Monoxygenase X, a Member of the Copper-dependent Monoxygenase Family Localized to the Endoplasmic Reticulum*

Received for publication, July 6, 2004, and in revised form, August 24, 2004
Published, JBC Papers in Press, August 26, 2004, DOI 10.1074/jbc.M407486200

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Based on sequence comparisons, MOX (monoxygenase X), is a member of the copper monoxygenase family that includes dopamine β-monoxygenase (DBM) and peptidylglycine α-hydroxylating monoxygenase (PHM). MOX has all of the residues expected to be critical for copper binding, and its cysteine residues can yield the intramolecular disulfide bond pattern observed in DBM. Although DBM and PHM function within the lumen of the secretory pathway, the published sequence for human MOX lacks a signal sequence, suggesting that it does not enter this compartment. We identified an upstream exon that encodes the signal sequence of human MOX. A retained intron yields minor amounts of transcript encoding MOX without a signal sequence. MOX transcripts are widely expressed, with the highest levels in the salivary gland and ovary and moderate levels in brain, pituitary, and heart. Despite the presence of a signal sequence, exogenous MOX is not secreted, and it localizes throughout the endoplasmic reticulum in both endocrine or non-endocrine cells. Neither appending green fluorescent protein to its C terminus nor deleting the hydrophobic domain near its C terminus facilitates secretion of MOX. MOX is N-glycosylated, is tightly membrane-associated, and forms oligomers that are not disulfide-linked. Based on its sequence and localization, MOX is predicted to hydroxylate a hydrophobic substrate in the endoplasmic reticulum.

The copper/ascorbate-dependent monoxygenases constitute a small, but essential family of enzymes that use molecular oxygen and ascorbate to catalyze the hydroxylation of their substrates (EC 1.14.17.x) (1). The defining member of this family of enzymes was dopamine β-monoxygenase (DBM), also known as dopamine β-hydroxylase or DBH; EC 1.14.17.1 (2, 3) (see Fig. 1). Hydroxylation of the β-carbon of dopamine consumes 1 mol of oxygen and 2 mol of ascorbate, yielding norepinephrine plus 2 mol of semidehydroascorbate. DBM was purified, sequenced, and studied in detail before it was cloned (4, 5). The second member of this family, peptidylglycine α-hydroxylating monoxygenase (PHM; EC 1.14.17.3), catalyzes the α-hydroxylation of the C-terminal Gly residue in many different secreted peptides (6–8). The 315-amino acid catalytic core of PHM, defined by truncation mutagenesis, is 28% identical to the corresponding region of DBM (9). Structural studies on the catalytic core of PHM defined the active site and revealed key roles for six copper-binding ligands (10–12). Enzymes homologous to DBM and PHM are not found in yeast or bacteria.

Although the reactions catalyzed by DBM and PHM use very different substrates and produce very different products, the chemistry involved is similar and both enzymes produce products stored in regulated secretory granules and used for intercellular communication. Both enzymes are essential for survival. Genetically engineered mice lacking DBM are not viable (13). Mice lacking PHM develop massive edema at about embryonic day 14, with no live progeny produced (14). Drosophila lacking a functioning PHM gene generally die as late embryos (15). Both enzymes function in the lumen of the secretory pathway, and both require adequate supplies of substrate, reduced ascorbate, and copper. The vesicular monoamine transporters (VMAT1 and VMAT2) couple uphill transport of dopamine to efflux of protons, to deliver dopamine to the lumen of the secretory pathway (16, 17). The peptidylglycine substrates of PHM are produced from prepropeptides synthesized in the endoplasmic reticulum and subject to endo- and exoproteolytic processing as they progress through the secretory pathway (6, 18).

A third family member, monoxygenase X (MOX), was identified in a search for genes whose expression was altered in senescent human fibroblasts (19). Although key active site residues are conserved, the published sequence for human MOX has no signal sequence, making it difficult to see how it could function in a manner similar to DBM or PHM. A screen for genes involved in neural crest development yielded chicken MOX (also known as dopamine β-hydroxylase-related, DBHR) (20), which includes an N-terminal signal sequence. A potential homolog identified in the mouse genome (BAAB95089), also includes an N-terminal signal sequence. No substrate has been identified for human or chicken MOX.

Because both DBM and PHM produce essential signaling molecules, it was tempting to speculate that MOX plays a similarly important role in signaling. Although very little is known about the MOX protein, transcripts encoding hMOX are prevalent in brain, kidney, and lung, with levels increasing in some lines of senescent fibroblasts (19, 20). In the developing chick embryo, MOX transcripts are expressed in newly differentiating neural crest cells, most migrating neural crest cells, and in non-neuronal tissues such as the myotome. The properties of the MOX protein have not been explored.

