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### Research Article

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# A Novel Form of Hereditary Myeloperoxidase Deficiency Linked to Endoplasmic Reticulum/Proteasome Degradation

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## Abstract

Myeloperoxidase (MPO) deficiency is a common inherited disorder linked to increased susceptibility to infection and malignancy. We identified a novel missense mutation in the MPO gene at codon 173 whereby tyrosine is replaced with cysteine (Y173C) that is associated with MPO deficiency and assessed its impact on MPO processing and targeting in transfectants expressing normal or mutant proteins. Although the precursor synthesized by cells expressing the Y173C mutation (MPO<sub>Y173C</sub>) was glycosylated, associated with the molecular chaperones calreticulin and calnexin, and acquired heme, it was neither proteolytically processed to mature MPO subunits nor secreted. After prolonged association with calreticulin and calnexin in the endoplasmic reticulum, MPO<sub>Y173C</sub> was degraded. Furthermore, the 20S proteasome inhibitor *N*-acetyl-L-leucyl-L-leucyl-L-norleucyl inhibited its degradation, suggesting that the proteasome mediates proteolysis of MPO<sub>Y173C</sub> and, thus, participates in quality control in this novel form of hereditary MPO deficiency. (*J. Clin. Invest.* 1998. 101:2900–2909.) Key words: myeloperoxidase • polymorphonuclear leukocyte • endoplasmic reticulum • proteasome • molecular chaperone

## Introduction

Myeloperoxidase (MPO)<sup>1</sup> (donor: H<sub>2</sub>O<sub>2</sub> oxidoreductase, EC 1.11.1.7) is a 150-kD hemoprotein stored exclusively in the azurophilic granules of monocytes and neutrophils (PMNs). During PMN activation, MPO is released into the phagosome

or extracellular space where, in the presence of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) generated by the PMN NADPH-dependent oxidase and chloride ions, hypochlorous acid and other highly toxic species are generated (1–5).

MPO is synthesized only during the promyelocytic stage of myeloid cell development and the 75–80-kD primary translation product is modified in the endoplasmic reticulum (ER) by cotranslational N-linked glycosylation followed by deglycosylation to generate the 90-kD apopMPO (6–8). During its maturation and processing, the 90-kD precursor MPO interacts transiently with the molecular chaperones calreticulin (CRT) (9) and calnexin (CLN) (10). After heme insertion, proMPO is processed further in a post-ER, pregranule compartment resulting in mature 59- and 13.5-kD subunits that are linked by a disulfide bond to form a single heterodimer (6–8). Two heterodimers are linked by an additional disulfide bond to form the mature molecule that is stored in azurophilic granules (6–8). In addition to the enzyme destined for the azurophilic granule, a significant fraction of normal proMPO enters the secretory pathway where it is modified in the Golgi complex en route to extracellular release (11, 12).

The MPO–H<sub>2</sub>O<sub>2</sub>–halide system represents the most efficient oxygen-dependent microbicidal mechanism in PMNs (1). PMNs that lack MPO or in which MPO activity is inhibited with sodium azide have retarded bactericidal activity and absent candidacidal function in vitro (7, 13). In addition, MPO-deficient subjects have been reported to have increased susceptibility to infection and malignancy (7). We previously identified a missense mutation in exon 10 of the MPO gene as the molecular basis for one form of inherited MPO deficiency. In the mutant protein, tryptophan replaces arginine at codon 569 (11) and this substitution results in defective processing of the protein such that heme is not inserted into the apoenzyme. As a result, proMPO is not made and the mature subunits of MPO are not generated (11). However, this mutation does not account for all cases of MPO deficiency (14) and the precise mechanism for the arrest in maturation has not been identified.

In this report, we describe a genotypically distinct form of MPO deficiency characterized by a single missense mutation predicting an amino acid substitution of cysteine for tyrosine at codon 173 (Y173C) and assess the impact of this mutation on the biosynthesis and maturation of MPO. The results indicate that CRT and CLN function to retain misfolded mutant MPO precursor in the ER for eventual degradation by the cytosolic proteasome and, thus, extend the quality control repertoire of these ER proteins to include soluble proteins destined for the lysosome.

