Myeloperoxidase Biosynthesis by a Human Promyelocytic Leukemia Cell Line: Insight Into Myeloperoxidase Deficiency

By William M. Nauseef

The biosynthesis and processing of myeloperoxidase (MPO), a cationic enzyme present in the azurophilic granules of human polymorphonuclear leukocytes (PMNs), were studied in the human promyelocytic leukemia cell line, HL-60. HL-60 cells produce large quantities of enzymatically active MPO that has the same electrophoretic behavior as MPO isolated from normal PMNs. Mature MPO is a glycoprotein of approximately 150,000 molecular weight (mol wt) composed of two heavy-light protomers (αβ) with subunits of 59,000 and 13,500 mol wt, respectively, under reducing conditions. The primary translation product of MPO messenger RNA (mRNA) isolated from HL-60 was a single polypeptide of mol wt 80,000. In HL-60 cells labeled with [35S]-methionine, the labeled MPO isolated by immunoprecipitation had a mol wt of 89,000. Treatment of this 89-kilodalton (kDa) species with endoglycosidase H produced a 79-kDa peptide, suggesting that the 89-kDa protein contained high-mannose side chains. The 89-kDa species had no detectable peroxidase activity. During chase experiments some of the 89-kDa peptide was processed to smaller species of mol wt 39,000, 59,000, and 13,500, although a fraction of the 89-kDa peptide remained unprocessed after a chase of 100 hours. In addition, a small amount of the 89-kDa peptide appeared in the medium without any of the processed smaller peptides. These studies suggest (1) that the primary translation product in MPO biosynthesis is an 80-kDa peptide that undergoes cotranslational cleavage of the signal peptide and glycosylation to produce an 89-kDa pro-MPO, (2) that pro-MPO is a single polypeptide containing the α and β subunits of MPO and contains endoglycosidase H–susceptible high-mannose side chains, and (3) that posttranslational modification of pro-MPO results in targeting to the lysosome and proteolytic maturation of pro-MPO to active enzyme. In light of the previous observation that MPO-deficient and normal PMNs contain an 89-kDa protein immunochemically related to MPO, these studies on MPO biosynthesis indirectly support the hypothesis that defective posttranslation processing by pro-MPO may underlie hereditary MPO deficiency.

Myeloperoxidase (MPO), a cationic glycoprotein present in the azurophilic granules of human polymorphonuclear leukocytes (PMNs), plays an important role in PMN microbicidal activity. Numerous investigators have characterized the function of MPO in oxygen-dependent events following PMN stimulation. The structure of MPO has likewise been extensively studied, and although some disagreement exists, most studies suggest that MPO is composed of a pair of identical heavy-light protomers with a combined mol wt of approximately 140 to 150,000. The two protomers are each composed of a 59-kDa α subunit and a 13.5-kDa β subunit and are linked along their long axis by a single disulfide bond joining the two heavy or two light subunits. MPO contains 2% to 4% carbohydrate, and a recent study of the subunit structure indicates that the 59-kDa subunit is glycosylated, whereas the 13.5-kDa subunit has no carbohydrate.

Hereditary MPO deficiency has been identified as a relatively common defect, present in one of 2,000 individuals in the population. Although initial studies suggested that MPO deficiency is the result of a structural gene defect inherited as an autosomal recessive trait, recent family studies suggest that the mode of inheritance is more complex. In vitro studies of PMN function from such individuals demonstrate delayed bactericidal and an absence of candidacidal action. PMNs from individuals with functional MPO deficiency do not contain normal subunits of MPO, as determined by polyacrylamide gel electrophoresis (PAGE) and immunoblotting. However, both MPO-deficient and normal PMNs contain an 89-kDa peptide that is immunochemically related to MPO. The presence of this large peptide immunochemically related to MPO in both MPO-deficient and normal PMNs suggests that MPO deficiency may be due to defective processing of an MPO precursor, which results in aberrant packaging of MPO into the azurophilic granules. The goals of this work are to characterize the events in MPO synthesis and to determine the steps that are critical for correct transfer of nascent MPO from its intracellular site of synthesis to its final intracellular destination in the azurophilic granule. With these results it will be possible to examine the nature of the defect in hereditary MPO deficiency.

For these studies the human promyelocytic leukemia cell line HL-60 was used to examine MPO synthesis because PMNs are terminally differentiated cells with little, if any, protein synthesis. Undifferentiated HL-60 cells produce 47.5 μg of MPO per 10^6 cells, and the MPO produced has a normal subunit structure as determined by immunoblot analysis. This cell line thus provides a useful system for the study of MPO synthesis. Data presented here are consistent with the hypothesis that MPO deficiency is due not to the absence of the gene for MPO synthesis but to a defect in its expression.

From the Department of Medicine, Veterans Administration Medical Center and University of Iowa, Iowa City. Submitted March 8, 1985; accepted Oct 14, 1985.

