Caenorhabditis elegans and Human Dual Oxidase 1 (DUOX1) “Peroxidase” Domains

INSIGHTS INTO HEME BINDING AND CATALYTIC ACTIVITY

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The seven members of the NOX/DUOX family are responsible for generation of the superoxide and \( \text{H}_2\text{O}_2 \) required for a variety of host defense and cell signaling functions in non-phagocytic cells. Two members, the dual oxidase isoforms DUOX1 and DUOX2, share a structurally unique feature: an N-terminal peroxidase-like domain. Despite sequence similarity to the mammalian peroxidases, the absence of key active site residues makes their binding of heme and their catalytic function uncertain. To explore this domain, we have expressed CeDUOX11–589, a dual oxidase domain from the nematode *Caenorhabditis elegans* (CeDUOX11–589) and human (hDUOX11–593) DUOX1 “peroxidase” domains. Evaluation of these proteins demonstrated that the isolated hDUOX11–593 does not bind heme and has no intrinsic peroxidase activity. In contrast, CeDUOX11–589 binds heme covalently, exhibits a modest peroxidase activity, but does not oxidize bromide ion. Surprisingly, the heme appears to have two covalent links to the protein despite the absence of a second conserved carboxyl group in the active site. Although the N-terminal dual oxidase motif has been proposed to directly convert superoxide to \( \text{H}_2\text{O}_2 \), neither DUOX1 domain demonstrated significant superoxide dismutase activity. These results strengthen the *in vivo* conclusion that the CeDUOX1 protein supports controlled peroxidative polymerization of tyrosine residues and indicate that the hDUOX1 protein either has a unique function or must interact with other protein factors to express its catalytic activity.

The purposeful generation of reactive oxygen species within phagocytic cells has long been recognized as a component of their antibacterial defense system (1, 2). Reactive oxygen species generation is mediated by a membrane-bound NADPH oxidase (NOX)\(^3\) and is activated by a diverse number of stimuli. The NOX enzymes catalyze the NADPH-dependent one-electron reduction of oxygen to superoxide (\( \text{O}_2^- \)) (3). It has long been debated whether the generation of similar species in other cell types is also an intentional, physiologically controlled process or is an accident of aerobic respiration. This controversy has been clarified by identification of the NOX/DUOX family of NADPH oxidases. The seven members of this family (NOX 1–5 and DUOX1 and 2) have been shown to produce the reactive oxygen species utilized for functions as varied as cellular signaling, host defense, and thyroid hormone biosynthesis (4–8). The latter function is specifically attributed to the DUOX members of this family.

DUOX1 and 2 (formerly also known as ThOX1 and 2 for thyroid oxidase) were first identified in the mammalian thyroid gland (9, 10). This localization is not exclusive because both can also be found in nonthyroid tissues; DUOX1 is prominent in airway epithelial cells (11) and DUOX2 in the salivary glands and gastrointestinal tract (4, 12, 13). Homologs of each DUOX have also been identified in lower organisms, including *Caenorhabditis elegans* and *Drosophila melanogaster* (14). The human isoforms are 83% homologous, ~190 kDa in size (after glycosylation), and are located in close proximity, because they are configured head-to-head on human chromosome 15 (15, 16). The glycosylation of both DUOX1 and 2 is extensive, contributing ~30 kDa to the total apparent protein mass (17). Recent investigation has uncovered that maturation factors DUOX1 and DUOX2 are required to achieve heterologous expression of each DUOX in full-length, active form (18).

Structurally, DUOX1 and 2 are characterized by a defining N-terminal, extracellular domain exhibiting considerable sequence identity with the mammalian peroxidases, a transmembrane (TM) segment appended to an EF-hand calcium-binding cytosolic region and a NOX2 homologous structure (six TMs tethered to NADPH oxidase; see Fig. 1A) (10). Both isoforms have a conserved calcium-binding site in the N-terminal peroxidase domain, mimicking that found in MPO, LPO, EPO, and TPO. Interestingly, although homologous to these heme-containing peroxidases, the peroxidase-like domains of the DUOX proteins lack some of the highly conserved amino acid residues that are thought to be essential for heme binding and/or peroxidase catalysis (see Fig. 1B) (16).