## Methods

**Materials.** [<sup>35</sup>S]Methionine/cysteine (7.18 mCi/0.5 ml) and [<sup>14</sup>C]δ aminolevulinic acid ([<sup>14</sup>C]δ ALA) (0.25 mCi/2.5 ml) were obtained from Amersham Life Sciences Products (Arlington Heights, IL). Heat-

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1. *Abbreviations used in this paper:* ALA, aminolevulinic acid; ALLM, *N*-acetyl-L-leucinal-L-leucinal-L-methional; ALLN, *N*-acetyl-L-leucyl-L-leucyl-L-norleucyl; CFTR, cystic fibrosis transmembrane conductance regulator; CLN, calnexin; CRT, calreticulin; endo H, endoglycosidase H; ER, endoplasmic reticulum; MPO, myeloperoxidase; MPO<sub>Y173C</sub>, the precursor synthesized by cells expressing Y173C; *N*-GF, *N*-glycanase F; TBS, Tris-buffered saline; TM, tunicamycin; WT-MPO, K562 cells transfected with cDNA encoding normal MPO; Y173C, missense mutation in MPO gene at codon 173 whereby tyrosine is replaced by cysteine.

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killed, formalin-fixed protein A bearing *Staphylococcus aureus* (Pansorbin cells) were purchased from Calbiochem (San Diego, CA). Restriction endonucleases, T4 DNA ligase, calf-intestinal alkaline phosphatase (CIP), *TaqI* polymerase, and corresponding buffers were obtained from either Promega Co. (Madison, WI) or New England Biolabs, Inc. (Beverly, MA). Oligonucleotide primers were obtained from the DNA Core Facility at the University of Iowa (Iowa City, Iowa).  $H_2O_2$  was purchased from Mallinckrodt Chemical Inc. (Paris, KY). Sequenase version 2.0/dideoxy sequencing kit, FBS, and RPMI 1640 ( $\pm$ L-methionine) were purchased from Gibco Life Technologies (Gaithersburg, MD). *N*-glycanase F (*N*-GF) and endoglycosidase H (endo H) were obtained from Boehringer Mannheim (Indianapolis, IN). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

**Identification of a missense mutation in patient BS.** Heparinized, venous blood was obtained from individuals for routine screening in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the University of Iowa (Iowa City, Iowa), and PMNs were isolated as described previously, using Hypaque-Ficoll density gradient separation after dextran sedimentation (15). Related individuals with variations of partial to complete MPO deficiency were identified both by enzymatic analysis and immunoblotting and then screened via exon mapping as described previously (11, 16). Nucleotide sequencing of MPO exon 4 in patients BS, GS, CJ, and AJ revealed an A/G missense mutation at nucleotide 681 (data not shown).

**Site-directed mutagenesis.** The Y173C mutation was created using PCR as described previously (11). A full-length cDNA for MPO previously cloned into pBluescript SK (Stratagene, La Jolla, CA) was used as template for site-directed mutagenesis. Overlap extension with PCR using forward primers ( $^{249}$ TGGAAGTCTCCGACCGTATACCGTCTTA $^{278}$ ) and ( $^{675}$ ACAAATGCCGCCACCATCACCGAT $^{699}$ ) and reverse primers ( $^{667}$ CCTCGTCTGTTTACCGCGTGG $^{688}$ ) and ( $^{943}$ GGTGAAGTCGAGGTCGTGG $^{961}$ ) generated a 696-bp cDNA fragment (position of the nucleotide replacement is underlined) with an A/G substitution predicting a Y173C replacement in MPO. The fragment was digested with *StuI* and *BclI* (product, 493 bp), cloned into the identical region of MPO cDNA in pBluescript, and then sequenced to confirm the correct nucleotide substitution. Subsequently, MPO<sub>Y173C</sub> cDNA in pBluescript was digested with *NotI* and *HindIII* and cloned into the mammalian expression vector pREP10 (Invitrogen, San Diego, CA).

**Stable transfections in K562 cells.** Both K562 cells transfected with cDNA encoding normal MPO (WT-MPO) and MPO<sub>Y173C</sub> were generated from transfected K562 cell lines (American Type Culture Collection, Rockville, MD) as described previously (11). K562 cells were grown in RPMI 1640 containing penicillin-streptomycin, 2 mM L-glutamine, and 10% FBS and passaged when confluent.  $8 \times 10^6$  log-phase cells were washed in unsupplemented RPMI 1640, resuspended in 0.5 ml RPMI 1640, and electroporated (300 V, 960  $\mu$ Fd) with 20  $\mu$ g pREP10 plasmid containing either WT-MPO or MPO<sub>Y173C</sub> DNA and 10  $\mu$ g of salmon sperm carrier DNA in Hepes/NaCl. Stable transfectants were selected using 150  $\mu$ g/ml hygromycin (Calbiochem) 48 h after transfection and stable lines expressing the desired protein for up to 6 wk were obtained 72 h after selection.

