Myeloperoxidase Precursors Incorporate Heme

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Myeloperoxidase of neutrophil granulocytes is synthesized as a larger molecular weight precursor, which is processed to yield mature polypeptides with molecular weights of 62,000 and 12,000. We have investigated the incorporation of heme into myeloperoxidase of the human promyelocytic HL-60 cell line labeled with 5-amino[14C]levulinic acid. Myeloperoxidase was isolated by immunoprecipitation followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and radiolabeled myeloperoxidase was visualized by fluorography. A 3-h pulse labeling with 5-amino[14C]levulinic acid resulted in labeling of the M, 90,000 and M, 82,000 precursor polypeptides. During subsequent chase of the label, conversion to mature radioactive heavy M, 62,000 subunit was observed but no radioactivity was associated with the mature small M, 12,000 subunit. Peptide mapping after proteolytic cleavage with V8 proteinase showed that 5-amino[14C]levulinic acid was associated with a single M, 23,000 polypeptide while multiple radioactive fragments were visible after proteolytic cleavage of myeloperoxidase biosynthetically labeled with [14C]leucine. That 5-amino[14C]levulinic acid was specifically incorporated into heme of myeloperoxidase was also demonstrated by dissociation under reducing conditions which yielded 14C-labeled heme as indicated by reversed phase high pressure liquid chromatography. The ionophore monensin and the base chloroquin, which block processing of myeloperoxidase, did not affect the incorporation of 5-amino[14C]levulinic acid, further supporting the notion that the incorporation of heme is independent of final processing of the polypeptide. Our data establish that heme is incorporated into myeloperoxidase already at the level of the precursor and that processing yields a heme-containing heavy subunit and a heme-free small subunit.

Myeloperoxidase (1, 2) is a major constituent of the lysosomal-like azurophil granules of neutrophil leukocytes (3, 4). The enzyme is a heme-containing glycoprotein that plays a role in the oxygen-dependent bactericidal action of neutrophils (5). Production of myeloperoxidase is closely linked to myeloid differentiation and it is synthesized at the promyelocytic stage of maturation (6). Myeloperoxidase is a tetramer with M, 120,000-160,000 and consists of two heavy chains and two light chains (7-10). Two heme groups associated with the heavy subunits (8) are present in the mature holoenzyme (1, 11).

Like lysosomal enzymes, myeloperoxidase is synthesized as a larger precursor polypeptide chain which has a molecular weight of 85,000-90,000 (12-15). During the intracellular transport the precursor is processed in prelysosomal and lysosomal structures to yield mature heavy (M, 62,000) and light (M, 12,000) subunits (13, 16). Another myeloperoxidase chain of M, 82,000 was postulated to be an intermediate form (13). One report showed an initial precursor with M, 75,000, which was processed to mature heavy and light subunits (17). Recently it was reported that also the M, 75,000 species may be an intermediate form and a direct precursor to mature subunits (18). There is no real consensus about the subunit structure of myeloperoxidase; the presence of two different heavy subunit peptides has been proposed (19). Myeloperoxidase may, like lysosomal enzymes, be directed to lysosomes by the mannose 6-phosphate-dependent pathway as [32P]phosphate incorporated into myeloperoxidase was susceptible to the digestion with endo-β-N-acetylglucosaminidase H (14, 16) indicating that oligosaccharide side chains are modified by phosphorylation as in lysosomal enzymes (20).

That lysosomal enzymes and myeloperoxidase are synthesized as larger precursors may play a role in sorting events for directing transport to lysosomes. It could also play a role to protect the cell against destructive action because the precursor may not be catalytically active. It is unknown if acquisition of peroxidase activity requires the prior shortening of myeloperoxidase precursor chains. We have now addressed this question by investigation of the incorporation of the heme group, necessary for catalytic activity, using biosynthetic labeling with 5-amino[14C]levulinic acid followed by immunoprecipitation of myeloperoxidase. The human promyelocytic cell line HL-60 was utilized for biosynthetic labeling. We report that 5-amino[14C]levulinic acid is incorporated into the precursor forms of myeloperoxidase. Pulse-chase labeling demonstrated that processing yields large heme-containing and small heme-free subunits. Peptide mapping after proteolytic cleavage with V8 proteinase showed that heme was present in a single peptide.