Functionally, mature DUOX enzymes appear to produce \( \text{H}_2\text{O}_2 \) in contrast to other NOX family members that produce superoxide. This activity is regulated by \( \text{Ca}^{2+} \) concentration through triggered dissociation of NOXA1 and possibly other as yet unidentified interacting proteins (19). Because the N-terminal peroxidase domain is the structural feature that differenti-
ates the dual oxidases from the NOX proteins, it may be directly responsible for the conversion of superoxide to $\text{H}_2\text{O}_2$. To investigate this crucial domain, we report here the first expression, purification, and characterization of the Homo sapiens (hDUOX1$_{1–593}$) and C. elegans (CeDUOX1$_{1–589}$) DUOX1 peroxidase domains. We demonstrate that heme is covalently bound to CeDUOX1$_{1–589}$ (two covalent bonds are suggested by heme hydroxylation studies), whereas hDUOX1$_{1–593}$ does not stably bind this co-factor. Both domains share overall sequence similarity with the mammalian peroxidases (specifically LPO), but only CeDUOX1$_{1–589}$ exhibits peroxidase activity, as measured with either ABTS or tyrosine ethyl ester as the substrate. We also demonstrate that neither DUOX1 domain has significant superoxide dismutase or halide oxidizing activity.

**EXPERIMENTAL PROCEDURES**

*Materials and General Methods*—Sf9 cells (Invitrogen) were grown in ExCell 420™ medium (SAFC Biosciences) supplemented with glutamine (2.7 g/liter). High Five™ cells were grown in Express Five™ medium (Invitrogen) supplemented with glutamine (2.7 g/liter) and 10% fetal bovine serum. Both cell lines were kept in suspension at 27 °C (100 rpm) and maintained at densities between 0.5 $\times$ 10$^6$ and 2 $\times$ 10$^6$ cells/ml. Trifluoroacetic acid, formic acid, $\text{H}_2\text{O}_2$ (30% w/w), ABTS, LPO (bovine), CaCl$_2$, and 5.5-dimethyl-1,3-cyclohexanedione were purchased from Sigma-Aldrich. T4 DNA ligase and restriction endonucleases were obtained from New England Biolabs. DNA sequencing was performed by Elim Biopharmaceuticals. The entire gene insert was completely sequenced for each plasmid construct. For protein identification, mass spectra of all trypsin digests were obtained on a QSTAR-XL hybrid QqTOF mass spectrometer (Applied Biosystems; supplemental Fig. S1). Digests were obtained on a QSTAR-XL hybrid QqTOF mass spectrometer (Applied Biosystems; supplemental Fig. S1). For protein identification, mass spectra of all trypsin digests were obtained on a QSTAR-XL hybrid QqTOF mass spectrometer (Applied Biosystems; supplemental Fig. S1).

**Determination of hDUOX1 Peroxidase Domains**

**Heme Staining Protocol**—Detection of heme-protein complexes from SDS–PAGE was achieved by incubation with a solution containing tetramethylbenzidine and the addition of $\text{H}_2\text{O}_2$ for color development, as previously described (22). Experimental details are included in the *supplemental data*.

**HPLC Analysis of Covalent Heme Binding**—Samples were analyzed by direct injection onto a 150 × 4.6-mm C4 reversed phase column (Vydac 214MS) on an Agilent 1200 series HPLC instrument. The protein was eluted with a linear gradient of 20–50% acetonitrile in water (0.1% trifluoroacetic acid) over 30 min (1 ml/min) with detection at 215 and 400 nm. For covalent heme binding as a function of $\text{H}_2\text{O}_2$, CeDUOX1$_{1–589}$ (26 μM) was incubated with $\text{H}_2\text{O}_2$ (26–520 μM) for 5 min at 25 °C, pH 8.0. Excess $\text{H}_2\text{O}_2$ was then consumed by incubation with catalase (5 units) for 5 min. The resulting samples were chromatographed as described above, with the proportion of covalently bound heme determined by the percentage of the total 400-nm integration attributed to the peak(s) that co-eluted with the 215-nm absorbing protein peak (27–30 min).

**CeDUOX1$_{1–589}$ Dihydroxyheme Isolation**—CeDUOX1$_{1–589}$ (2 mg/ml, 70 μl) was digested by incubation for 1 h with trypsin

Table S1 and subcloned into TOPO vector pCR 2.1 (Invitrogen; pJLM05). This plasmid construct was subsequently digested with BamHI and EcoRI, and the gene was inserted by ligation into pAcGP67-b (pJLM08).

The original source of the *C. elegans* *duox1* 1–589 gene was a ProQuest *C. elegans* cDNA Library (Invitrogen). The gene encoding the peroxidase domain of CeDUOX1 (residues 1–589) was amplified from the cDNA library using primers JLM-Bac-Celegans1F and JLM-BacC-Celegans1R that introduced BamHI and PstI sites onto the 5’ and 3’ ends of the *duox1* 1–589 open reading frame (supplemental Table S1). The PCR product was subcloned into TOPO vector pCR 2.1 (Invitrogen; pJLM022). This plasmid construct was subsequently digested with BamHI and PstI, and the gene was inserted by ligation into pAcGP67-b (pJLM023).