**Metabolic labeling and immunoprecipitation of MPO and MPO<sub>Y173C</sub>.** Metabolic labeling of transfected cell lines was performed as described previously (11). Briefly,  $2 \times 10^6$  WT-MPO- and/or MPO<sub>Y173C</sub>-transfected K562 cells were washed in PBS and then reseeded in 6-well tissue culture dishes in methionine-free RPMI 1640 (containing 10% dialyzed FBS) to  $5 \times 10^5$  cells/ml. When indicated, proteasome inhibitors (from 50 mM stocks in DMSO) were added during the incubation in methionine-free media and were present for the duration of the chase period. For experiments in which it was necessary to inhibit glycosylation, cells were incubated for 4 h in the presence of 5  $\mu$ g/ml tunicamycin (TM) before metabolic labeling. After incubation at 37°C for 60 min in methionine-free media, cells were pulse-labeled for 60 min with 25  $\mu$ Ci/ml [ $^{35}$ S]methionine and then

chased for indicated time periods with 1 mM cold methionine. Cells were centrifuged at 500 g for 6 min at 25°C and then clarified culture media were saved for parallel immunoprecipitations. Cells were resuspended in lysis buffer (Tris-buffered saline [TBS], pH 7.5, containing 0.5 mg/ml leupeptin and pepstatin A, 0.5% CETAB, and 1.0% Triton X-100) with 1.0–2.0 mM PMSF and solubilized on ice for 20 min. Insoluble material was removed by briefly clarifying the lysate in a microcentrifuge (15 s at 12,000 rpm), which was then precleared with 7  $\mu$ l of nonimmune rabbit serum (rotation at 4°C for 30 min) followed by 50  $\mu$ l of washed Pansorbin protein A (rotation at 4°C for an additional 30 min). The lysate was clarified again to remove immune complexes (5 min at 12,000 rpm) and then diluted to 1.26 ml with 50 mM Tris-Cl (pH 7.4) containing 190 mM NaCl and 2.5% Triton X-100 (dilution buffer). Generally, 5–7  $\mu$ l of primary antibody (7  $\mu$ l of rabbit anti-MPO antibody, 7  $\mu$ l of rabbit anti-CRT antibody, or 5  $\mu$ l of rabbit anti-CLN antibody [kindly provided by Dr. David B. Williams, University of Toronto, Toronto, Canada]) was added to cell lysates or to 1.26 ml of cell-conditioned culture medium, and the immunoprecipitates were rotated for 4 h or overnight at 4°C. Subsequently, 50  $\mu$ l of washed Pansorbin protein A were incubated with the immune complexes for 30 min and then pelleted as above. Immune complexes were washed sequentially in TBS (pH 7.5) containing 0.5% Triton X-100, TBS containing 0.5% Triton X-100 and 2.0 M urea, TBS containing 0.5% Triton X-100 and 1 mg/ml BSA, and once in TBS alone.

For sequential immunoprecipitations used in the analysis of CLN-MPO or CRT-MPO complexes, Pansorbin protein A immune complexes obtained after precipitation with either CRT or CLN antibodies were washed, resuspended in 100  $\mu$ l of TBS containing 1.0% SDS, heated to 100°C for 5 min, and then cooled immediately on ice. After clarification as described above, supernatants were transferred to fresh tubes, diluted 1:10 in dilution buffer, and then precipitated with antibody to MPO as described above. Samples were resolved by 5–20% SDS-PAGE (see below).

For analysis of heme incorporation into WT-MPO or MPO<sub>Y173C</sub>, cells were prepared as described above, pulse-labeled with 14.3  $\mu$ Ci/ml of [ $^{14}$ C] $\delta$  ALA for 24 h in RPMI 1640 containing 5% FBS, and MPO was immunoprecipitated.