**EXPERIMENTAL PROCEDURES**

**Materials**

L-[14C]Leucine (342 mCi/mmol), 5-amino[14C]levulinic acid hydrochloride (52 mCi/mmol), and EN'PHANCE were from Du Pont-New England Nuclear. Protein A-Sepharose CL-4B and the ProRPC HR 5/2 reversed phase C18/C8 column were from Pharmacia P-L Biochemicals. Phenylmethanesulfonyl fluoride, chloroquin, monensin, and V8 proteinase from *Staphylococcus aureus* were from Sigma. Bio-Gel and acrylamide/bisacrylamide (29:1) were from Bio-Rad. Heme prepared from hemoglobin was a gift from Dr. J. O. Jepson, Malmö Hospital, Sweden.

**Methods**

Cell Culture—HL-60 cells (passages 20-40) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine
were washed and resuspended in RPMI 1640 with 10% FBS at a density of 10^6/ml and incubated for different chase periods.

Labeling with 5-Amino[^14C]Leucinulic Acid—Cells were incubated at a density of 2 × 10^6/ml for the appropriate time in leucine-free minimum essential medium (Eagle's) with 10% PBS supplemented with 30 μCi of [^14C]leucine/ml. They were washed and resuspended in RPMI 1640 medium with 10% FBS at a density of 10^6 cells/ml for different chase periods.

Immunoprecipitation—Extraction was with a radioimmunoprecipitation assay (RIPA) buffer consisting of 0.15 M NaCl, 30 mM Hepes (pH 7.3), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS. The extracts were kept on ice for 1 h and centrifuged at 32,000 × g for 1 h, and 300-500 μl of the supernatant was mixed with 15 μl of anti-myeloperoxidase serum produced by immunization of rabbits (7). After being on ice for at least 1 h, 40 μl of Protein A-Sepharose (200 mg/ml) solution in RIPA buffer was added for collection of the immunoprecipitate. Protein A-Sepharose samples were washed five times with RIPA buffer and the pellet was resuspended in 50 μl of water and 15 μl of electrophoresis sample buffer (0.4 M Tris/HCl (pH 6.8), 50% glycerol, 10% SDS, 5% mercaptoethanol), boiled for 5 min, and used for electrophoresis.

SDS-PAGE and Fluorography—SDS-PAGE was performed on a linear gradient of 5 to 20% polyacrylamide gel with 3% stacking gel as described earlier (13). Apparent M_r values were determined by use of the following [^3H]-methylated standards (Du Pont-New England Nuclear): cytochrome c (M_r, 12,300), carbonic anhydrase (M_r, 30,000), ovalbumin (M_r, 46,000), bovine serum albumin (M_r, 69,000), and phosphorylase b (M_r, 97,400). Gels were fixed in 10% trichloroacetic acid, 30% methanol for 1 h and treated with EN3HANCE for 1 h, and 300-500 μl of the supernatant was mixed with 15 μl of anti-myeloperoxidase serum. Lanes were eluted from Protein A-Sepharose at 60 °C for 15 min with 1.0 M guanidine hydrochloride containing 0.1 M sodium phosphate, pH 7.3, 4% SDS-PAGE and Fluorography—SDS-PAGE was performed on a linear gradient of 4 to 10% polyacrylamide gel with 3% stacking gel as described above. The samples were then diluted with 1 volume of 10% glycerol supplemented with 5 μg of V8 proteinase immediately before electrophoresis, which was performed at 10 mA/gel and was interrupted for 30 min when the dye marker was close to the bottom of the stacking gel. Electrophoresis was then continued at 25 mA/gel followed by fluorography.

Myeloperoxidase Subunits and Heme Separation by Gel Filtration—The samples used were obtained by labeling HL-60 cells with [^14C]leucine or 5-amino[^14C]leucinulic acid followed by cell extraction and immunoprecipitation as described above. The samples were then diluted with 1 volume of 10% glycerol supplemented with 5 μg of V8 proteinase immediately before electrophoresis, which was performed at 10 mA/gel and was interrupted for 30 min when the dye marker was close to the bottom of the stacking gel. Electrophoresis was then continued at 25 mA/gel followed by fluorography.