Supported by Biomedical Research Support Grant RR 05372 from the Biomedical Research Support Branch, Division of Research Facilities and Resources, National Institutes of Health; Merit Review grant from the Veterans Administration; a Smith, Kline and French Young Investigator award awarded by the Infectious Disease Society of America; Basil O'Connor Starter Research Grant 5-468 awarded by the March of Dimes Birth Defects Foundation; and Grant AI-20866 from the US Public Health Service.

Presented in part at the annual meeting of the American Association of Immunologists, June 1984, St Louis.

Address reprint requests to Dr William M. Nauseef, Department of Internal Medicine, University of Iowa Hospitals and Clinics, Iowa City, IA 52242.
cotranslational or posttranslational processing of the precursor protein of MPO, resulting in a failure to package the enzyme correctly into the azurophilic granule.

**MATERIALS AND METHODS**

*Reagents.* Diisopropylfluorophosphate (DFP), leupeptin, pepstatin A, phenylmethylsulfonylfluoride (PMSF), cetyltrimethylammonium bromide (CETAB), Triton X-100, EDTA, and molecular weight markers for polyacrylamide gels were obtained from Sigma Chemical Co (St Louis). Suspensions of formalin-fixed *Staphylococcus aureus* (Cowan strain) were used as a source of protein A in immunoprecipitation and were obtained from Bethesda Research Laboratories (Gaithersburg, Md). Cowan cells were heated to 95 °C for 30 minutes in 10% 2-mercaptoethanol and 3% sodium dodecyl sulfate (SDS) as previously described and stored as 10% suspensions in phosphate-buffered saline at −20 °C until use. Immediately prior to use, the 10% suspension of Cowan cells was washed sequentially with 1-mL volumes of 0.5% Triton X-100 (TX-100) in 10 mmol/L Tris buffer, pH 7.5, with 150 mmol/L NaCl (TBS), 2 mol/L urea in 5% TX-100 in TBS, 1 mg/mL bovine serum albumin in 0.5% TX-100 in TBS, and TBS before reconstitution to 10% suspension in TBS. RPMI 1640 Select Amine Kit (GIBCO, Grand Island, NY) was used to prepare the methionine-free medium that was supplemented with 1 mmol/L pyruvate and 10% heat-inactivated fetal calf serum (FCS) supplied by the Tissue Culture Facility of the Cancer Center at the University of Iowa. [35S]-methionine of high specific activity (1,495 Ci/mmole) was purchased from Amersham Corp (Arlington Heights, Ill). Endoglycosidase H was obtained from Dr Frank Maley (Albany, NY). Micrococcal nuclease (15,000 U/mL) was obtained from Worthington Biochemical Corp (Freehold, NJ).

**HL-60 cells.** The human promyelocytic cell line HL-60 was obtained from Dr Harry L. Malech of Yale University and grown in RPMI 1640 medium supplemented with 1 mmol/L pyruvate and 10% heat-inactivated FCS.

**Polyacrylamide gel electrophoresis.** Samples were solubilized in gel sample buffer and placed in a boiling water bath for two minutes. Bromphenol blue tracking dye in 400 μL of total RNA and 85 to 100 μCi of [35S]-methionine. In all experiments translation mixtures to which no RNA was added served as controls.

*Precipitations.* Protein was precipitated from 10% trichloroacetic acid and counted to estimate the total amount of [35S]-methionine incorporated into protein. Immunoprecipitations were carried out in two steps: the first to remove any labeled proteins that nonspecifically bind to rabbit serum or to Cowan cells and the second to precipitate specifically labeled protein related to MPO. Samples were diluted in a microfuge tube to 150 μL with TBS, 7 μL of nonimmune rabbit serum was added, and the sample was incubated at 4 °C for 30 minutes. Cowan cells (100 μL of a washed 10% suspension) were added and the sample vortexed and incubated at 4 °C for 30 minutes. Cowan cells were removed by centrifugation (12,000 g for one minute) and the supernatant fluid (approx. 230 μL) was transferred to another 1.5-mL microfuge tube. The sample was brought to 2% SDS by the addition of 35.4 μL of 5% SDS, placed in a boiling water bath for two minutes, and then diluted with 1.06 mL (4 vol) of dilution buffer (30 mmol/L Tris-HCl 190 mmol/L NaCl, 2.5% TX-100, 6 mol/L EDTA, pH 7.4). The sample was vortexed, 5 μL of rabbit anti-MPO antiserum previously described previously 2 and added, and the sample incubated at 4 °C for five to 16 hours. Cowan cells (100 μL of a 10% suspension) were added and the sample incubated at 4 °C for 30 minutes. The suspension was layered atop a 400-μL cushion of 1 mol/L sucrose in TBS and centrifuged (12,000 g for five minutes in a Beckman Microfuge 12) to pellet the Cowan cells. The supernatant fluid was aspirated and discarded and the pellet washed sequentially with 1 mL of 0.5% TX-100/TBS, 2 mol/L urea in 0.5% TX-100/TBS, 1 mg/mL BSA in 0.5% TX-100/TBS, and TBS. After the final wash the pellet was resuspended in 100 μL of gel sample buffer (62.5 mmol/L Tris HCl, 190 mmol/L NaCl, 6 mmol/L EDTA, 2.3% SDS, 2.5% TX-100, 5% 2-mercaptoethanol, pH 6.8), heated for two minutes in a boiling water bath to release the antigen-antibody complex from the protein A on the Cowan cells, vortexed while hot, and centrifuged to remove Cowan cells. A 20-μL aliquot of the supernatant fluid was solubilized in 200 μL Scintigent (Fisher Chemical Co, St Louis) and 2.5 mL of scintillation fluid (19 g 2,5-diphenyloxazole, 439 mg dimethyl-2,5-diphenyloxazole, 1,165 mL TX-100 in 2,330 mL toluene) to count activity of [35S]. The remainder of the sample was electrophoresed into an SDS polyacrylamide gel.