**Generation of Recombinant Baculoviruses**—Plasmids pJLM08 and pJLM023 were co-transfected with viral baculogold DNA into Sf9 insect cells, according to the manufacturer’s instructions (BD Biosciences). The resulting viruses were plaque-assayed to generate high-titer recombinant baculovirus stocks amplified (two rounds of amplification in Sf9 cells) from single viral populations.

**Expression and Purification of hDUOX1$_{1–593}$**—In a typical overexpression, 1 liter of High Five™ cells (2.0 $\times$ 10$^6$ cells/ml) supplemented with 250 μM 5-aminolevulinic acid (Fluka) were infected with the hDUOX1$_{1–593}$-His$_6$ viral stock at a multiplicity of infection of 5 for 3 days at 27 °C. After 72 h of infection, cell suspensions were concentrated and purified by nickel affinity chromatography (nickel-nitrilotriacetic acid–agarose; see supplemental data for purification details); the purified protein was stored at −20 °C. The protein stock concentration was determined by Bradford assay in triplicate (21).

**Expression and Purification of CeDUOX1$_{1–589}$**—CeDUOX1$_{1–589}$-His$_6$ was overexpressed and purified as described above for hDUOX1$_{1–593}$-His$_6$; the purified protein was stored at −20 °C, and its concentration was determined by Bradford assay.

**Heme Staining Protocol**—Detection of heme-protein complexes from SDS–PAGE was achieved by incubation with a solution containing tetramethylbenzidine and the addition of $\text{H}_2\text{O}_2$ for color development, as previously described (22). Experimental details are included in the *supplemental data*.

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(1 mg/ml, 2 μl) at room temperature, followed by the addition of proteinase K (Fermentas; 20 mg/ml, 3 μl); the digest was continued overnight at 37 °C. As a control, hemin was purchased from Sigma-Aldrich; a stock solution was created by dissolving 1–2 mg in 200 μl of 1 N NaOH, followed by the addition of 9.8 ml of 20 mM phosphate buffer, 0.4 M NaCl, pH 8.0); 2.5 μl stock was diluted into 25 μl of buffer for analysis. Digested protein (40 μl) and porphyrin (25 μl) separations were achieved on a Vydac 214MS reversed phase C4 column (150 × 4.6 mm) using water containing 0.1% trifluoroacetic acid (solvent A) and acetonitrile containing 0.09% trifluoroacetic acid (solvent B) on an Agilent 1200 series HPLC instrument. All of the samples were eluted at a flow rate of 1 ml/min, with a 3-min isocratic wash of 15% solvent B, followed by a linear gradient of 15–43% solvent B over 31 min, monitored at 400 nm. The same gradient and column were utilized for analysis of the observed peaks by LC-MS.

Reduction and CO Binding Studies of CeDUOX11–589—The ferrous and CO bound absorbance spectra for CeDUOX11–589 (6 μM) were collected in 100 mM phosphate buffer, pH 7.0; 800 μl of total sample volume. To achieve reduction, sodium dithionite (8 μl, 100 mM) was added to an argon flushed enzyme sample in a sealed cuvette. After reduction, the enzyme was incubated with CO for 30 s, and the spectra were collected until saturation was demonstrated.

Cyanide Binding to CeDUOX11–589—The cyanide (CN−) bound absorbance spectrum for CeDUOX11–589 (6 μM) was collected via titration with KCN in 100 mM phosphate buffer, pH 7.0. A stock solution of KCN (10 mM) was utilized to titrate from 12.5 to 225 μM until enzyme saturation was demonstrated. A titration curve was established by plotting the change in absorbance (∆A), from subtraction of difference spectra maxima (423 nm) and minima (402 nm), against CN− concentration; a dissociation constant was determined from this plot at half the maximum ∆A observed.

ABTS Activity Assay Comparison of the DUOX1 Homologs—Peroxidase activity was measured using 500 nM ABTS incubated with 500 nM LPO, hDUOX11–593, or CeDUOX11–589 in 50 mM phosphate buffer (pH 7.0, treated with Chelex-100 resin (Bio-Rad)). The reactions were initiated by the addition of H2O2 (50 μM). Each reaction was monitored by recording the absorbance intensity at 414 nm as a function of time. The molar extinction coefficient for ABTS at 414 nm is ε = 36,800 M−1 cm−1 (23).

