed in duplicate.

To further characterize the ability of *N*-bromoamines to mediate tyrosine ring bromination, we incubated *N*-bromotaurine with L-tyrosine at different pHs and subsequently quantified 3-bromotyrosine and 3,5-dibromotyrosine formation (Figure 4). L-Tyrosine ring bromination occurred at all pHs examined. Interestingly, 3,5-dibromotyrosine was formed in particularly high yield at physiological pH, resulting in the near quantitative formation of C−Br bonds (with respect to moles of reactive halogen consumed) at pH 7.4 (Figure 4). We further examined the time course and stoichiometry of *N*-bromoamine reduction by L-tyrosine by incubating the amino acid with N^{α}-acetyl-N^{ϵ}-bromolysine (a low-molecular weight surrogate for a protein-bound *N*-bromoamine) for various times at 37 °C. Reactions were stopped by addition

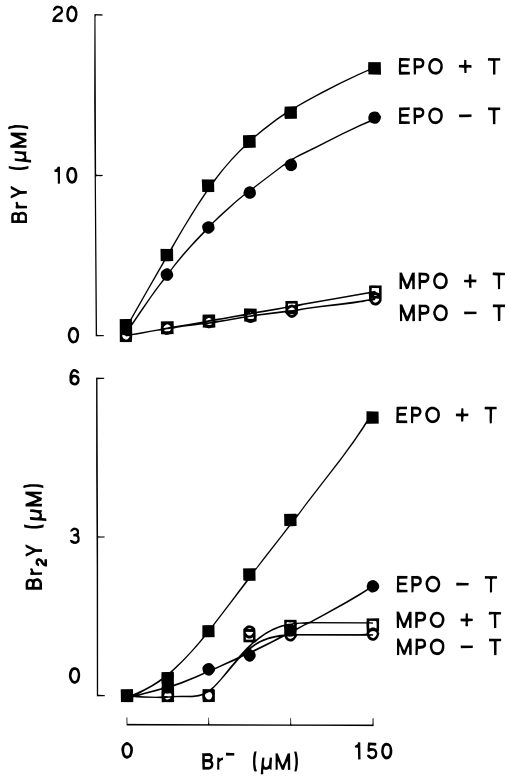

FIGURE 6: Bromide dependence of the L-tyrosine oxidation by eosinophil peroxidase and myeloperoxidase. L-Tyrosine (500 μM) was incubated with either eosinophil peroxidase (EPO) or myeloperoxidase (MPO) (14.2 nM each), H_2O_2 (100 μM), NaCl (100 mM), and the indicated concentration of Br^- (as the NaBr salt) in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C for 60 min. The contents of 3-bromotyrosine and 3,5-dibromotyrosine formed were then determined by reverse phase HPLC as described in Experimental Procedures. In the indicated samples, 20 mM taurine (T) was also included. Results represent the mean of duplicate determinations from a typical experiment performed in quadruplicate.

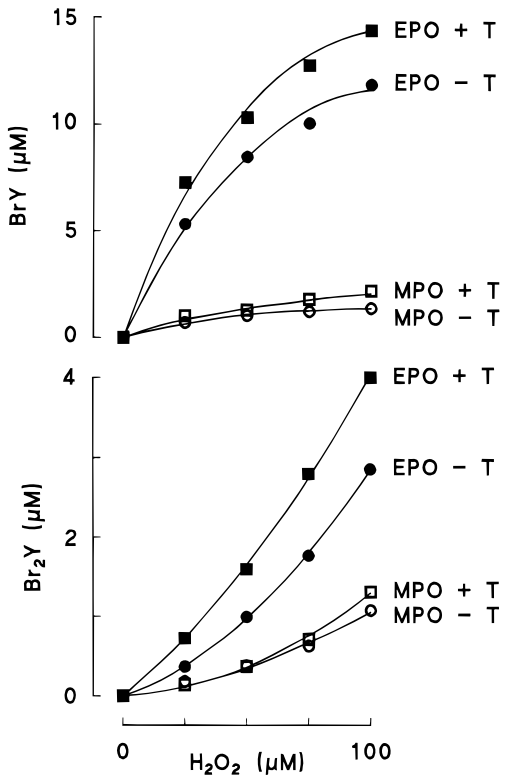

FIGURE 7: H_2O_2 dependence of L-tyrosine oxidation by isolated eosinophil peroxidase and myeloperoxidase at plasma levels of halides. L-Tyrosine (500 μM) was incubated with either eosinophil peroxidase (EPO) or myeloperoxidase (MPO) (14.2 nM each), NaCl (100 mM), NaBr (100 μM), and the indicated concentration of H_2O_2 in sodium phosphate buffer (20 mM, pH 7.0) at 37 °C for 60 min. Where indicated, taurine (20 mM) was also included. Following reaction, the contents of 3-bromotyrosine and 3,5-dibromotyrosine were determined by reverse phase HPLC as described in Experimental Procedures. Results represent the mean of duplicate determinations from a typical experiment performed in quadruplicate.