* This work was supported by National Institutes of Health Grant DK-32949. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby indicated as marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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The abbreviations used are: DBM, dopamine β-monoxygenase; PHM, peptidylglycine α-hydroxylating monoxygenase; MOX, monoxygenase X; DBHR, dopamine β-hydroxylase-related; h, human; m, mouse; GPP, green fluorescent protein; CSFM, complete serum-free medium; DBHIL, dopamine β-hydroxylase-like; PHMxs, peptidylglycine α-hydroxylating monoxygenase, catalytic core; PAM, peptidylglycine α-amidating monoxygenase; EST, expressed sequence tag; ER, endoplasmic reticulum; TES, N-tris (hydroxymethyl)methyl-2-aminethane-sulfonic acid.

This paper is available on line at http://www.jbc.org

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We undertook these studies to determine whether a human MOX signal sequence could be identified. Monoxygenase family members present in Caenorhabditis elegans and Drosophila were identified. We next determined the sites of expression of MOX in adult mouse tissues and compared the pattern of MOX expression to that of DBM and PHM. Using several antisera to MOX, we characterized the expression of exogenous MOX in endocrine and non-endocrine cells. The unique features of MOX suggest that it functions in the endoplasmic reticulum.

**Materials and Methods**

Isolation of RNA and Reverse Transcription-PCR—Tissues dissected from adult male and female C57Bl/6 mice were extracted with TRIzol reagent (Invitrogen) for preparation of RNA. cDNA was prepared from total RNA (1 μg) using SuperScript II reverse transcriptase (Invitrogen). This cDNA (5% of the product) was amplified using primers specific for mMOX, mDBM, mPHM, and mDBH. For MOX: forward primer, 5'-TCATCTGAGTTGAGGCTGCT; reverse primer, 5'-GGTA-

We sequenced the PCR products with a forward and a reverse primer. The PCR products were subcloned into the TA-cloning vector (Invitrogen) and the plasmid was sequenced in the Molecular Core at the University of Connecticut Health Center. To visualize MOX in live cells, transient expression of exogenous MOX in adult mouse tissues and compared the pattern of MOX expression to that of DBM and PHM. Using several antisera to MOX, we characterized the expression of exogenous MOX in endocrine and non-endocrine cells. The unique features of MOX suggest that it functions in the endoplasmic reticulum.

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catalase, and apoferritin (200 samples). Cytochrome samples were either treated with dithiothreitol (1 mM for DBM; 10 mM for PHM) or not. Gradients were centrifuged for 5 h at 4 °C and the bottom of the gradient was used to recover protein. After centrifugation, samples were collected starting from the top of the gradient. MOX was immunoprecipitated from each fraction using CT45 and fractionated on a 10% SDS gel. For DBM, equal aliquots of each fraction were fractionated on a 10% SDS gel and detected with a DBM antibody (1:1000, Ab3125).

RESULTS

Distinguishing Features among Copper Monoxygenase Family Members—Although their catalytic cores are highly homologous, the topologies predicted for MOX, DBM, and PAM are distinctly different (Fig. 1). Although mouse MOX is predicted to have a cleaved signal sequence, human MOX lacks a signal sequence (19). Both mouse and human MOX have a stretch of 17 hydrophobic residues near their C termini. DBM has a signal anchor, and its disulfide-linked dimers form tetramers (26). PAM is a Type 1 membrane protein with an N-terminal signal sequence and a transmembrane domain near its C terminus (27). Similar disulfide bonding patterns have been identified in DBM and PAM, and these Cys residues are conserved in MOX (Fig. 1).

Both the human genome and the Drosophila genome contain three copper monoxygenase family members (Table I). Consistent with their size and predicted topologies, the catalytic core of MOX is more similar to DBM than to PHM. The mouse genome contains a fourth copper monoxygenase family member, DBHL, which is slightly more similar to MOX than to DBM. Although the intron/exon boundaries of mDBHL are similar to those of hMOX and hDBM, a gene resembling mDBHL could not be identified in the human genome. The functions of two of the Drosophila copper monoxygenases have been studied: the catalytic core of dPHM is 39% identical to that of hPHM, and the catalytic core of Drosophila tyramine β-hydroxylase is 50% identical to that of hDBM (Table I). The third family member in Drosophila is almost equally identical to hMOX and hDBM, a gene resembling mDBHL.
enzyme is hydroxylating tyramine to produce octopamine (28).