***N*-GF and endo H digestions.** 20  $\mu$ l of sucrose gradient fractions was denatured by heating in the presence of 1% SDS and then diluted 10 times with the appropriate enzyme buffer. Digestions with *N*-GF or endo H were performed using 0.5 mU *N*-GF and 10 mU endo H, respectively, for 1 h at 37°C as per the manufacturer's instructions. Alternatively, immunoprecipitated samples were washed and then resuspended in the appropriate enzyme buffer, heat-denatured in the presence of 0.2% SDS, and then digested. 6 $\times$  SDS-sample buffer was added to digestions and samples were resolved by SDS-PAGE.

**MPO activity.** K562 cells or transfected K562 cells were seeded at  $2 \times 10^5$  cells/ml  $\pm$  2  $\mu$ g/ml hemin chloride and then assayed for peroxidase activity 48 h later as previously described (11). Briefly, cells were washed in PBS and then resuspended in TBS (pH 7.5) containing 0.1% Triton X-100 to  $6 \times 10^7$  cells/ml and kept on ice until use. Reaction mixtures included 40  $\mu$ l of lysed cells and 25  $\mu$ l of 10 mg/ml orthodiansidine in 10 mM sodium phosphate (pH 6.0) containing 0.03%  $H_2O_2$ . Kinetic consumption of  $H_2O_2$  was monitored continuously for 60 s at 460 nm using a spectrophotometer (DV 640; Beckman Instruments, Palo Alto, CA) and  $V_{max}$  was calculated automatically.

**Subcellular fractionation of transfected K562 cells.** K562 cells transfected with WT-MPO or MPO<sub>Y173C</sub> constructs were seeded at  $2 \times 10^5$  cells/ml in culture medium containing 2  $\mu$ g/ml of hemin chloride and cultured for 60 h.  $1.5 \times 10^7$  cells were washed once in 30 ml of PBS and  $N_2$ -cavitated in 0.5 ml of relaxation buffer as described by Borregaard et al. (17). Cavitates were loaded atop a 10–60% continuous sucrose gradient, centrifuged at 35,000 rpm (SW41 rotor; Beckman Instruments) (151,000 g  $r_{av}$ ) for 15 h at 4°C, and 0.5-ml fractions were collected from the bottom of each tube. Assays for cytochrome c re-

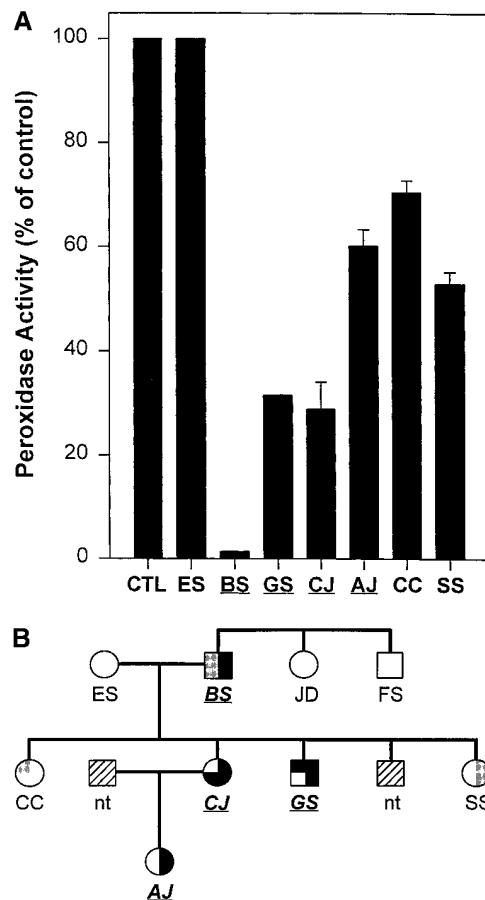
ductase and  $\beta$ -glucuronidase, markers for the ER and lysosome, respectively, were performed as described previously (18, 19). The distribution of protein in the gradient was measured by the BCA protein assay (Pierce, Inc., Rockford, IL). To analyze WT-MPO, MPO<sub>Y173C</sub>, CRT, and  $\beta$ -COP distribution, 20  $\mu$ l of each fraction was mixed 1:1 with 2 $\times$  SDS sample buffer, resolved by 10% SDS-PAGE, and then immunoblotted with rabbit anti-MPO, anti-CRT, or anti- $\beta$ -COP (clone M3A5; Sigma Chemical Co.) antibodies. Immunoblots were developed using an enhanced chemiluminescence detection system (SuperSignal Substrate; Pierce, Inc.) according to the manufacturer's instructions.