of a molar excess of methionine, and then the content of methionine sulfoxide (an indicator of reactive halogen remaining) and the total number of moles of C−Br bonds formed (the number of moles of 3-bromotyrosine + 2 × the number of moles of 3,5-dibromotyrosines) were determined. Each mole of *N*-bromoamine reduced resulted in the near stoichiometric formation of a stable C−Br bond (Figure 5). Collectively, these results demonstrate that *N*-bromoamines readily promote tyrosine ring bromination. They also suggest that the scavenging of EPO-generated HOBr/OBr^- by primary amines, abundant moieties in biological fluids, will serve as a mechanism for trapping and "funneling" reactive halogen to stable ring-brominated forms of tyrosine.

Eosinophil Peroxidase Generates Stable Ring-Brominated Tyrosine Species at Plasma Levels of Halides in High Yield. The ability of isolated EPO and MPO to form 3-bromotyrosine and 3,5-dibromotyrosine in the presence of physiological levels of halides was evaluated. EPO efficiently catalyzed formation of the ring-brominated forms of L-tyrosine even in the presence of a vast molar excess of Cl^- (Figures 6 and 8). Inclusion of a molar excess of taurine in reaction mixtures augmented ring bromination by EPO, consistent with *N*-bromoamines being intermediates in the reaction. In contrast, MPO failed to generate significant levels of ring-brominated tyrosine species at physiological levels of halides, even in the presence of excess amines (Figures 6 and 8).

In a parallel set of experiments, EPO and MPO were incubated with L-tyrosine, plasma levels of halides (100 mM Cl^- and 100 μM Br^-), and low doses of H_2O_2 similar to those which might be generated following phagocyte activation (*1*, *3*). Again, EPO, but not MPO, promoted tyrosine ring bromination in relatively high yield (Figures 7 and 8), and addition of excess primary amine-containing species (e.g., taurine) further augmented ring bromination (Figure 7). At higher levels of H_2O_2, the levels of 3-bromotyrosine and 3,5-dibromotyrosine production by EPO decreased, and addition of excess taurine to these reaction mixtures reversed this effect. These results are consistent with reduction of HOBr by excess H_2O_2 in the absence of added amine (*15*). In contrast, use of high levels of H_2O_2 ($\geq$100 μM) or Br^- ($>$200 μM) in incubations containing MPO demonstrated significant increases in the extent of formation of ring-brominated species, and addition of excess taurine to these mixtures inhibited ring bromination. These results suggest that under in vitro conditions of supraphysiological levels of H_2O_2 and Br^-, halide exchange (HOCl + Br^- = HOBr + Cl^-; *16*) accounted for MPO-dependent formation of the brominating intermediate (i.e., addition of taurine trapped HOCl as an *N*-chloroamine, preventing the oxidation of Br^- by secondary nonenzymatic reactions; *15*). Collectively, these

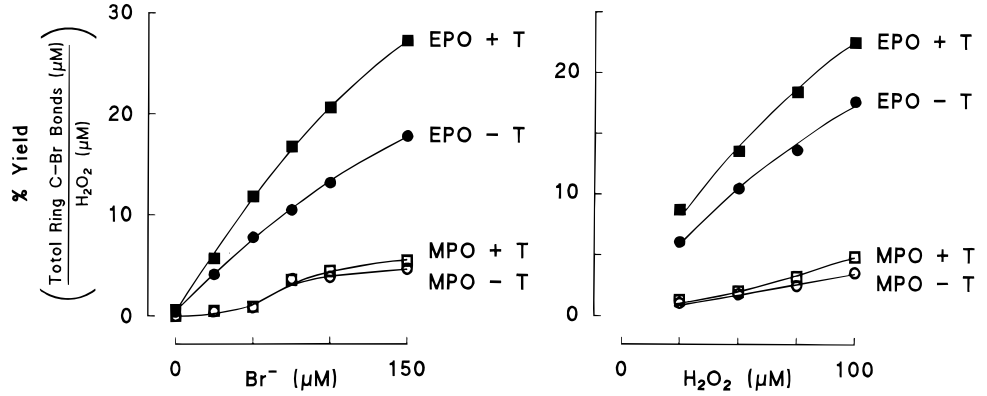

FIGURE 8: Yield of L-tyrosine bromination by eosinophil peroxidase and myeloperoxidase. The overall yields of L-tyrosine ring bromination (C−Br bonds) mediated by EPO and MPO in the experiments described in Figures 6 and 7 were calculated as the sum of the number of moles of 3-bromotyrosine + 2 × the number of moles of 3,5-dibromotyrosine formed divided by the number of moles of oxidant (H_2O_2) added to the reaction mixture. Results represent the mean of duplicate determinations for a typical experiment performed in quadruplicate.