Peroxidase Activity Assay(s) with Tyrosine Ethyl Ester—Enzyme peroxidase activity was measured using tyrosine ethyl ester as a substrate (1 mM) in phosphate-buffered saline (pH 7.4, treated with Chelex-100 resin (Bio-Rad)). LPO, CeDUOX11–589 and hDUOX11–593 (1 μM) were assayed; each reaction was initiated with the addition of H2O2 (100 μM). Initial reaction kinetics of tyrosine cross-linking were monitored using a Fluorolog 3 Spectrofluorometer (Horiba Jobin Yvon), with excitation at 295 nm and emission monitored at 414 nm using 1-nm excitation and emission slits at room temperature. After 1 h of reaction time, LPO and CeDUOX11–589 assays were further analyzed by direct injection onto a 50 × 2.1-mm C18 reversed phase column (Waters X TerraMS) on an Agilent 1200 series HPLC instrument. The assays were characterized utilizing an isocratic gradient of 9% acetonitrile in water (0.1% formic acid), followed by a linear gradient of 9–10% acetonitrile in water (0.1% formic acid) over 10 min (0.2 ml/min) with detection at 280 nm and fluorescence detection by excitation at 295 nm and emission at 414 nm. The same gradient and column were utilized for analysis of the observed peaks by LC-MS.

Superoxide Dismutase Activity Assays(s)—SOD activity was measured by the percentage reaction inhibition rate of enzyme (SOD, hDUOX11–593, or CeDUOX11–589) with WST-1 substrate (a water soluble tetrazolium dye) and xanthine oxidase using a SOD assay kit (Fluka; 19160) according to the manufacturer’s instructions. Each end point assay was monitored by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. All of the enzyme stocks were made in 50 mM HEPES buffer, pH 7.0, 5% glycerol (to allow for CaCl2 addition); each assay was performed in triplicate.

Bromide Oxidation Activity Assay(s)—5,5-Dimethyl-1,3-cyclohexanedione (1 mM, 50 μl), NaBr (2 mM, 50 μl), and enzyme (50 mM LPO, 50 μl; 5 mM CeDUOX11–589 or hDUOX11–593, 50 μl) were mixed in a quartz cuvette. To this solution was added 850 μl of H2O2 (0.52 mM in 50 mM citrate buffer, pH 4.4). The resulting solution was mixed, and the absorbance at 292 nm was recorded as a function of time. The initial velocity was calculated from the change in absorbance between 10 and 30 s; the molar extinction coefficient of 5,5-dimethyl-1,3-cyclohexanedione at 292 nm is ε = 20,000 M−1 cm−1 (24).

RESULTS

Design and Expression of Soluble DUOX1 Peroxidase Domain Constructs—hDUOX1 and CeDUOX1 are large membrane-bound proteins (~180 kDa) predicted to contain an extracellular N-terminal domain tethered by a transmembrane helix to a C-terminal region with six TM domains, two cytosolic EF hand motifs, and an NADPH oxidase domain (Fig. 1A). This N-terminal extracellular domain is homologous to classical peroxidases such as MPO and TPO (43% sequence similarity) (17); however, neither the hDUOX1 nor the CeDUOX1 peroxidase domain contains the full complement of heme-binding and catalytic residues, as shown in Fig. 1B. This partial sequence alignment with the classical peroxidases demonstrates that both DUOX homologs lack the catalytic distal histidine residue (Ser108, hDUOX1; and Tyr107, CeDUOX1) and the aspartate residue that in MPO and LPO covalently binds to the heme co-factor through an ester linkage (Leu107, hDUOX1; and Ala104, CeDUOX1) (25, 26). hDUOX1 lacks additional critical peroxidase residues: the glutamate that forms the second covalent bond to the heme (Arg241) and the proximal histidine residue (Ser331). Both of these amino acids are conserved in the CeDUOX1 homolog. Despite sequence similarity, the only critical residue of the heme-binding region conserved among mammalian peroxidases and the DUOX proteins is the catalytic arginine residue (Arg210, hMPO; Arg238, hDUOX1; and Arg235, CeDUOX1). Soluble constructs are required to elucidate the function of this region for both DUOX homologs.