RESULTS AND DISCUSSION

HL-60 cells were incubated with 5-aminol[^14C]leucinulic acid for 3 h and the label was chased for 4, 7, and 20 h. Biosynthetic labeling was also performed by incubation with [^14C]leucine.

The abbreviations used are: FBS, fetal bovine serum; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RIPA, radioimmunoprecipitation; HPLC, high pressure liquid chromatography.

The cell extracts were immunoprecipitated with anti-myeloperoxidase serum. The fluorograms obtained after SDS-PAGE are shown in Fig. 1. Incubation experiments with [^14C]leucine (Fig. 1, lane A) showed labeling of the precursors (M_r, 90,000 and 82,000) and the two mature chains (M_r, 62,000 and 12,000). After 3-h labeling with 5-aminol[^14C]leucinulic acid, the isotope was found to be incorporated into myeloperoxidase precursors of M_r, 90,000 and 82,000 (Fig. 1, lane B). There was no unspecific precipitation with myeloperoxidase-absorbed anti-myeloperoxidase serum or preimmune serum (Fig. 1, lanes C, D, H, and I). Between 4 and 7 h of chase, radioactivity

![Fig. 1. Pulse-chase labeling of myeloperoxidase with [^14C]leucine and 5-aminol[^14C]leucinulic acid. HL-60 cells were pulsed with 5-aminol[^14C]leucinulic acid for 3 h and the label was chased 4, 7, and 20 h. [^14C]leucine labeling of cells was performed as a 2-h pulse followed by chase for 20 h. After isotopic labeling, extraction, immunoprecipitation with anti-myeloperoxidase serum, and SDS-PAGE were performed as described in the text. Lane A, [^14C]leucine-labeled cell extracts treated with anti-myeloperoxidase serum. Lanes B, C, and D, extracts of 5-aminol[^14C]leucinulic acid-labeled cells treated with anti-myeloperoxidase serum, absorbed anti-myeloperoxidase serum, and nonimmune serum, respectively. Lanes E and F, anti-myeloperoxidase-treated extracts of 5-aminol[^14C]leucinulic acid-labeled cells chased for 4 and 7 h, respectively. Lanes G, H, and I, extracts of 5-aminol[^14C]leucinulic acid-labeled cells chased for 20 h and treated with anti-myeloperoxidase serum, absorbed anti-myeloperoxidase serum, and nonimmune serum, respectively. The position of myeloperoxidase precursor polypeptides M_r 90,000 (90) and M_r 82,000 (82), the mature polypeptides M_r 62,000 (62) and M_r 12,000 (12), as well as the M_r 40,000 (40) presumed degradation product is indicated to the left.](image1)

![Fig. 2. Peptide mapping of 5-aminol[^14C]leucinulic acid and [^14C]leucine-labeled myeloperoxidase. HL-60 cells were labeled with 5-aminol[^14C]leucinulic acid and [^14C]leucine, respectively, followed by peptide mapping of immunoprecipitated myeloperoxidase performed as described in the text. Lanes A and B show the peptide maps of[^14C]leucine-myeloperoxidase labeled for 3 h (lane A) and after 20-h chase of the label (lane B). Lanes C and D depict the peptide maps of 5-aminol[^14C]leucinulic acid-myeloperoxidase labeled for 3 h (lane C) and after 20-h chase of the label (lane D). The position of molecular weight markers cytochrome c (12 × 10^3), carbonic anhydrase (30 × 10^3), and ovalbumin (46 × 10^3) is indicated to the left. The position of the radioactive fragment obtained from 5-aminol[^14C]leucinulic acid-labeled myeloperoxidase in lanes C and D is also indicated (25 × 10^3).](image2)
of $M_{r}, 90,000$ precursor decreased while the radioactivity associated with the $M_{r}, 82,000$ polypeptide increased (Fig. 1, lanes E and F). During subsequent chase for 20 h 5-amino$^{14}$C]levulinic acid labeled myeloperoxidase was converted to the mature heavy subunit of 62,000 (Fig. 1, lane G). Thus, the results indicated that the precursor polypeptides of myeloperoxidase incorporated heme. Accumulation of radioactivity in the heavy mature subunit of myeloperoxidase during chase demonstrated that the heme groups are associated with the larger $M_{r}, 62,000$ subunit of mature myeloperoxidase. The small $M_{r}, 12,000$ subunit lacked labeling with 5-amino$^{14}$C]levulinic acid, demonstrating that it lacks heme. The previously reported lack of spectral evidence for heme in subcellular fractions containing precursor myeloperoxidase (21) is most likely explained by the limit of assay sensitivity since only very small amounts of precursor polypeptide are expected to be present.