*Separation of subcellular components.* HL-60 cells were harvested, washed, and resuspended in 20 mL of relaxation buffer (100 mmol/L KCl, 3 mmol/L NaCl, 1 mmol/L adenosine triphosphate (ATP) (Na₉), 3.5 mmol/L MgCl₂, 10 mmol/L piperazine-N,N'-Gis buffer. After multiple washes in Burridge buffer, the paper was dried and exposed to X-Omatic film in a cassette with an intensifying screen.

*Preparation of RNA.* Total cellular RNA was prepared by the method of Chirgwin et al² from pellets of HL-60 cells harvested from cultures in suspension. The total RNA obtained, estimated by the absorbance at 260 nm (A₂₆₀ = 1 for 42 μg/mL), was 3.05 μg per 10⁶ cells. Later experiments using a phenol-chloroform extraction of HL-60 cell pellets gave qualitatively the same results but a higher yield of biologically functional RNA as judged by in vitro translation.

*In vitro protein synthesis.* The RNA isolated from HL-60 cells was translated in vitro according to the method of Hunt and Jackson³ using a rabbit reticulocyte-lysate system and [35S]-methionine. Lysates of rabbit reticulocytes were prepared in the laboratory of Dr Edward C. Heath, Department of Biochemistry, University of Iowa. Lysates were prepared without addition of exogenous methionine and treated with micrococcal nuclease⁴ to inhibit translation of endogenous RNA and to increase dependence of translation on exogenously added RNA. Typical translations were done in a volume of 55 μL and contained 5 to 15 μg of total RNA and 85 to 100 μCi of [35S] methionine. In all experiments translation mixtures to which no RNA was added served as controls.

*Immunoautoradiography.* Samples were electrophoresed into 10% polyacrylamide gels prior to electroblotting to nitrocellulose paper according to the method of Towbin et al⁰ and processed as described previously.¹ The nitrocellulose paper to which proteins had been transferred was exposed sequentially to 3% BSA in Burridge buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% NaN₃, pH 7.4), anti-MPO antiserum diluted 1:40 in 3% BSA—Burridge buffer, and [125I]-protein A (200,000 cpm/mL) in 3% BSA—Burridge buffer. After multiple washes in Burridge buffer, the paper was dried and exposed to X-Omatic film in a cassette with an intensifying screen.

From www.bloodjournal.org by guest on September 14, 2017. For personal use only.
of Percoll according to the technique of Borregaard et al. Fractions (1.0 mL) were collected and Percoll removed by ultracentrifugation (180,000 g for 120 minutes) prior to further analysis.

Biochemical analysis. Fractions from the Percoll gradient were assayed for biochemical markers of subcellular organelles. Alkaline phosphatase (plasma membrane) activity was assayed in a 1-mmol/L MgCl₂, 30-mmol/L sodium barbital buffer (pH 10.5) as the hydrolysis of p-nitrophenyl phosphate (1 mg/mL). Lysozyme (granules) was assayed spectrophotometrically at 450 nm as the disappearance of turbidity of 0.2 mg/mL of Micrococcus lysodeikticus in 67 mmol/L sodium phosphate buffer at pH 6.2.

Galactosyl transferase (Golgi) was measured as described by Beau- fay et al. and modified by Brown and Swank.