Initially, the full-length human and C. elegans DUOX1 proteins were truncated using the TMHMM Server version 2.0 transmembrane helix algorithm to identify the primary
sequences likely to compose their entire extracellular peroxidase domains (27). Based on this analysis, we designed constructs of hDUOX1 (residues 1–593) and CeDUOX1 (residues 1–589) for expression in Escherichia coli. N- and C-terminal His6-tagged and glutathione S-transferase-tagged constructs were created but attempts at expression under various conditions did not yield soluble protein (data not shown). As a result, our expression efforts shifted to a baculovirus system. This technique often yields recombinant proteins with the proper folding, oligomerization, and post-translational modifications, thus generating a protein structurally and functionally similar to its native counterpart. Both truncated DUOX1 homologs were inserted into baculovirus expression vector pAcGP67-b, which allows for secretion of the recombinant protein (resulting in the N-terminal addition of five amino acids to the expressed protein as part of the secretion signal sequence; supplemental Fig. S1). N- and C-terminal His6-tagged constructs of the DUOX1 proteins were co-transfected with baculovirus DNA into Sf9 cells to obtain high titer viral stocks for infection (29).

To examine the extent of heme attachment to CeDUOX11–589, an HPLC system was utilized under acidic conditions that favor dissociation of noncovalently bound heme from the apoprotein (29). As shown in Fig. 2C, only 60% of the prosthetic heme co-elutes with the 215-nm absorbing protein peak; this is consistent with results for LPO expressed in baculovirus (29). Investigation of this recombinant LPO covalent heme binding has shown the process to be autocatalytic, as opposed to requiring an enzymatic system such as that required for covalent heme attachment in cytochrome c (30–32). To determine whether a similar autocatalytic process is responsible for heme oxidation and covalent incorporation in CeDUOX11–589, the protein was preincubated with varying concentrations of H2O2, and the extent of covalent heme binding was evaluated by HPLC. Fig. 2D highlights the results of H2O2 treatment, clearly demonstrating that the covalent binding reaction is autocatalytic, because the fraction of protein-bound heme increases as a function of H2O2 concentration. A maximum for the amount of heme incorporation (81%) was achieved with 5 equivalents of H2O2; the Soret maximum shifted from 408 to 410 nm at this percentage of incorporation after H2O2 treatment.
With covalent heme binding established as a major interaction between co-factor and enzyme, the nature of this linkage in CeDUOX1–589 was examined. Mammalian peroxidases form covalent linkages to the 1- and 5-methyl groups of the heme porphyrin through conserved aspartate and glutamate residues (Fig. 1B). The result of enzymatic digestion and LC-MS analysis of the classic peroxidases with two covalent bonds (LPO, TPO, and EPO) is a 1,5-dihydroxyheme derivative. Mutation studies of heme proteins (horseradish peroxidase F41E, horseradish peroxidase S73E, LPO D225E, and LPO E375D) to investigate the autocatalytic nature and sequence of covalent bond formation have also led to non-native linkages at the 3- and 8-methyl positions (33, 34). Because of single covalent bond formation in each mutant enzyme, once digested (trypsin/Pronase or proteinase K), each liberated heme co-factor was found as a mono- or dihydroxyheme (1-OH, 3-OH, 5-OH, or 8-OH) derivative. For further investigation of the nature of covalent heme binding, CeDUOX1–589 was digested and analyzed by HPLC and LC-MS to determine whether a monohydroxy- or dihydroxyheme derivative would result. A heme derivative was isolated (m/z 650, t = 13.2 min), and determined, both by retention time and mass, to be dihydroxyheme, suggestive of two covalent protein-heme linkages (Fig. 3). This covalent bond character, although consistent with that of the known mammalian peroxidases, was highly unexpected in the case of CeDUOX1–589 because the aspartate residue required for covalent heme binding is not conserved in this enzyme. It is clear that a different carboxyl residue or a different amino acid altogether is involved in formation of the second covalent bond to the heme.

Structural Stability of the DUOX1 Proteins—Circular dichroism measurements were performed on hDUOX1–593 and CeDUOX1–589 to confirm that the protein constructs are properly folded (supplemental Fig. S2). The far-UV spectra (195–250 nm) for both homologs support an α-helical structure, with prominent troughs at 208 and 220 nm, characteristic of LPO and other mammalian peroxidases (35–37). For further insight into the stability of hDUOX1–593 and CeDUOX1–589, Trp fluorescence emission spectra were collected for each upon excitation at 292 nm, exhibiting broad emission bands with λmax at 341 and 348 nm, respectively (supplemental Fig. S2). The observed wavelength maxima shift to 351 nm upon thermal unfolding (80 °C; supplemental Fig. S2) and decrease in fluorescence intensity, consistent with quenching caused by solvent exposure upon unfolding. Chemical denaturation of both proteins using 7 M guanidine hydrochloride resulted in a further red shift of the λmax to ~360 nm; this result suggests that the DUOX peroxidase domain(s) retain some tertiary structure upon thermal denaturation, consistent with previous studies on LPO (37).