Knowledge of the crystal structure of PHMcc and biochemical modification of DBM provide another means of analyzing MOX (Table II). The six ligands that interact with the two essential copper atoms in PHM are completely conserved among all family members. The active site residues that interact with the peptidylglycine substrate have been identified (1, 12). In addition, molecular modeling has predicted residues in DBM that may interact with its substrate, dopamine (1). When the homologous residues in MOX are identified, it is striking that all are hydrophobic. For example, Arg<sup>240</sup>, which binds the peptide carboxylate in PHMcc, is Gln<sup>395</sup> in hDBM and Leu<sup>319</sup> in hMOX. Similarly, Asn<sup>316</sup> in PAM is another Leu in MOX. If the active site of MOX resembles that of PHM, its substrate is unlikely to be charged or highly polar.

**Expression of MOX Is Widespread**—We next set out to determine where MOX was expressed and to compare its expression pattern to those of PAM and DBM (Fig. 2). Total RNA prepared from the indicated adult mouse tissues was reverse-transcribed, and the cDNA used as a PCR template (Fig. 2). Gene-specific primers were designed for PAM (420 bp), DBM (520 bp), MOX (420 bp), and DBHL (702 bp). MOX transcripts were readily identified in a wide variety of adult tissues. Levels were highest in the salivary gland and ovary, with lower levels in olfactory bulb, cerebellum/brain stem, parietal cortex, pituitary, atrium, ventricle, adrenal, thymus, testis, and kidney. Expression of MOX is widespread among all family members.

**hMOX Can Include an N-terminal Signal Sequence**—Following the identification of human MOX (hMOX; GenBank<sup>TM</sup> accession number AY007239), homologous genes were identified in mouse (mMOX; GenBank<sup>TM</sup> accession number: BAA95089), and chicken (GenBank<sup>TM</sup> accession number AF327450) (20). Based on SMART and Signal P analyses, both mouse MOX (mMOX) and Gallus MOX include N-terminal signal sequences (smart.embl-heidelberg.de; www.cbs.dtu.dk/services/SignalP-2.0); cleavage of the mMOX signal sequence is predicted to occur after Gly<sup>19</sup> (Fig. 3A, open arrow). In addition to lacking another 67 amino acid residues common to mouse (Fig. 3A) and chicken MOX. After this region of divergence at the N terminus, the amino acid sequences of hMOX and mMOX are 81% identical.

To search for an N-terminal signal sequence for hMOX, we used the nucleotide sequence unique to mMOX to search for homologous sequences in the human EST data base. A clone (GenBank<sup>TM</sup> accession number AI751100) encoding an amino acid sequence 76% identical to that of mMOX-(1–100) was identified (Fig. 3B). SMART and Signal P analyses of this sequence revealed that the first 19 residues should function as a signal sequence, with cleavage occurring after Gly<sup>19</sup> (Fig. 3B, open arrow).

The gene encoding hMOX is situated on chromosome 6q (19) and, like DBM, includes multiple exons (Fig. 3C). Intron 3, the longest intron, spans 43.9 kb at the Pro<sup>156</sup>-Val<sup>157</sup> junction, whereas intron 2, the shortest, spans only 170 nucleotides. Blast analysis of the human genome using the unique sequence encoded by human EST AI751100 identified an additional exon upstream of those previously thought to define hMOX (Exon 0, Fig. 3C). We will refer to forms of hMOX that include this signal sequence as the long form of hMOX, the form originally identified by Chambers <i>et al.</i> (19), will be referred to as the short form of hMOX. The sequence unique to the N terminus of the short form of hMOX (S1a, Fig. 3C) is encoded by the intron that separates the first two exons of the human MOX gene.

**The Major Form of hMOX Has a Signal Sequence**—To compare expression of the long and short forms of the hMOX transcript, we paired three different sense primers with an antisense primer in the region common to both the short and long forms of hMOX (Fig. 4A). When paired with a sense primer in the common region, both forms of hMOX yield a 250-bp fragment (Common). When paired with a forward primer in exon 0, the only long form of hMOX yields a product (620 bp). When paired with a forward primer situated in the intron between exons 0 and 1, only the short form of hMOX yields a product (600 bp). Total RNA from adult human brain was reverse-transcribed, and PCR amplification was carried out using the three primer pairs for the same number of cycles. Very little product was observed using the primer pair specific for the short form of hMOX described originally (19) (Fig. 4B). Consistent with this observation, similar amounts of product