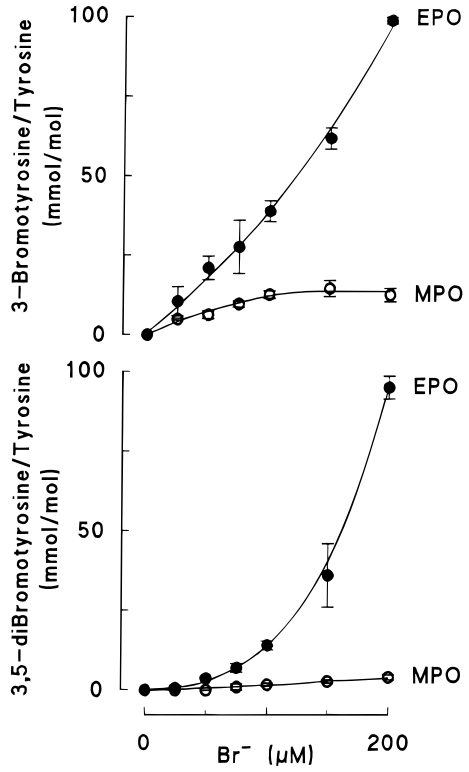

FIGURE 9: Bromide dependence of protein tyrosyl residue bromination by eosinophil peroxidase and myeloperoxidase. BSA (0.4 mg/mL) was incubated with either eosinophil peroxidase (EPO) or myeloperoxidase (MPO) (28.4 nM each), H_2O_2 (100 μM), NaCl (100 mM), and the indicated concentration of Br^- (as the NaBr salt) in sodium phosphate buffer (20 mM, pH 7.0) for 1 h at 37 °C. Following reaction, proteins were desalted and subjected to acid hydrolysis, and the contents of 3-bromotyrosine and 3,5-dibromotyrosine were determined as described in Experimental Procedures. Data represent the mean ± standard deviation from results of four independent experiments.

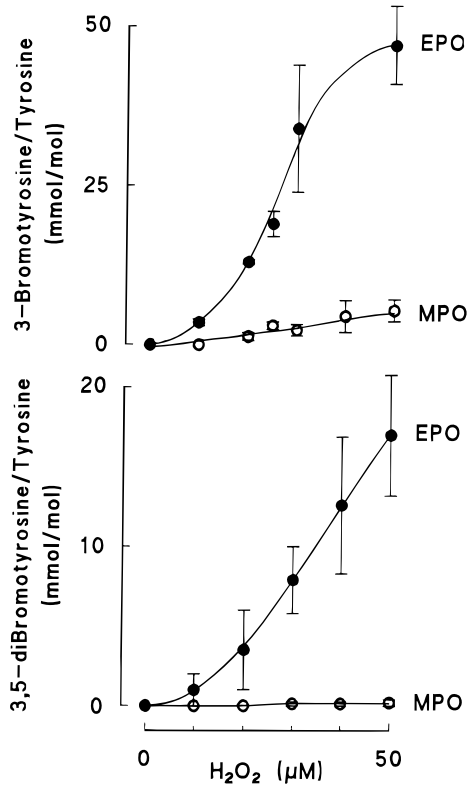

FIGURE 10: H_2O_2 dependence of protein tyrosyl residue bromination by eosinophil peroxidase and myeloperoxidase at plasma levels of halides. BSA (0.4 mg/mL) was incubated with either eosinophil peroxidase (EPO) or myeloperoxidase (MPO) (28.4 nM each), NaCl (100 mM), NaBr (100 μM), and the indicated concentration of H_2O_2 in sodium phosphate buffer (20 mM, pH 7.0) for 60 min at 37 °C. Following reaction, proteins were desalted and subjected to acid hydrolysis, and the 3-bromotyrosine and 3,5-dibromotyrosine contents were determined as described in Experimental Procedures. Data represent the mean ± standard deviation from results of four independent experiments.

results demonstrate that at plasma levels of halides and physiologically plausible levels of H_2O_2, EPO, but not MPO, readily promotes aromatic bromination of L-tyrosine.