Biosynthetic labeling. HL-60 cells were harvested by centrifugation and resuspended at 3 × 10⁶/mL in a methionine-free RPMI 1640 medium with 1 mmol/L pyruvate and 10% heat-inactivated FCS that had been dialyzed against phosphate-buffered saline (pH 7.4) in 6,000 mol wt cut-off dialysis tubing prior to addition to the RPMI. Cells were incubated for 30 minutes at 37 °C in an atmosphere of 5% CO₂ in methionine-free medium prior to addition of [³⁵S]-methionine (100 μCi/mL). In pulse-chase experiments an excess of unlabeled methionine (1 mmol/L) was added at the end of the labeling interval, and incubation was continued for the designated period of time. At the end of the incubation, cells were recovered by centrifugation (1,400 g for ten minutes). Cell pellets were solubilized in phosphate-buffered (10 mmol/L potassium phosphate, 150 mmol/L NaCl at pH 7.0) saline with 10 mmol/L EDTA, 1 mmol/L PMSF, 100 μg/mL leupeptin, 100 μg/mL pepstatin A, 0.5% CETAB, and 0.1% Tx-100 on ice for 30 minutes. The solubilized cells were clarified by ultracentrifugation (100,000 g for 60 minutes in a type 50 Ti Beckman rotor [Beckman Instruments, Fullerton, Calif]) and the supernatant fluid frozen at −80 °C or subjected to analysis immediately.

Endoglycosidase H treatment. Antigen-antibody complexes were released from Cowan cells by adding 50 μL of 0.2% SDS and 5% 2-mercaptoethanol and heating the suspension in a boiling water bath for two minutes. Free Cowan cells were removed by centrifugation (12,000 g for one minute) and the supernatant diluted to 500 μL with 150 mmol/L citrate buffer (pH 5.3) to reduce the concentration of SDS to 0.2%. Five microliters of 100 mmol/L PMSF was added to inhibit proteolysis during the deglycosylation. A quantity of 50 ImU of endoglycosidase H (endo H) was added and the sample incubated at 37 °C for 24 hours with constant agitation. As control, 50 μL of citrate buffer was added to an identical sample. At the end of the incubation period, 50 μL of 100% trichloroacetic acid (TCA) was added and the sample placed on ice for 30 minutes. The resultant precipitate was washed twice in 500 μL of 50% methanol and then dissolved in 100 μL of gel sample buffer, heated in a boiling water bath for two minutes, and electrophoresed as will be described.

RESULTS

Use of HL-60 cell line. Because PMNs isolated from venous blood synthesize little or no protein, it was necessary to use the HL-60 cell line to analyze MPO biosynthesis. To verify that HL-60 cells were appropriate for these studies, the MPO subunits synthesized by HL-60 cells were studied by immunoblotting. MPO purified from human PMNs as previously described, normal PMNs, and HL-60 cells were electrophoresed into a 10% polyacrylamide gel under reducing conditions, followed by electrophoretic separation of separated proteins to nitrocellulose paper. Autoradiography of the resulting immunoblot (Fig 1) demonstrated that purified MPO (lane 1), HL-60 cells (lane 2), and normal PMNs (lane 3) contain the same MPO subunit peptides of 59 kDa (α subunit) and 13.5 kDa (β subunit). In addition, the anti-MPO antiserum also identified an 89-kDa peptide present in HL-60 cells and normal PMNs. When the antiserum was adsorbed with purified MPO prior to treatment of the nitrocellulose paper, this 89-kDa peptide as well as the normal subunits of MPO were no longer detected (data not shown). These observations indicate that the 89-kDa species is immunochemically related to normal MPO and that HL-60 cells provide a suitable model to study MPO biosynthesis.

In vitro translation. To identify the primary translation product of MPO mRNA, RNA isolated from HL-60 cells was translated in a micrococcal nuclease-treated rabbit reticulocyte lysate system and the products immunoprecipitated. The primary translation product was an 80-kDa peptide (Fig 2) that represented 0.16% of the total TCA-precipitable counts when translation was performed for 60 minutes. The addition of 3% polyethylene glycol or 50% (NH₄)₂SO₄ to the supernatant remaining after the immunoprecipitation failed to recover additional MPO-related peptides. This finding confirms recent reports of the primary translation product of MPO mRNA from HL-60 cells. This result demonstrates that the nonglycosylated peptide backbone of the precursor of MPO has a molecular weight of 867.
sufficient size (80 kDa) to account for the α and β subunits (59 + 13.5 kDa) covalently linked into a single polypeptide. After HL-60 cells were induced to differentiate, either by culturing for three days in conditioned medium containing γ-interferon or by culturing for five days in 1.3% dimethyl sulfoxide, their isolated RNA failed to translate for any MPO-related material in vitro (data not shown). Of note, these induced cells still produced functional RNA since the total TCA-precipitable counts from translations primed with [\(^{35}\)S]-methionine. Products were immunoprecipitated with anti-MPO and subjected to SDS-PAGE followed by autoradiography of the dried gel. The primary translation product for MPO synthesis was a single peptide that migrated with a molecular weight of 80 kDa under reducing conditions. Residual activity of endogenous RNA in the reticulocyte lysate accounted for the other labeled proteins seen (lane 1).