**TABLE II**

| Rat PHM: Cu<sup>II</sup>, Cu<sup>III</sup> | Monooxygenase X Localized to the ER |
|--------------------------------------|-----------------------------------|
| **Y**<sup>79</sup> | **H**<sup>107</sup> | **L**<sup>110</sup> | **L**<sup>205</sup> | **R**<sup>240</sup> | **H**<sup>297</sup> | **T**<sup>304</sup> | **E**<sup>387</sup> | **M**<sup>391</sup> | **Y**<sup>396</sup> |
| hMOX | M | L | L | L | L | L | L | L | Y |
| mMOX | M | L | L | L | L | L | L | L | Y |
| dMOX | M | L | L | L | L | L | L | L | Y |
| hDBM | M | L | L | L | L | L | L | L | Y |
| mDBM | M | L | L | L | L | L | L | L | Y |
| dDBM | M | L | L | L | L | L | L | L | Y |
| hPHM | M | L | L | L | L | L | L | L | Y |
| mPHM | M | L | L | L | L | L | L | L | Y |
| dPHM | M | L | L | L | L | L | L | L | Y |
| c.e.PH | M | L | L | L | L | L | L | L | Y |
| mDBHL | M | L | L | L | L | L | L | L | Y |

**FIG. 2.** MOX transcripts are expressed in a wide variety of adult tissues. DNA prepared from the adult mouse tissues indicated was reverse-transcribed and used for PCR. Primer sets specific for mouse MOX, DBM, PAM, and DBHL were used to compare patterns of expression. PCR amplification: PAM, 25 cycles; DBM, 30 cycles; MOX, 35 cycles; DBHL with nested PCR, 30 cycles followed by 25 cycles. Actin levels in all samples were comparable (data not shown).
were observed using the primer pair specific for the long form of hMOX or the primer pair that amplifies both isoforms of hMOX.

We next used the common primer pair to evaluate expression of MOX in other human tissues (Fig. 4C). MOX transcripts were most prevalent in adult and fetal brain and spinal cord, with lower levels in cerebellum and fetal liver and barely detectable levels in placenta. In all of these tissues, PCR using primer pairs specific to the long and short forms of hMOX revealed a pronounced preponderance of the long form (data not shown). The major MOX transcript encodes a protein with a predicted cleavable N-terminal signal sequence, meaning that MOX, like DBM and PHM, should function within the lumen of the secretory pathway.

Despite Having a Signal Sequence, MOX Is Not Secreted—To explore the properties of MOX, we expressed it transiently in pEAK RAPID cells, which lack regulated secretory granules, and in AtT-20 corticotrope tumor cells, which have secretory granules. We developed antisera to several synthetic mMOX peptides (Fig. 5A). Antiserum (CT164) to the more N-terminal peptide identified a 72-kDa band, approximately the mass predicted for mMOX (69-kDa) (Fig. 5B). The signal was not shown by preimmune serum, and was blocked when the antigenic peptide was included with the antibody (data not shown). Antiserum generated to the more C-terminal peptide (CT45) detected native MOX but failed to reveal a signal on Western blots.

When spent medium was harvested from transfected cells expressing MOX, no secreted MOX was detected (Fig. 5B). For comparison, cells transiently transfected with PAM-3, a soluble, secreted protein, were analyzed at the same time (Fig. 5B); secretion of PAM-3 was readily detected. To explore the reason for this unexpected result, we constructed two fusion proteins: MOX-GFP, with GFP appended to the C terminus of MOX, and MOX-Arg596-GFP, with MOX truncated just before the C-terminal hydrophilic stretch and GFP appended (Fig. 5A). Neither variant of MOX was secreted; removing the C-terminal hydrophilic stretch did not facilitate the secretion of MOX (Fig. 5B). Cells expressing MOX-GFP consistently contained a protein the size of MOX, suggesting that proteolysis separated MOX from the appended GFP.