Eosinophil Peroxidase Brominates Protein Tyrosine Residues in High Yield at Plasma Levels of Halides. To further explore the role of EPO in promoting tyrosine bromination, we examined the ability of the isolated enzyme to generate 3-bromotyrosine and 3,5-dibromotyrosine on intact proteins. Bovine serum albumin (BSA) was incubated with either EPO or MPO, H_2O_2, plasma levels of Cl^- (100 mM), and varying levels of Br^- (0−200 μM), and then the content of ring-brominated tyrosine residues formed was determined as described in Experimental Procedures. At all concentrations of Br^- examined, EPO promoted bromination of protein tyrosyl residues (Figures 9 and 11). In contrast, little if any detectable brominated tyrosine species were formed by isolated MPO (Figures 9 and 11). In parallel experiments, BSA was incubated with either EPO or MPO, plasma levels

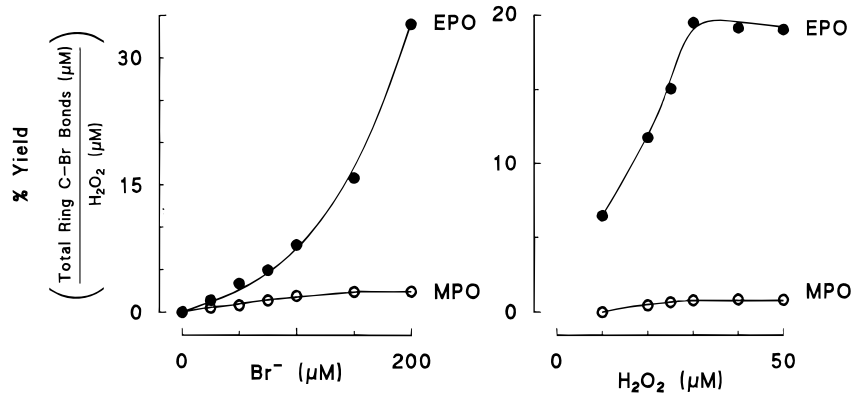

FIGURE 11: Overall yield of protein tyrosyl residue bromination by eosinophil peroxidase and myeloperoxidase at physiological concentrations of halides and H_2O_2. The overall yield of total ring bromination (number of moles of C−Br bonds) of protein tyrosyl residues in BSA oxidized by EPO and MPO for the experiments described in the legends of Figures 9 and 10 were calculated as described in the legend of Figure 8. Data represent the mean from results of four independent experiments.

of halides (100 mM Cl^- and 100 μM Br^-), and low doses of H_2O_2 (0−50 μM). Again, EPO promoted protein tyrosyl residue bromination, but MPO failed to generate any significant brominated products (Figures 10 and 11). Examination of the overall yield of the reaction (Figure 11) confirmed that EPO, but not MPO, efficiently incorporated halogen into stable ring-brominated forms (C−Br bonds) at plasma levels of halides. Collectively, these results demonstrate that 3-bromotyrosine and 3,5-dibromotyrosine are excellent candidate molecular markers for identifying sights of EPO-catalyzed protein oxidative damage in vivo.

DISCUSSION

Markers of proteins damaged by EPO would provide powerful tools for studying the importance of activated eosinophils in the origins of tissue injury. Substantial evidence implicates eosinophil activation, EPO secretion, and oxidative damage in the cellular injury characteristic of disorders such as asthma, allergic inflammatory conditions, certain cancers, and parasitic infections (*1*−*3*). However, direct demonstration that EPO promotes oxidative damage to tissues in vivo is lacking. Our goal was therefore to identify stable and specific products of EPO-mediated protein oxidative damage which might eventually serve as molecular "fingerprints" to define the role of these cells in mediating tissue injury in vivo.

Eosinophils are distinct among phagocytic cells in their ability to form brominating oxidants in the presence of physiological concentrations of halides (*8*, *9*). The unique ability of these leukocytes to preferentially generate brominating agents is due to the unusual substrate preference of EPO (*9*, *15*, *29*). Because EPO is the only known pathway for selective formation of brominating intermediates in humans at plasma levels of halides, brominated compounds have the potential to serve as sensitive and specific molecular markers for EPO-dependent tissue damage. In this study, we have identified 3-bromotyrosine and 3,5-dibromotyrosine as stable, specific, and abundant products formed in proteins oxidized by EPO in the presence of plasma levels of halides. Structural confirmation of the ring-brominated adducts was achieved by multiple independent methods, including ESI-MS, GC−MS, and both ^1H and ^{15}N NMR spectroscopy. EPO, but not MPO, readily formed protein-bound 3-bromotyrosine and 3,5-dibromotyrosine at neutral pH and physiological levels of halides. Thus, detection of these products in vivo, particularly during inflammatory conditions where eosinophils are abundant, would implicate a role for EPO in oxidative tissue damage. Although eosinophils are the only known cell type which synthesizes EPO, other phagocytic cells, such as neutrophils, mast cells, and basophils (*59*−*62*), have been shown to be capable of binding and internalizing this highly cationic protein, and may thus promote tissue injury through EPO-dependent formation of reactive brominating species. Furthermore, although MPO did not brominate protein tyrosine residues appreciably in the presence of plasma levels of Cl^- and Br^- (Figure 11), this abundant leukocyte peroxidase may contribute to protein bromination, particularly at sites where Br^- is abundant and Cl^- is limiting.