Fig 2. Synthesis of MPO in vitro. A rabbit reticulocyte lysate was supplemented with amino acids (except methionine) and treated with micrococcal nuclease to inhibit translation of endogenous RNA. The lysate was primed with no RNA (lane 1) or 15 μg of RNA isolated from HL-60 cells (lane 2) and proteins labeled with [\(^{35}\)S]-methionine. Products were immunoprecipitated with anti-MPO and subjected to SDS-PAGE followed by autoradiography of the dried gel. The primary translation product for MPO synthesis was a single peptide that migrated with a molecular weight of 80 kDa under reducing conditions. Residual activity of endogenous RNA in the reticulocyte lysate accounted for the other labeled proteins seen (lane 1).

After HL-60 cells were induced to differentiate, either by culturing for three days in conditioned medium containing γ-interferon or by culturing for five days in 1.3% dimethyl sulfoxide, their isolated RNA failed to translate for any MPO-related material in vitro (data not shown). Of note, these induced cells still produced functional RNA since the total TCA-precipitable counts from translations primed with RNA from induced cells was higher than control lysates with no RNA added. Thus, this suggests that under the induction conditions used, differentiation was associated with cessation of transcription of the gene for MPO, indicated by the absence of MPO synthesis.

Analysis of subcellular fractions. HL-60 cells were disrupted by nitrogen cavitation and the subcellular organelles separated on a density gradient of Percoll. The immunoblots of individual fractions are shown in Fig 3 and demonstrate that the 89-kDa peptide and the normal subunits of MPO sedimented into separate subcellular fractions. Samples from fractions 17 to 19 from the gradient were electrophoresed in lanes 1 to 3 of the immunoblot shown in Fig 3 and comigrated with the major peak of peroxidase activity. The only MPO peptide detected in this fraction was the 59-kDa α subunit, whereas the β subunit ran at the dye front in the 10% acrylamide gel. Fractions 22 and 23, electrophoresed in lanes 4 and 5 of the immunoblot, contained both the α subunit and the 89-kDa peptide. These fractions contained a small amount of peroxidase activity and comigrated with activity of galactosyl transferase, a marker for trans-Golgi. Immunoblots of fractions 25 and 26 contained only the 89-kDa peptide (lanes 6 and 7) and cosedimented with peak activities of galactosyl transferase and alkaline phosphatase and had both 89-kDa and 59-kDa peptides identified by the immunoblot. Fractions 25 and 26 were the least dense fractions in the gradient and had no peroxidase activity but peak activities for galactosyl transferase and alkaline phosphatase. The immunoblot of these fractions, lanes 6 and 7, contained predominantly the 89-kDa peptide. The 13.5-kDa species migrated with the dye front in a 10% gel.

Fig 3. Immunoperoxidase analysis of MPO peptides in subcellular fractions separated on a gradient of Percoll. Percoll was removed from fractions collected from the gradient prior to separation by SDS-PAGE in a 10% polyacrylamide gel and immunoblotting. Samples from fractions 17 to 19 were electrophoresed in lanes 1 to 3. These fractions contained one of the two major peaks of peroxidase activity and in the immunoblot contained the 59-kDa subunit of MPO. Fractions 22 and 23 were electrophoresed in lanes 4 and 5. These fractions contained the remaining peroxidase activity in the gradient as well as galactosyl transferase and alkaline phosphatase and had both 89-kDa and 59-kDa peptides identified by the immunoblot. Fractions 25 and 26 were the least dense fractions in the gradient and had no peroxidase activity but peak activities for galactosyl transferase and alkaline phosphatase. The immunoblot of these fractions, lanes 6 and 7, contained predominantly the 89-kDa peptide. The 13.5-kDa species migrated with the dye front in a 10% gel.

Further work on improving resolution of species in the less dense regions of the gradient is underway, and the identity of subcellular organelles containing 59-kDa and 89-kDa species cannot be absolutely ascertained from this analysis. It is clear from these initial studies that (1) the 89-kDa peptide and mature MPO were in separate subcellular compartments; (2) the 89-kDa peptide was in fractions from the gradient that were less dense than those containing lysosomes and cosedimented with biochemical markers for Golgi and plasma membranes; and (3) the 89-kDa peptide has no peroxidase activity, whereas fractions with the 59-kDa α subunit also had peroxidase activity.

Pulse labeling experiments. Uptake of [\(^{35}\)S]-methionine by HL-60 cells starved in a methionine-free medium prior to labeling was rapid, and immunoprecipitable material was detectable after five minutes of labeling (data not shown). In pulse-chase experiments cells were labeled for 30 minutes because these conditions resulted in the greatest number of counts incorporated into an MPO-related peptide without
Since first identified as verdoperoxidase by Agner in 1941, MPO has been studied extensively, and its important 205-kDa role in the microbicidal activity of PMNs via the MPO-H2O2-halide system has been firmly established. Despite 1 1 6-kDa extensive investigation of the structure2 and function of 97-kDa 205-kDa 66-kDa 116-kDa i 2 processing of the initial product to more mature species. The earliest labeled peptide immunoprecipitated was an 89-kDa peptide (Fig 4, lane 1).