To understand why MOX was not secreted, we prepared

**Fig. 3.** The human genome encodes an isoform of MOX with a signal sequence. A, SMART analysis (www.cbs.dtu.dk/services/SignalP-2.0) predicts that mMOX (BAJ85089) has a cleaved signal sequence at its N terminus, with signal peptidease cleaving after Gly19 (open arrow). In contrast, hMOX (AY007239) lacks a signal sequence. The region unique to mMOX is underlined. After this unique region, mMOX and hMOX are 87% identical at the amino acid level. The locations of the exon/intron boundaries in the hMOX gene are indicated (black arrows); the exon/intron boundary in mouse MOX that does not align with a boundary in human MOX is marked by a filled diamond (black arrow). C, the genomic DNA encoding this alternate N terminus (Exon 0) is located 26.4 kb upstream of the genomic sequence encoding the published N terminus of hMOX. The transcript encoding hMOX without an N-terminal signal sequence (short form, S1α) is indicated by a thick line. Exons are indicated by boxes and introns by lines (dotted lines when not drawn to scale). AI751100 is the GenBank number for the hEST clone encoding hMOX with a signal sequence.
crude soluble and particulate fractions from pEAK RAPID cells transiently expressing MOX, MOX-GFP, MOX-Arg596-GFP, an integral membrane protein (PAM-1) or a soluble, secreted protein (PAM-3). The crude particulate fraction was washed with 0.1 M Na2CO3, pH 11.0, to remove peripheral proteins (25), and the carbonate pellet and supernatant were examined along with the soluble fraction. Almost all of the MOX was recovered in the carbonate pellet (Fig. 5C). As expected, intact PAM-1 was recovered in the carbonate pellet, whereas cleavage products lacking a transmembrane domain were found in the soluble fraction. PAM-3, which is secreted efficiently, was present in the soluble fraction and in the carbonate pellet. Like PAM-3, MOX-GFP was present in the soluble fraction and the carbonate pellet (Fig. 5C). Removing the C-terminal hydrophobic region of MOX before appending GFP had little effect on the partitioning of MOX among these different fractions (Fig. 5C). Although appending GFP to MOX or to MOX-Arg596 increased its solubility and diminished the amount of protein recovered in the carbonate pellet, neither MOX-GFP fusion protein was secreted (Fig. 5B).

MOX is Localized to the Endoplasmic Reticulum—To explore the localization of MOX, we used differential centrifugation to fractionate pEAK RAPID cells transiently expressing MOX (Fig. 6A). MOX was largely recovered in the low speed pellet (P1), the crude nuclear fraction, and P2, an ER- and plasma membrane-enriched fraction, with detectable amounts in the high speed P3 microsomal pellet. MOX was not detected in the cytosolic fraction (Fig. 6A, left). Western blot analysis for an ER marker, protein disulfide isomerase, confirmed that protein disulfide isomerase was concentrated in the P2 fraction (Fig. 6A, right).

We used several different expression vectors to examine the subcellular localization of exogenous MOX in AtT-20 mouse corticotrope tumor cells and in pEAK RAPID cells using immunofluorescence (Fig. 6B). We expressed MOX, MOX-GFP, and MOX-Arg596-GFP. Each MOX protein exhibited the same behavior when expressed in AtT-20 cells (Fig. 6B) or in pEAK RAPID cells (data not shown). We first compared the localization of MOX-GFP (Fig. 6B) to that of MOX (Fig. 6D). MOX-GFP visualized by GFP fluorescence or staining with MOX antibody after fixation and permeabilization yielded the same diffuse, reticular pattern (Fig. 6B, MOX versus MOX-GFP). MOX yielded a similar diffuse reticular pattern (Fig. 6D). MOX-GFP was partially co-localized with BiP, an ER marker (Fig. 6C); MOX-GFP was more concentrated in the perinuclear region of the cell, with BiP more evenly distributed throughout the ER. When cells expressing MOX were visualized for ACTH, a secretory granule marker (Fig. 6D), or syntaxin 6, a trans-Golgi network marker, were visualized simultaneously using monoclonal antisera (red, syntaxin staining is indicated by the white arrowhead). Merged images are shown in the bottom panels. Images were taken with a 60× oil objective and a deconvolved image through the middle of each cell is shown. Scale bar is 10 μm.
were incubated with medium containing [35S]Met for 30 min. One well expressed in pEAK RAPID cells. Two days later, replicate wells of cells pEAK RAPID cells transiently expressing exogenous MOX and immunoprecipitation to determine whether MOX under-
sults, indicating that localization of MOX to the ER is not cell
Expression of MOX in pEAK RAPID cells yielded similar re-
lular localization when expressed in AtT-20 cells (18).

PAGE and fluorography (Fig. 7A) samples were fractionated by SDS-PAGE and visualized with *). No newly synthesized MOX was detected in the medium and newly synthesized MOX was visualized by fluorography; *, nonspe-
cific band. No newly synthesized MOX was detected in the medium (data not shown). Similar results were obtained in 3 separate incubations. B, pEAK RAPID cells expressing EGFP or mMOX were extracted as above (A); equivalent amounts of the soluble (Sol) and particulate (Part) samples were fractionated by SDS-PAGE and visualized with MOX antibody CT164. C, Longer Exposure, the arrow points to 69-kDa MOX. C, pEAK RAPID cells transiently expressing mMOX were treated with tunicamycin (2 μg/ml) as indicated for 3.5 h before incubation with [35S]Met for 30 min. Immunoprecipitates (CT45) were prepared and analyzed as above. Tunicamycin pre-treatment eliminated the 72-kDa form of MOX from both the soluble and particulate fractions; *, non-
specific band. D, MOX immunoprecipitated from the soluble and par-
ticulate fractions after a 30-min Pulse or a 3-h Chase was digested with endoglycosidase H (Endo H) as indicated and then fractionation by SDS-PAGE. The N-linked oligosaccharide attached to MOX remained sensitive to endoglycosidase H throughout the Chase.