One remarkable feature of EPO-catalyzed bromination of protein tyrosine residues is its overall high yield at neutral pH and plasma levels of halides. The rapid rate of formation of *N*-bromoamines in aqueous solution (*19*, *44*) would suggest that these species would be formed rather than products of aromatic bromination. Alternatively, *N*-bromoamines of free α-amino acids would be anticipated to undergo a deamination and decarboxylation reaction, yielding an aldehyde, as is seen with HOCl-dependent oxidation of free α-amino acids (*34*, *35*), and HOBr-mediated oxidation of the nonaromatic amino acid L-serine (*34*). The high yield and neutral pH preference for ring bromination of protein tyrosyl residues are thus distinguished from those observed with MPO-dependent ring chlorination reactions, which occur in significantly lower yield and with a strong acid pH dependence (*12*, *30*, *34*, *35*, *39*, *41*, *42*, *63*). This difference is due to the ability of *N*-bromoamines, but not *N*-chloroamines (*17*, *64*), to readily promote aromatic halogenation at neutral pH. In fact, at pH 7.4, reduction of *N*-bromoamine intermediates by tyrosine resulted in the near stoichiometric formation of stable C−Br bonds in ring-brominated adducts (Figure 5). Protein-bound and free primary amines are some of the most abundant nucleophilic moieties that reactive halogenating species will encounter in vivo (*25*). The combined concentration of primary amino groups in serum or the cytosol of leukocytes (e.g., neutrophils and eosinophils) is approximately 50 or 150 mM, respectively (*30*, *65*). The ability of *N*-bromoamines formed to readily promote aromatic bromination of tyrosine is thus anticipated to prolong the

effective half-life of brominating agents formed in vivo and to funnel brominating equivalents toward stable ring-brominated adducts of tyrosine. Moreover, it has been suggested that the abundance of the taurine within phagocytes (~20 mM) serves to trap oxidized forms of halides and protect critical intracellular targets from oxidation and halogenation (*15*, *65*). The facile reduction of *N*-bromoamines resulting in aromatic halogenation reactions raises the interesting possibility that high intracellular levels of the β-amino acid will instead promote post-translational modification of accessible intracellular tyrosine resides through bromination.

The biological consequences of bromotyrosine formation by EPO are unknown. Several marine organisms and prokaryotes have peroxidases (e.g., bromoperoxidase and chloroperoxidase) which are used in the biosynthesis of brominated natural products (*47*, *66*, *67*). A variety of brominated tyrosine analogues have been described in these organisms, and many are known to possess antitumor, viricidal, or bacteriocidal properties (*68*−*74*). Eosinophils play an essential role in vivo, destroying pathogenic microorganisms, parasites, and tumor cells. It is therefore tempting to speculate that the EPO−H_2O_2−Br^- system of eosinophils may similarly promote some cytostatic, antimicrobial, or antimalignant effects through formation of bromotyrosine derivatives.

Eosinophils have evolved enzymatic mechanisms for generating an arsenal of reactive species which are critical to host defenses; however, these agents also have the potential to harm healthy tissue. Although EPO is believed to mediate oxidative damage and cellular injury in disorders where eosinophil activation occurs, direct demonstration of EPO-dependent oxidative damage is lacking. This study demonstrates that 3-bromotyrosine and 3,5-dibromotyrosine are stable and specific oxidation products formed by the action of EPO on target proteins. Their detection in vivo might thus serve to identify sites of eosinophil activation and the role of reactive brominating species in protein oxidative damage during inflammatory diseases.

ACKNOWLEDGMENT

We thank Mrs. Mimi Passalaqua for assistance in the preparation of the manuscript. ESI-MS experiments were performed at the Cleveland Mass Spectrometry Core Facility. NMR spectroscopy was performed at the Cleveland Structural Biology NMR Core Facility and the Washington University High Resolution NMR Facility.

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BI982401L