**SDS-PAGE and autoradiography.** Immunoprecipitations were routinely resuspended in 60  $\mu$ l of SDS sample buffer containing 2-mercaptoethanol and heated for 3 min at 100°C. After clarification (5 min at 12,000 rpm), 30  $\mu$ l of sample was resolved by 5–20% SDS-PAGE and gels were fixed, dried, and then exposed to Kodak X-Omat film for 2–6 h ( $[^{35}\text{S}]$ methionine) or 14 d ( $[^{14}\text{C}]\delta$  ALA) at  $-80^\circ\text{C}$ . For comparing reduced versus nonreduced proteins, samples prepared with or without 25 mM of DTT were resolved by 8% SDS-PAGE.  $[^{35}\text{S}]$ Methionine gels were quantitated using a PhosphorImager (SF; Molecular Dynamics, Sunnyvale, CA) and  $[^{14}\text{C}]\delta$  ALA gels via a densitometer (model GS-9000U Flying Spot; Shimadzu, Kyoto, Japan).

## Results

**Identification of a genotypically distinct form of MPO deficiency.** During routine laboratory screening, a 27-yr-old female (SS) was found to have partial deficiency of MPO. Immunoblot analysis of PMNs isolated from SS revealed the presence of 90-kD MPO precursors and  $\sim 25\%$  of the normal amount of mature MPO subunits (data not shown). Amplicons of exon 10 were resistant to digestion with BglII, indicating SS did not possess the R569W missense mutation previously identified as a cause of hereditary MPO deficiency (11, 16). The father of SS, BS, was completely MPO-deficient and his PMNs lacked mature 59-kD MPO species and peroxidase activity (Fig. 1 A). Genomic sequencing of BS's DNA identified a guanine/adenine substitution at nucleotide 681 ( $\text{A}^{681}\text{G}$ ) in exon 4 of one allele, whereby tyrosine would be replaced by cysteine at codon 173 (Y173C). The same mutation was present in family members CJ, GS, and AJ (Fig. 1 B). Like BS, all three of these offspring were heterozygous for Y173C and had reduced PMN peroxidase activity and correspondingly reduced levels of mature MPO (Fig. 1, A and B). Based on the distribution of peroxidase deficiency and of the Y173C mutation in his family tree, BS must have two mutant alleles, Y173C and an abnormality that has not yet been unidentified (Fig. 1, A and B). In contrast to BS, GS, CJ, and AJ have partial MPO deficiency and, thus, must possess one allele encoding normal MPO.

The Y173C substitution lies between C167 and C180 (positions 1 and 14 in the mature 13.5-kD subunit), two residues demonstrated by crystal structure analysis of MPO to be linked by a disulfide bond (20). Interposition of an additional cysteine at position 173 could form novel and/or might disrupt normal disulfide bonds during biosynthesis and, thus, create misfolded MPO precursors (Fig. 2 A). To assess the presence of additional or absence of normal disulfide linkages, electrophoretic mobilities of WT-MPO and MPO<sub>Y173C</sub> were compared by nonreducing SDS-PAGE. Migration of precursor MPO<sub>Y173C</sub> was retarded slightly in comparison to that of WT-MPO precursor, consistent with the presence of aberrant disulfide bonds in MPO<sub>Y173C</sub> (Fig. 2 B). Disruption of normal disulfide bonds might perturb structural determinants required for