All of our data suggest that MOX is a protein of the endoplasmic reticulum. The uniform, reticular staining pattern ob-
served for both MOX-GFP and MOX suggests that the protein does not form insoluble aggregates, a concern for any exoge-
nous, overexpressed protein. DBM, the most homologous copper monooxygenase family member, exhibits a similar subcell-
ular localization when expressed in AIT-20 cells (18). Expression of MOX in pEAK RAPID cells yielded similar re-
sults, indicating that localization of MOX to the ER is not cell type-specific.

MOX Is N-Glycosylated—We next utilized metabolic labeling and immunoprecipitation to determine whether MOX under-
goes any post-translational modification. Multiple wells of pEAK RAPID cells transiently expressing exogenous MOX were incubated with medium containing [35S]Met for 30 min and either harvested immediately or incubated with fresh medium lacking [35S]Met for 30 min, 1 h, or 4 h (Fig. 7A). Medium was collected, and cell extracts were separated into a soluble fraction (Soluble) and a crude particulate fraction (Particulate). Following solubilization of the particulate fraction with detergent, MOX was immunoprecipitated and analyzed by SDS-
PAGE and fluorography (Fig. 7A).

After the 30-min pulse, 69- and 72-kDa forms of MOX were identified in the soluble fraction. The molecular mass predicted for mMOX, 69 kDa, matches the smaller band in the soluble fraction. Even when the pulse-labeling period was shortened to 10 min, two forms of MOX were detected (data not shown). The particulate fraction contained only the 72-kDa form of MOX. The major change noted during the chase incubation was a decrease in the amount of labeled MOX. Although 72-kDa MOX in the particulate fraction had a half-life of ~4.6 h, both forms of soluble MOX had a slightly shorter half-life (3.8 h for soluble 72-kDa MOX and 2.9 h for soluble 69-kDa MOX). Medium collected from the transfected cells was analyzed together with cell extracts; consistent with the Western blot analysis shown in Fig. 5, [35S]Met-labeled MOX was never detected in the spent medium (data not shown). Consistent with these different half-lives, Western blot analysis revealed a prevalence of 72-kDa MOX in the particulate fraction (Fig. 7B); the soluble fraction contained about one-third as much 72-kDa MOX and a barely detectable amount of 69-kDa MOX (Fig. 7B, Longer Exposure, arrow).

The sequence of mMOX includes four potential N-glycosylation sites (Asn-Xaa-Ser/Thr): Asn114-Ser-Thr, Asn247-Asp-Ser, Asn476-Leu-Thr, and Asn517-Leu-Ser; all four sites are conserved in human MOX (Fig. 1). Only the first site is outside the region of the catalytic core; based on the structure of PHMec (1), the other three sites would be located in loops connecting various β-strands and might be surface-accessible. To determine whether MOX is N-glycosylated, transfected pEAK RAPID cells were pre-treated with tunicamycin, a potent inhibitor of N-glycosylation (30). Pre-treatment with tunicamycin eliminated the 72-kDa form of MOX from both the soluble and particulate fractions, leaving only the 69-kDa form of MOX (Fig. 7C). More of the newly synthesized MOX was recovered from the soluble fraction following tunicamycin treatment.

Because MOX is N-glycosylated, we used endoglycosidase H to assess the maturity of its N-linked oligosaccharide chains. If MOX remains in the endoplasmic reticulum, as suggested by our pulse/chase and immunofluorescence localization studies, its N-linked oligosaccharide chains might remain endoglycosi-
dase H-sensitive. MOX was immunoprecipitated from the soluble and particulate fractions of transiently transfected cells labeled for 30 min (Pulse) or labeled and then chased for 3 h (Chase). The immunoprecipitated MOX was digested with endoglycosidase H or buffer control before analysis by SDS-PAGE and fluorography. In both the soluble and particulate fractions, endoglycosidase H treatment completely eliminated the 72-kDa form of MOX, converting it into a 69-kDa protein (Fig. 7D). Although MOX present after the 30-min Pulse might not have had time to mature, MOX present after the 3-h chase remained endoglycosidase H-sensitive.