Small Ligand Binding to CeDUOX1–589—CO and CN− molecules bind tightly to mammalian peroxidases (38, 39). Both maxima suggest a folded protein structure resulting in protection of Trp residues from solvent exposure. The DUOX1 homologs contain a large number of Trp residues (hDUOX1–593, 19 Trp; CeDUOX1–589, 11 Trp); both the disparity in the number of Trp residues and quenching by the heme chromophore likely contribute to the difference in emission intensity. The observed wavelength maxima shift to ~351 nm upon thermal unfolding (80 °C; supplemental Fig. S2) and decrease in fluorescence intensity, consistent with quenching caused by solvent exposure upon unfolding. Chemical denaturation of both proteins using 7 M guanidine hydrochloride resulted in a further red shift of the λmax to ~360 nm; this result suggests that the DUOX peroxidase domain(s) retain some tertiary structure upon thermal denaturation, consistent with previous studies on LPO (37).
cation with the heme iron in the distal binding pocket. To determine whether the binding pocket of CeDUOX1–589 will allow such interactions, steady-state absorbance spectra were collected and are shown in Fig. 4. Ferric CeDUOX1–589 exhibits a Soret band maximum at 408 nm with bands at 533 and 623 nm in the visible region (Fig. 4, A and B, and supplemental Table S2). Upon reduction with sodium dithionite, CeDUOX1–589 undergoes a red shift to 425 nm, with Q band maxima at 533 and 557 nm. Exposure of the reduced protein to CO leads to the formation of a complex with the ferrous heme iron, demonstrated by a blue shift of the Soret band to 419 nm, with Q bands at 539 and 563 nm.

Cyanide adduct formation, in contrast to CO, results in a stable complex with the ferric heme iron. Consistent with other mammalian peroxidases, upon formation of the Fe$^{3+}$/CN$^{-}$ adduct, the Soret band of CeDUOX1–589 exhibited a red shift (Fig. 4C). In the case of LPO (consistent with the other classical mammalian peroxidases; supplemental Table S2), upon complexation with CN$^{-}$, the Soret maxima experiences a large shift of 20 nm, from 412 to 432 nm; CeDUOX1–589 exhibits a smaller shift of 7 nm to a Soret maxima of 415 nm at saturation (40). The reduced magnitude of the shift may be related to a difference in the properties of the heme binding pocket and/or the presence of a tyrosyl residue in place of the distal histidine. The hydrogen bonding interactions of the ligated cyanide are likely to differ significantly with a distal tyrosine rather than an imidazole residue. To further characterize the cyanide binding event, a titration was used to monitor the UV-visible spectral shift upon the addition of potassium cyanide (12.5–225 μM) to 6 μM CeDUOX1–589. This study established a dissociation constant for cyanide binding of 25 μM; this value is consistent with dissociation constants found for mammalian peroxidases LPO (42 μM) and TPO (10 μM) at neutral pH (39, 41).

Activity Studies for the DUOX Peroxidase Domain(s)—Because of the absence of several residues that are conserved in peroxidases, including the distal histidine residue, the actual catalytic function of the hDUOX1 and CeDUOX1 peroxidase domains remains uncertain. It has been shown through site-specific mutagenesis that the N-terminal region of DUOX1 may not act as a classical peroxidase but rather as a SOD (8, 12). In an effort to clarify the function of these domains, now available as purified and structurally...
sound proteins, we examined each homolog with regard to its peroxidase and SOD activity.

Catalytic activity studies were first conducted with ABTS, a substrate commonly used to evaluate peroxidase activity. In contrast to a standard peroxidase, LPO (3.3 \pm 1.0) \times 10^9 \text{ mol oxidized per min/mol of enzyme}) hDUOX1\textsubscript{1–593} exhibited no peroxidase activity, whereas CeDUOX1\textsubscript{1–589} had a modest activity of 14.1 \pm 0.8 \text{ mol oxidized per min/mol of enzyme}.