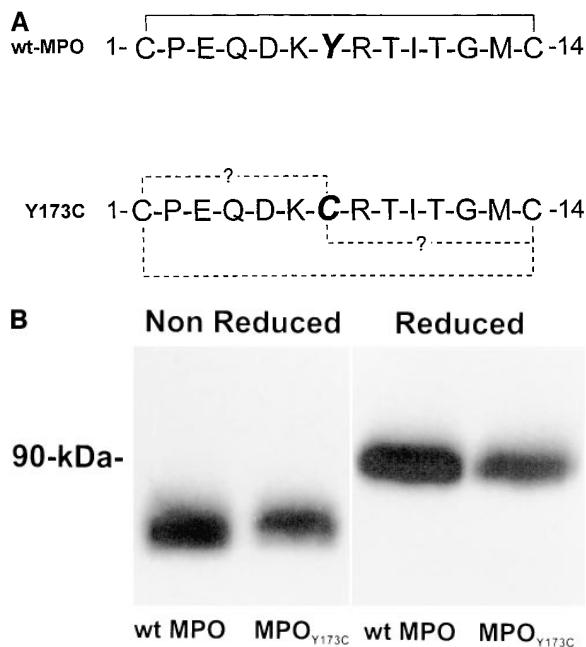


**Figure 1.** Hereditary MPO deficiency resulting from the Y173C mutation. (A) Peroxidase activity of PMNs from members of the S family with inherited MPO deficiency compared with that of healthy control subjects (CTL). (B) Sequencing of genomic DNA from members of the S family identified the Y173C mutation. Subjects with Y173C (black) were compound heterozygotes, the other allele being normal (white) or an unidentified mutation (gray). The peroxidase activity of PMNs from a given subject is represented by the relative amount of the symbol that is white (e.g., ES has 100% activity whereas BS has no activity). Cross-hatched squares indicate individuals not tested (nt). Results are expressed as the mean  $\pm$  SD of two to three separate trials.

proper folding and therefore compromise normal biosynthesis and/or intracellular compartmentalization.

**Processing of MPO<sub>Y173C</sub>.** To characterize the impact of Y173C on MPO biosynthesis and processing, we expressed MPO<sub>Y173C</sub> and WT-MPO in the human hematopoietic cell line K562. Both WT-MPO and MPO<sub>Y173C</sub> transfectants produced similar quantities of 90-kD MPO after pulse-labeling and chase intervals as long as 8 h (Fig. 3 A). However, the chase period revealed three significant differences in the cellular fate of MPO<sub>Y173C</sub> in comparison with that of normal MPO.

First, cells expressing normal MPO constitutively secreted a fraction of their MPO precursor, as demonstrated previously (11, 12). The secreted MPO precursor was partially resistant to digestion with endo H, indicating these species traversed the Golgi and underwent modification of their oligosaccharide sidechains from high-mannose to complex forms (Fig. 3 B). In



**Figure 2.** Comparison of disulfide bonds in WT MPO and MPO<sub>Y173C</sub>. (A) The crystal structure of MPO (20) indicates that a disulfide bond exists between residues C1 and C14 (C167 and C179 of the 90-kD precursor) in the mature 13.5-kD MPO light subunit. The Y173C mutation (enlarged and italicized) is interposed between the linked cysteines and might perturb normal WT-MPO disulfide bridging. (B) WT-MPO and MPO<sub>Y173C</sub> transfectants were pulse-labeled with [<sup>35</sup>S]methionine, immunoprecipitated with αMPO, and then subjected to either reducing or nonreducing SDS-PAGE as indicated. Results comparing reducing versus nonreducing SDS-PAGE are representative of three separate experiments.

contrast, cells expressing MPO<sub>Y173C</sub> failed to secrete any MPO-related peptide even after 24 h of chase (Fig. 3 A). Cell-associated MPO precursors either from WT-MPO or MPO<sub>Y173C</sub> transfectants remained endo H-susceptible (Fig. 3 B). The inability of the MPO<sub>Y173C</sub> transfectants to secrete MPO species did not reflect differential glycosylation as initial glycosylation of both MPO<sub>Y173C</sub> and WT-MPO appeared identical when the products of transcription-translation assays in the presence of microsomal membranes were analyzed (Fig. 3 C). Taken together these findings suggest that in contrast to the normal processing events, the 90-kD MPO precursor synthesized in MPO<sub>Y173C</sub> transfectants did not enter the secretory pathway and did not undergo maturation of the oligosaccharide side-chains in the Golgi.

Second, proteolytic maturation of precursor forms into subunits of lysosomal MPO differed in the two cell lines. After 8 h of chase, significant amounts of precursor had been processed to mature MPO in cells expressing WT-MPO, demonstrated by immunoprecipitation of the 59-kD heavy subunit of mature MPO after 24 h of chase (Fig. 3 D, left). In contrast, cells expressing MPO<sub>Y173C</sub> did not generate subunits of mature MPO, an observation consistent with the phenotype of affected patients. Based on the failure to generate the lysosomal form of MPO in the MPO<sub>Y173C</sub>, we anticipated that the cells would also fail to incorporate heme into apopromMPO and would, thus, lack peroxidase activity.