Exogenous MOX Forms Oligomers—All three copper monooxygenase family members have a single Cys residue in their signal or signal/anchor sequence (Fig. 1). MOX shares the 12 Cys residues that form intrachain disulfide bonds in DBM, the major change noted during the chase incubation was a decrease in the amount of labeled MOX. Although 72-kDa MOX in the particulate fraction had a half-life of ~4.6 h, both forms of soluble MOX had a slightly shorter half-life (3.8 h for soluble 72-kDa MOX and 2.9 h for soluble 69-kDa MOX). Medium collected from the transfected cells was analyzed together with cell extracts; consistent with the Western blot analysis shown in Fig. 5, [35S]Met-labeled MOX was never detected in the spent medium (data not shown). Consistent with these different half-lives, Western blot analysis revealed a prevalence of 72-kDa MOX in the particulate fraction (Fig. 7B); the soluble fraction contained about one-third as much 72-kDa MOX and a barely detectable amount of 69-kDa MOX (Fig. 7B, Longer Exposure, arrow).

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bic stretch, might be predicted to form an interchain disulfide bond. The pH-dependent association of disulfide-bonded DBM dimers yields tetramers (31). To explore the quaternary structure of MOX, extracts of transfected cells were applied to linear sucrose gradients (Fig. 8A). As for the metabolic labeling experiments, extracts were separated into crude soluble and particulate fractions; proteins in the particulate fraction were sol-
ubilized by incubation with 1% Triton X-100 (TMT buffer). Aliquots of both fractions were then applied to 5–20% sucrose gradients, and MOX was analyzed by immunoprecipitation. DBM secreted from stably transfected Chinese hamster ovary cells was analyzed for comparison. As expected, secreted DBM was largely tetrameric, and preincubation with dithiothreitol disrupted these tetramers (Fig. 8B). In contrast, the behavior of...
MOX solubilized from the particulate fraction was unaffected by thiol-reducing reagents. Most of the MOX was recovered in large aggregates, whereas the MOX remaining in the gradient fractionated as a monomer (Fig. 8A).

**DISCUSSION**

*The Dominant Form of Human MOX Has a Signal Sequence*—MOX (hMOX; short form, GenBank™ accession number AY007239) was first identified by differential screening of senescent human fibroblasts (19). Because the predicted protein lacked a signal peptide sequence, it seemed unlikely that MOX could function in a manner similar to the other members of this copper-dependent monooxygenase family, DBM and PAM. Following the identification of hMOX, homologous genes were identified in mouse (mMOX; GenBank™ accession number BAA956089) and chicken (GenBank™ accession number AF327450, also called DBHR) (20). Based on SMART and Signal P analyses, both mMOX and chicken MOX have cleavable N-terminal signal sequences. Using the N-terminal amino acid sequence of mMOX to search the human EST database, we identified the first 19 residues as a signal sequence. Human MOX cDNA with an N-terminal signal sequence was also identified by bioinformatic methods (GenBank™ accession number AF359094) (32). Using reverse transcription-PCR, we established that the form of hMOX with an N-terminal signal sequence is by far the most prevalent form. The hMOX signal sequence is eliminated when transcription starts in the intron between exons 0 and 1.

*MOX Shows a Broad Tissue Distribution*—MOX and DBHL, a gene identified in the course of sequencing the mouse T-cell receptor locus (33), are new members of the copper monooxygenase family. Mouse DBHL is the most divergent family member, even lacking some highly conserved residues, and the human genome contains no DBHL gene and no human EST was identified. MOX is more similar to DBM than to PHM. However, from the tissue distribution point of view, MOX is more like PAM, with expression in a number of different tissues in the adult mouse and in human. In the adult mouse, PAM is most prevalent in pituitary, atrium, ventricle, lung, and adrenal, whereas MOX is most prevalent in the salivary gland and ovary, which express low levels of PAM. In contrast, expression of DBM and DBHL is highly restricted, with DBM transcripts detectable only in the adrenal and cerebellum/brain stem, and DBHL only in mouse thymus and testis.

The expression pattern observed for MOX early in avian development suggests that MOX plays a role in the production of important signaling molecules (20). MOX transcripts are first detected at the neural plate border (stage 7), where neural crest induction occurs, and are expressed throughout the development of the neural crest (20). MOX transcripts are also detected in the myotome. The expression of MOX during development of the mouse embryo has not yet been studied. DBM, the only enzyme that can hydroxylate the β-carbon of dopamine to yield norepinephrine, serves as a marker for the noradrenergic system and is first detected in avian embryos at stage 18 (4, 34). PHM, the only enzyme that can hydroxylate the C-terminal Gly residue in peptidylglycine intermediates to yield amidated product peptides, serves as a marker for peptidergic systems (1, 35). PAM is expressed in the cardiogenic region of the developing mouse embryo at embryonic day 9 and appears in the developing nervous system, limb mesoderm, and mesenchyme (36).