Pulse-chase experiments demonstrated that this 89-kDa was very slowly processed into normal MPO subunits (Fig 4). Thirty minutes after the addition of cold methionine, a fraction of the 89-kDa species was processed to a 47-kDa peptide intermediate (data not shown) that was further processed to a 39-kDa peptide, the molecular weight of the α subunit of MPO when purified MPO is electrophoresed under nonreducing conditions.3 After five hours of chase the major peptides immunoprecipitated had molecular weights of 89,000, 39,000, and 47,000 (lane 2). Subsequently, the α and β subunits of native MPO were seen after 20 hours of chase, migrating at 59,000 and 13,500 (lane 3). Of note, the predominant species immunoprecipitated were 89-kDa and 39-kDa peptides after a prolonged period of chase (100 hours, lane 5). Samples of immunoprecipitated supernatant contained small amounts of the 89-kDa peptide (data not shown).

Endoglycosidase treatment. Endo H is an enzyme that digests glycoproteins with high-mannose side chains at the proximal N-acetylglucosamine-N-acetylglucosamine link- age.44 To characterize the glycosylated intermediates of MPO biosynthesis, immunoprecipitates of extracts from cells pulsed for 30 minutes were treated with endo H. The autoradiography of immunoprecipitated protein incubated in the presence (lane 2) of endo H is shown in Fig 5. The 89-kDa species migrated as a peptide of 79 kDa after treatment with endo H, indicating that the 89-kDa peptide contains high-mannose side chains. In addition, treatment with endo H reduced the 59-kDa subunit to 53 kDa, whereas the 39-kDa species was essentially unchanged. The 39-kDa peptide migrates as a broad band even after digestion with endo H, suggesting that the oligosaccharide side chains on this peptide are heterogeneous and only a fraction are of the high-mannose type. When the autoradiograph is developed sufficiently long to visualize well the 13.5-kDa subunit (not shown), it appeared unaltered by endo H digestion. These observations suggest that the precursor to MPO is glycosylated in the Golgi by the addition of high-mannose side chains to the nascent peptide. In addition, some of the high-mannose side chains are not processed in the Golgi and are present in the α subunit of mature MPO.

DISCUSSION

Since first identified as verdoperoxidase by Agner in 1941,44 MPO has been studied extensively, and its important role in the microbicidal activity of PMNs via the MPO-H2O2-halide system has been firmly established.46 Despite extensive investigation of the structure4 and function46 of

![Fig 4. Synthesis of MPO by HL-60 cells. HL-60 cells were starved in methionine-free RPMI 1640 with 10% dialyzed FCS for 30 minutes prior to the addition of [35S]-methionine (100 µCi/mL). Cells were labeled for 30 minutes, after which time a sample was taken (zero chase, lane 1) and cold methionine (final concentration, 1 mmol/L) was added. Samples were taken at 5, 20, 44, 68, and 100 hours (lanes 2 to 6 respectively), immunoprecipitated, reduced, and electrophoresed into a gradient gel (8% to 14% acrylamide and 0% to 14% glycerol). Initially, an 89-kDa peptide was labeled, and it was processed to a 47-kDa intermediate and a 39-kDa peptide that was apparent at five hours of chase (lane 2). After 20 hours of chase (lane 3), the α (59 kDa) and β (13.5 kDa) subunits of mature MPO were identified with a gradual decrease in the 89-kDa peptide.

![Fig 5. Digestion of immunoprecipitated MPO peptides by endo H. Labeled HL-60 cells that had been chased for 68 hours were immunoprecipitated. The immunoprecipitates were incubated at 37 °C for 24 hours without (lane 1) or with (lane 2) 50 mU of endo H. Endo H digested the 89-kDa peptide to 79 kDa, the 59-kDa peptide to 53 kDa, and the 39-kDa peptide to 37 kDa, indicating that these glycoproteins contain high-mannose side chains. After more prolonged exposure (not shown), this autoradiograph demonstrated that the 13.5-kDa subunit was resistant to endo H digestion.]
MPO, studies of MPO biosynthesis are few in number,\textsuperscript{40, 41, 47} in part reflecting the terminally differentiated state of the PMN characterized by little, if any, protein synthesis.\textsuperscript{25} Currently there are at least three general reasons for an interest in MPO biosynthesis.