The biosynthetically labeled myeloperoxidase forms detected with $M_{r}, 40,000$–45,000 have been regarded as degradation products (13) or processing forms (15). 5-Amino$^{14}$C]levulinic acid was not detected in these products (Fig. 1, lanes B and E–G). Thus these forms of myeloperoxidase seem to lack prosthetic groups.

In order to establish the specificity of the 5-amino$^{14}$C]levulinic acid labeling of the heme group of myeloperoxidase, both peptide mapping and isolation of labeled heme were performed.

Myeloperoxidase, biosynthetically labeled with $^{14}$C]leucine and 5-amino$^{14}$C]levulinic acid, respectively, was utilized for peptide mapping. Fig. 2 demonstrates the pattern of proteolysis after treatment with V8 proteinase. Partial proteolysis of the $^{14}$C]leucine-labeled myeloperoxidase generated multiple labeled fragments of different molecular size. In contrast, myeloperoxidase labeled with 5-amino$^{14}$C]levulinic acid generated only one radioactive fragment with an apparent molecular weight of 23,000. The occurrence of a single peptide indicates that 5-amino$^{14}$C]levulinic acid is incorporated at one specific site of myeloperoxidase.

To further establish that 5-amino$^{14}$C]levulinic acid was specifically incorporated into the heme group, myeloperoxidase labeled with $^{14}$C]leucine and 5-amino$^{14}$C]levulinic acid, respectively, was dissociated by treatment with guanidine hydrochloride under reducing conditions (22) and subjected to gel filtration (Fig. 3A). Myeloperoxidase labeled with $^{14}$C]leucine was eluted in several peaks. SDS-PAGE of material from these peaks demonstrated that the 90- and 62-kDa forms of myeloperoxidase were eluted at approximately the same position. The 12-kDa subunit was separated from these larger forms of myeloperoxidase. The radioactivity of myeloperoxidase labeled with 5-amino$^{14}$C]levulinic acid and treated with guanidine hydrochloride under reducing conditions was eluted in two peaks. The first one was at the same position as the 90- and 62-kDa forms of myeloperoxidase, and the second was eluted near the total volume of the column where free heme is eluted. Analysis of the material of the second peak by HPLC on a ProRPC HR 5/2 column revealed that approxi-
imately 50% of the radioactivity was eluted close to the position of heme while the remainder was eluted like free 5-amino[14C]levulinic acid (Fig. 3B). These results indicate that 5-amino[14C]levulinic acid was specifically incorporated into heme of myeloperoxidase. An estimation revealed that a similar amount of 5-amino[14C]levulinic acid remained bound to the proform and the large subunit as was identified to be associated with free heme. It has been reported that one heme of myeloperoxidase may remain firmly bound to the large subunit even under dissociating conditions (22).

The effects of monensin, chloroquine, and NH4Cl on the processing of myeloperoxidase labeled with 5-amino[14C]levulinic acid were also examined. The ionophore monensin, which exchanges protons for Na+, and the base chloroquine, block processing probably by inhibition of transport through the Golgi apparatus (16). None of these agents interfered with the incorporation of 5-amino[14C]levulinic acid. In accordence with previous results, NH4Cl was found not to interfere with the processing, whereas both chloroquine and monensin blocked the processing leading to accumulation of the precursors of M, 90,000 and 82,000 (Fig. 4). The lack of effects of these agents on the incorporation of the heme group of myeloperoxidase indicates that the incorporation of heme is independent of final processing.

In conclusion, our results demonstrate for the first time that the incorporation of heme in myeloperoxidase occurs already at the level of the precursor polypeptide. The presence of heme is a prerequisite for peroxidase activity but it remains to establish if purified precursor myeloperoxidase has peroxidase activity or not.

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