**MOX Is a Luminal Protein but Is Not Secreted**—Because human MOX, like mouse and chicken MOX, has an N-terminal signal sequence, its secretion and localization in the secretory pathway were studied. None of the MOX antisera generated were sensitive enough to visualize endogenous MOX, so our conclusions are based on analyzing the expression of exogenous MOX. Attempts to express exogenous MOX in chick embryos were unsuccessful (20). Although exogenous MOX exhibited the same behavior in two very different cell types (non-endocrine and endocrine), data on the properties of endogenous MOX are needed. We never observed secretion of MOX. Consistent with this, both subcellular fractionation and immunocytochemistry indicate that MOX is localized to the ER, not to the trans-Golgi network or to secretory granules. Consistent with localization to the ER, the N-linked sugar attached to MOX remains sensitive to endoglycosidase H even 5 h after synthesis. Analyzing the mMOX sequence, we noticed a hydrophobic 17-amino acid stretch near the C terminus that might serve as a signal for addition of a glycosylphosphatidylinositol tail (37) (available at www.cbs.dtu.dk/services/SignalP/). Neither appending GFP to the C terminus nor removing this signal sequence, its secretion and localization in the secretory pathway were studied. None of the MOX antisera generated were sensitive enough to visualize endogenous MOX, so our conclusions are based on analyzing the expression of exogenous MOX. Attempts to express exogenous MOX in chick embryos were unsuccessful (20). Although exogenous MOX exhibited the same behavior in two very different cell types (non-endocrine and endocrine), data on the properties of endogenous MOX are needed. We never observed secretion of MOX. Consistent with this, both subcellular fractionation and immunocytochemistry indicate that MOX is localized to the ER, not to the trans-Golgi network or to secretory granules. Consistent with localization to the ER, the N-linked sugar attached to MOX remains sensitive to endoglycosidase H even 5 h after synthesis. Analyzing the mMOX sequence, we noticed a hydrophobic 17-amino acid stretch near the C terminus that might serve as a signal for addition of a glycosylphosphatidylinositol tail (37) (available at www.cbs.dtu.dk/services/SignalP/). Neither appending GFP to the C terminus nor removing this hydrophobic region altered the subcellular localization of MOX or resulted in MOX secretion. In addition, MOX remained membrane-associated after the particulate fraction was digested with phosphatidylinositol phospholipase C (1-phosphatidylinositol phosphohydrolase; Sigma) to release glycosylphosphatidylinositol-anchored proteins (data not shown) (38). Neither calnexin nor calreticulin, ER chaperones that bind monogluocosylated glycans (39, 40), were co-immunoprecipitated with MOX, suggesting that they are not responsible for retention of MOX in the ER.

**MOX Associates with Membranes**—MOX does not have a recognized ER retention signal (41). We used metabolic labeling to better understand its properties, revealing the presence of 69- and 72-kDa forms of MOX. Only the 72-kDa form is found in the particulate fraction. Because the 72-kDa form of MOX is absent from cells pre-treated with tunicamycin, and endoglycosidase treatment converts the 72-kDa form into a 69-kDa form, attachment of an N-linked oligosaccharide plays a role in...
its production. The 69-kDa form of MOX is found only in the soluble fraction, and its shorter half-life means that little of it is present at steady state (Fig. 7, A and B). We saw no evidence for conversion of 69-kDa MOX into 72-kDa MOX during chase incubations. Tunicamycin treatment results in the formation of membrane-associated 69-kDa MOX, indicating that N-glycosylation is not essential to the production of particulate MOX. The facts that newly synthesized MOX is not rapidly degraded and the distribution of MOX is uniform throughout the ER suggest that the behavior of the exogenous protein may accurately represent the behavior of the endogenous protein.

Particulate fractions were washed with carbonate to release peripherally associated proteins. The behavior of MOX and PAM-1, an integral membrane protein, were indistinguishable, with almost no full-length protein recovered in the soluble fraction and little removed by the carbonate wash. Appending GFP to the C terminus of MOX resulted in the recovery of some protein in the soluble fraction, making MOX mimic the behavior of PAM-3; the properties of MOX-GFP and MOX-Arg596-GFP to the C terminus of MOX plays a role in this process (45–48).

The Substrate for MOX May Be Hydrophobic—If MOX functions in a manner analogous to DBM and PHM, it will require the presence of copper to function properly, and it will consume hydrophobic Leu residues replace the charged or hydrophilic (Table II). We attempted to measure MOX-catalyzed ascorbate consumption by guest on July 24, 2018http://www.jbc.org/Downloaded from
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J. Biol. Chem. 2004, 279:48159-48167.
doi: 10.1074/jbc.M407486200 originally published online August 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407486200

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