First, there have been significant advances in understanding cotranslational and posttranslational events during protein synthesis by various types of cells.\textsuperscript{48-54} Of relevance to MPO biosynthesis are studies demonstrating the importance of posttranslational modification and phosphorylation of oligosaccharide side chains for the proper transport of lysosomal enzymes from the endoplasmic reticulum to the lysosome.\textsuperscript{48-50} Many of the lysosomal enzymes studied to date follow a similar pattern of production and processing. The primary translation product has an amino-terminal sequence that promotes insertion of the nascent peptide through the membrane and into the lumen of the endoplasmic reticulum. As cotranslational events, the insertion sequence is removed by a signal peptidase, and the nascent peptide is glycosylated at the amide nitrogen of certain asparagines in the peptide (N-linked glycosylation). Glycosylation is initiated by transfer en bloc of a high-mannose oligosaccharide chain (Glc\textsubscript{3}Man\textsubscript{9}GlcNAc\textsubscript{2}) from the lipid carrier dolichol to the asparagine. The oligosaccharide side chains are subsequently modified during passage through the Golgi where mannose-6-phosphate residues are incorporated into some of the side chains. These phosphomannosyl groups allow the glycoprotein to bind to the mannose-6-phosphate receptor on the lysosomal membrane and thereby translocate to the lysosome. Hasilek et al\textsuperscript{44} have recently demonstrated \textsuperscript{32}P incorporation by the \( \alpha \) subunit during MPO synthesis but were unable to demonstrate mannose-6-phosphate receptors. Newly synthesized glycosylated precursors of lysosomal enzymes, so-called pro forms, are generally larger than the corresponding mature enzyme, and proteolytic conversion to the mature form appears to be closely linked to transport to the lysosome.\textsuperscript{39, 51}

The second reason to examine MPO biosynthesis is that production of MPO is closely linked to myeloid differentiation.\textsuperscript{40, 41} Two recent studies have demonstrated that MPO synthesis by promyelocytic HL-60 cells ceases when the cells are chemically induced to differentiate to more mature cells, either macrophages or granulocytes.\textsuperscript{40, 41} The induced cells had no MPO-specific mRNA as assessed in an in vitro translation system, although functional RNA was still transcribed by induced cells. This suggests that cessation of transcription of the MPO gene is responsible for the termination of MPO synthesis seen during myeloid differentiation. These studies of MPO biosynthesis may provide information on the control mechanisms for protein synthesis during myeloid differentiation and insight into events associated with abnormal differentiation in neoplasia.

The third reason to study MPO biosynthesis is to examine the genetic basis for MPO deficiency, a common hereditary disorder of PMNs.\textsuperscript{12} In a previous study,\textsuperscript{1} sensitive immuno-blot analysis of peroxidase-deficient PMNs separated by PAGE demonstrated that MPO-deficient PMNs lack the normal \( \alpha \) and \( \beta \) chains of MPO but contain an MPO-related 89-kDa peptide that is also present in normal PMNs. The presence of this 89-kDa peptide is consistent with the hypothesis that MPO deficiency reflects a failure of developing myeloid cells to compartmentalize MPO into azurophilic granules, perhaps secondary to defective posttranslational processing of the enzyme precursor.

Studies presented here suggest that biosynthesis of MPO likely follows the same general pattern previously outlined for other lysosomal enzymes. The nonglycosylated primary translation product synthesized in vitro had a molecular weight of 80,000 and is thus a peptide of sufficient size to accommodate the \( \alpha \) and \( \beta \) chains of mature MPO (59 kDa + 13.5 kDa) linked covalently into a single precursor. The earliest form of MPO synthesized in vitro in pulse experiments was an 89-kDa protein identified within five minutes of labeling. Treatment of the 89-kDa peptide with endo H, an enzyme that cleaves between proximal \( N \)-acyethylglucosamine groups on high-mannose side chains, resulted in a 79-kDa peptide, demonstrating the presence of high-mannose side chains on the 89-kDa peptide. In preliminary experiments (data not shown), HL-60 cells labeled in the presence of tunicamycin, an antibiotic that prevents the initial transfer of the high-mannose oligosaccharide from dolichol to the nascent peptide backbone,\textsuperscript{39} produced a core peptide of 75 to 77 kDa. This core glycosylation occurred very early in biosynthesis and was very likely a cotranslational event whereby the signal peptide was removed from the 80-kDa primary translation product and the core peptide of approximately 77 kDa glycosylated at selected asparagines to produce the 89-kDa peptide, although there is no direct evidence for a signal peptide presented here. The 89-kDa species was identified in immunoblot analysis of subcellular fractions of HL-60 cells separated in a discontinuous gradient of Percoll. This peptide was localized in a subcellular organelle(s) that was less dense than lysosomes and that cosedimented with enzyme markers for Golgi and plasma membrane.