Tyrosine cross-linking has been established as the biological function of the DUOX enzymes in thyroid hormone biosynthesis for humans and in cuticle formation for C. elegans. Each domain was therefore assayed via fluorescence for dityrosine formation with tyrosine ethyl ester as a substrate (14). Similar results were obtained with this more natural substrate, i.e. no activity with hDUOX1\textsubscript{1–593} and modest activity with CeDUOX1\textsubscript{1–589} (Fig. 5A). To confirm that any increase in fluorescence observed was due directly to the formation of dityrosine, HPLC analysis of the reactions was performed to isolate the dityrosine product (Fig. 5B); separation isolated the fluorescent product formed (t = 8 min) from unreacted tyrosine ethyl ester (t = 2.8 min). LC-MS identified the fluorescent product as dityrosine (m/z 417).
As an alternative to peroxidase activity, the DUOX1 domains may function as SODs, converting two molecules of superoxide to O$_2$ and H$_2$O$_2$. The hDUOX1$_{1–593}$ peroxidase domain, which appears unable to bind heme, could conceivably bind a metal ion necessary for SOD activity. To determine whether any metal ions have co-purified with hDUOX1$_{1–593}$, the enzyme was submitted to metal analysis by inductively coupled argon plasma optical emission spectroscopy (Garratt Callahan Analytical Laboratory). This analysis showed that the only protein-associated metal ion was calcium, consistent with conservation of a calcium-binding site among the classical peroxidases and the DUOX family (47). Calcium does not belong to the known set of metals utilized by SODs to support their function (the mammalian enzymes utilize copper, zinc, and/or manganese (48–51)); therefore, this result suggests that hDUOX1$_{1–593}$ may also not function as a SOD. To further investigate, in the unlikely event calcium may be utilized for enzyme function, hDUOX1$_{1–593}$ and CeDUOX1$_{1–589}$ were evaluated for their ability to react with superoxide by a colorimetric inhibition assay utilizing WST-1 as a substrate (52, 53). Authentic superoxide dismutase was assayed as a control over a profile of concentrations (Fig. 5C). hDUOX1$_{1–593}$ and CeDUOX1$_{1–589}$ show less than 10% inhibition over concentrations from 10–120 ng/ml, over which SOD increases in inhibition strength to 70%. Superoxide dismutase demonstrates a high turnover rate even at low concentrations, which may not be shared by the DUOX enzymes. To ascertain whether any activity may be obtained at higher concentrations, each enzyme was assayed under the same conditions at 1 μM calcium, both with and without the addition of 100 μM CaCl$_2$. As shown in Fig. 5D, even at this higher concentration, no significant dismutase activity is exhibited by the DUOX1 homologs, in either the presence or the absence of additional calcium.

In sum, this collective activity data demonstrates hDUOX1$_{1–593}$ to be inactive as a peroxidase or superoxide dismutase, and CeDUOX1$_{1–589}$ to be inactive as a SOD but exhibiting low level peroxidase activity. This low activity level is realistic for CeDUOX1$_{1–589}$ in vivo and the DUOX family as a whole to prevent cellular oxidative stress and to function in a controlled manner toward polymerization of tyrosines and/or generation of signaling molecules. In contrast, mammalian peroxidases require high peroxidase activity levels, herein demonstrated by the high level of ABTS turnover and tyrosine ethyl ester cross-linking observed with LPO. These levels are necessary for in vivo generation of antimicrobial agents, i.e. hypohalides, from bromide, chloride, and thiocyanate oxidation. Specifically, LPO has the characterized ability to rapidly oxidize bromide at acidic pH (24, 54). Therefore, to support the theory that DUOX enzymes support controlled reactivity and should not oxidize halides, LPO was utilized as a control with both DUOX1 homologs to assay bromide oxidation. This study confirmed that neither DUOX peroxidase domain could oxidize bromide (LPO, (3.5 ± 0.7) × 10$^{-3}$ mol oxidized per min/mol enzyme; CeDUOX1$_{1–589}$ and hDUOX1$_{1–593}$, no significant oxidation). Although a low activity level appears native to CeDUOX1$_{1–589}$, the lack of an observed hDUOX1$_{1–593}$ function suggests that specific protein interactions may play a role in heme binding or in enhancing the activity of the protein.

**DISCUSSION**

The dual oxidase enzymes, the most recently discovered members of the NOX/DUOX family, have garnered much attention because of their unique N-terminal peroxidase-like domain(s). The focus on this region has so far been limited to cell suspension activity assays after expression in *E. coli* and RNA interference experiments with *C. elegans* for determination of cellular localization and function (14). Neither domain has been purified, leaving them largely uncharacterized. The inability to isolate this region from DUOX1 or DUOX2 for *in vitro* study leaves many questions regarding structure and function unanswered.

To better characterize these N-terminal regions, we have first focused on the single dual oxidase isoform, DUOX1. Here we report that expression and purification of the human and *C. elegans* DUOX1 peroxidase domains (hDUOX1$_{1–593}$ and CeDUOX1$_{1–589}$) was achieved in a recombinant baculovirus system. These purified constructs demonstrate distinctly different interactions with the heme prosthetic group, in accord with the fact that a different number of critical active site residues is missing in the two DUOX domains (Fig. 1B). Covalent heme association was found for CeDUOX1$_{1–589}$, which lacks fewer of the critical residues, but heme was not found to stably bind in purified hDUOX1$_{1–593}$.