When labeled HL-60 cells were chased with a 1,000-fold excess of unlabeled methionine, the 89-kDa peptide was slowly processed, first to an intermediate 47-kDa peptide that was converted to a 39-kDa peptide beginning within two hours of chase (data not shown). After five hours of chase, the \( \alpha \) and \( \beta \) subunits of mature MPO were immunoprecipitated, although the 39-kDa peptide remained prominent late into the chase phase. The 89 kDa was processed very slowly and was still present after 100 hours of chase. The relatively slow rate of processing of the MPO precursor has been noted by Hasilek et al who recently reported persistence of the precursor glycoprotein as late as 70 hours of chase.\textsuperscript{42}

In preliminary experiments, SV8 protease digestion of the 89-kDa species generated seven peptides of mol wt < 49,000, as did digestion under identical conditions of the immunoprecipitate after 24 hours of chase (data not shown). The persistence of the 89-kDa peptide late into the chase makes it impossible to compare a proteolytic digest of isolated 89 kDa with that of isolated 59-, 39-, and 13.5-kDa subunits. Kinetics of labeling in the pulse-chase experiments coupled with these findings are sufficient evidence for a product-precursor relationship between the 89-kDa peptide and mature MPO. In fact, few examples exist in the literature of such a relationship substantiated by peptide or sequence analysis of
the precursor and the product, although studies are currently underway toward that end.

Endo H treatment modified the 89-kDa glycoprotein to a 79-kDa peptide regardless of the time during the chase at which the 89-kDa material was immunoprecipitated, indicating persistence of the high-mannose side chains on the precursor and the product, although studies are currently underway toward that end.

Deglycosylation of the purified subunits of MPO using endo H demonstrated that the a subunit has susceptible high-mannose side chains and was deglycosylated to a 53-kDa peptide. In contrast, the b subunit appeared unaffected by endo H treatment. These findings extend a recent study by Olsen and Little in which they reported the results of deglycosylation of the purified subunits of MPO using trifluoromethanesulphonic acid. With this treatment the 89-kDa material was converted to 52 kDa, whereas the β subunit was unchanged. Therefore not only are the carbohydrate side chains in MPO limited to the α chain but they are primarily high-mannose oligosaccharides.

Purified MPO has subunits of 59 kDa and 13.5 kDa in a 1:1 ratio, but under nonreducing conditions has subunits of 59 kDa, 39 kDa, and 13.5 kDa in a 1:1:2 ratio. The 39-kDa species has been observed by numerous investigators studying MPO structure, but there is no consensus as to its relationship to the α and β chains of MPO. Investigators have attributed its presence to anomalous migration of unglycosylated 59-kDa polyacrylamide gels, boiling samples in SDS prior to electrophoresis, reducing samples for electrophoresis during or after heat treatment, proteolytic digestion of the 59-kDa peptide, and contamination of the MPO. Two-dimensional maps of chymotryptic digests of iodinated 59-kDa and 39-kDa species separated by PAGE are similar but not identical and clearly demonstrate that these species are very closely related. Data presented here demonstrate the differences in the carbohydrate composition of the 59-kDa and 39-kDa species, the latter being relatively insensitive to endo H. Although the interrelationship between the 59-kDa and 39-kDa species is still the subject of ongoing work, the 39-kDa peptide was the predominant species during pulse-chase experiments.

The data presented here provide a foundation for further examination of MPO synthesis, processing, and packaging in normal myeloid cells. The findings presented are consistent with the hypothesis that the genetic defect underlying MPO deficiency is different from that previously proposed. The presence of the 89-kDa MPO-related glycoprotein in MPO-deficient PMNs suggests that the deficient cells express the gene for MPO synthesis but that some event in the cotranslational/posttranslational processing is defective. This defect results in production of an aberrant form of MPO that, because of abnormal processing, is not packaged in the azurophilic granules during the promyelocytic stage of development. Small amounts of pro-MPO remain in the vestigial Golgi of peripheral blood PMNs where it appears as the faint band at 89 kDa when normal and MPO-deficient PMNs are analyzed by immunoblotting. MPO-deficient cells, which might be expected to contain larger amounts of pro-MPO as a reflection of faulty processing, may behave in a fashion akin to fibroblasts in culture.

REFERENCES


ACKNOWLEDGMENT

I wish to express my gratitude to the late Dr Edward Heath and Dr Kathy Anderson of the Department of Biochemistry, University of Iowa, for careful guidance during the initial phases of this work and valuable advice throughout, Dr Robert Karr for helpful suggestions, Dr Peter Densen for review of this manuscript, and Dr Robert A. Clark for insightful criticism and for reviewing this manuscript.
44. Trimble RB, Maley F: Optimizing hydrolysis of N-linked high-mannose oligosaccharides by endo-B-N-acetylglucosaminidase H. Anal Biochem 141:515, 1984
Myeloperoxidase biosynthesis by a human promyelocytic leukemia cell line: insight into myeloperoxidase deficiency

WM Nauseef