In view of the absence of heme binding, it is not surprising that hDUOX1$_{1–593}$ has no peroxidase activity. This result is consistent with the *in vivo* finding that DUOX2, but not DUOX1, supports thyroid hormone biosynthesis. Mutations in the DUOX2 isofrom have been conclusively shown to cause congenital hypothyroidism, a disorder that is not rescued by expression of DUOX1 in the thyroid (55–57). Clear evidence has also demonstrated that DUOX2 provides functional heme-dependent peroxidase activity in human respiratory tract epithelium; interestingly, this study also showed that treatment of epithelial cells with cytokines (interleukins 4 and 13) enhanced DUOX1 expression but did not induce measurable peroxidase activity (58). Investigations of the DUOX2 N-terminal region are in progress to determine its interaction with heme and to define its catalytic activities.

Our biophysical data (circular dichroism and Trp fluorescence) suggest that hDUOX1$_{1–593}$ is folded properly, yet as isolated is inactive as either a peroxidase or a superoxide dismutase. Possible explanations for this include the absence of a protein-protein interaction required for heme binding/catalytic activity, expression/retention as a nonfunctional vestigial region, or a unique as yet to be identified domain functionality. DUOX1 is not the dominant isoform in the thyroid; instead, it is found at high levels in respiratory tract epithelial cells and has been proposed to provide reactive oxygen species for airway homeostasis. Investigations of lung cancer cell lines identified the *diox(s)* as inactivated by hypermethylation, yet H$_2$O$_2$ production was rescued by the introduction of DUOX1 and its maturation factor DUOXA1. This suggests that DUOX1 may be active in lung tissue but requires other protein interactions for maturation (DUOXA1), transport, or activity (59, 60). Recently, the DUOX maturation factors (DUOXA1 and DUOXA2) were shown to form stable cell surface complexes with
the DUOX1 Peroxidase Domains

the DUOX proteins and may function as part of a H$_2$O$_2$-generating enzyme complex (61). Co-expression of hDUOX1–593 in the presence of DUOX1 or pull-down assays of the peroxidase domain with mammalian cell lysates may provide a better understanding of any specific protein interactions that modulate DUOX1 activity.

Consistent with the presence of a conserved heme-binding residue (Glu$^{238}$), CeDUOX1–589 has been found to covalently bind its prosthetic group. This interaction, combined with an ability to bind small ligands, suggests that the CeDUOX1–589 heme-binding pocket has a profile similar to that of the classical mammalian peroxidases, despite the absence of some typical catalytic residues. Furthermore, the demonstration that protein covalent binding is increased upon exposure to H$_2$O$_2$ and is thus autocatalytic, links it tightly with both LPO and TPO, both of which share this behavior (29, 62). Surprisingly, identification of a dihydroxyheme derivative upon enzymatic protein digestion of CeDUOX1–589 suggests that the protein may interact directly with the heme through two covalent bonds. This was unexpected based on sequence analysis of the heme-binding residues, because the amino acid required for the second covalent heme bond in enzymes such as LPO is not present in DUOX1 (Fig. 1B). It is plausible the hydroxylation may be the result of reaction with another residue (e.g. Tyr$^{109}$); site-specific mutagenesis studies will be required to confirm that a second covalent bond is actually present and to determine not only which residue is involved but also the nature of the bond.

Covalent heme binding itself demonstrates that CeDUOX1–589 has peroxidase activity, because autocatalytic binding is the result of a peroxidase reaction. However, when peroxidase activity is measured with two standard peroxidase substrates, ABTS and a tyrosine derivative, the peroxidase activity of CeDUOX1–589 is found to be much lower than that of LPO. This lower activity is consistent with the absence of a distal histidine residue that is critical for the high activity of most peroxidases (42–46). However, a low level of activity for the CeDUOX1 peroxidase domain may be desirable in the context of its function in the intact organism. CeDUOX1 is thought to function in polymerization of tyrosine residues in cuticle formation rather than in antibacterial defense. A low rate of peroxidase activity may be desirable in this role to prevent damage to the organism by an uncontrolled high level of peroxidase activity. It is instructive in this context that VPO1, another recently identified human peroxidase, has a much lower peroxidase activity than LPO despite the fact that it retains all the critical amino acid residues of a conventional peroxidase (54).

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