In comparison to untransfected K562 cells, cells expressing WT-MPO had significant peroxidase activity, which was augmented nearly three times when cells were grown in the presence of hemin chloride, as described previously (11) (Table I). Unexpectedly, lysates derived from MPO<sub>Y173C</sub> transfectants also exhibited MPO activity that was augmented two times when cells were grown in the presence of hemin, but this activity was significantly less than that of transfectants expressing normal MPO (Table I). In contrast to the observed phenotype in MPO-deficient individuals, these data suggest that MPO<sub>Y173C</sub> was processed to a heme-containing enzyme, albeit less efficiently than in transfectants expressing normal MPO. Thus, although MPO<sub>Y173C</sub> was not processed to mature MPO, MPO<sub>Y173C</sub> cells had detectable peroxidase activity, suggesting that some of the mutant apoprotein was processed to the enzymatically active, heme-containing proenzyme.

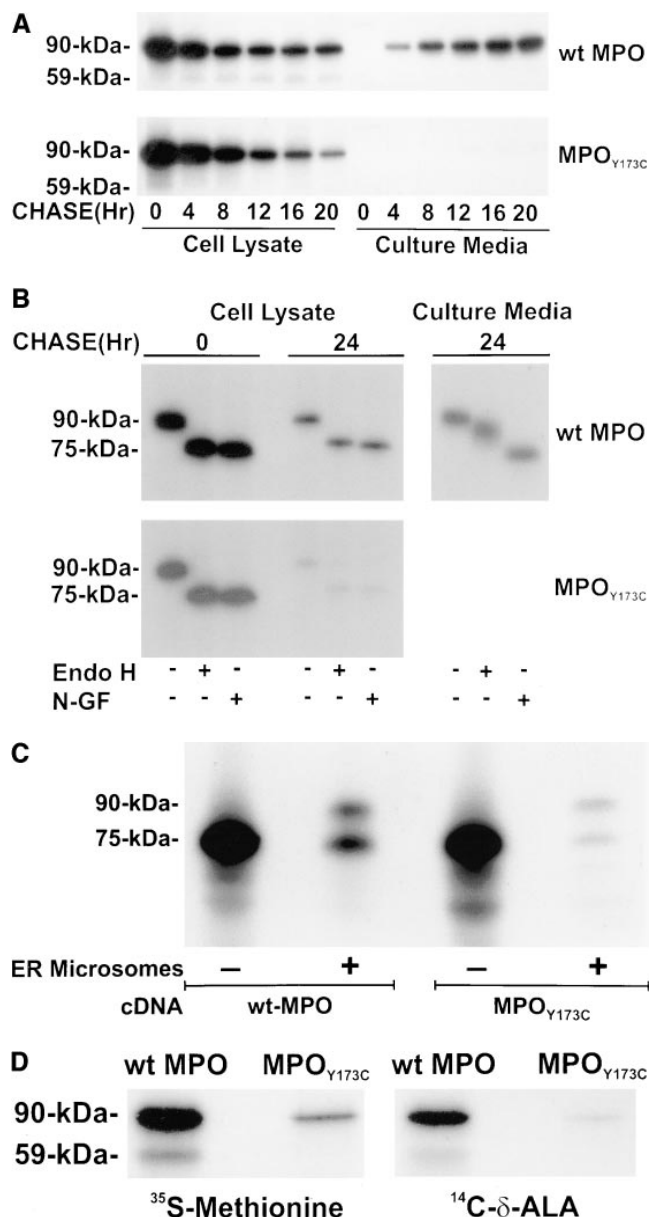
To test this hypothesis, we assessed heme insertion by following the incorporation of [<sup>14</sup>C]δ ALA-labeled prosthetic groups in MPO-related proteins. WT transfectants incorporated [<sup>14</sup>C]δ ALA into the functional heme groups of 90-kD promMPO and the 59-kD heavy subunit of mature MPO, as observed previously (Fig. 3 D) (11, 21). By comparison, MPO<sub>Y173C</sub> transfectants also incorporated heme into 90-kD apopromMPO, thereby generating promMPO, but not into a 59-kD mature MPO heavy subunit (Fig. 3 D). Less promMPO<sub>Y173C</sub> was detected in comparison to that in normal cells, either because heme insertion was less efficient in the mutant (~30.8% of that observed in normal cells) and/or because promMPO<sub>Y173C</sub> was less stable and/or had a shorter half-life than did WT-promMPO (Fig. 3 D). In either case, these data suggest that the peroxidase activity exhibited by MPO<sub>Y173C</sub> transfectants could be attributed to the presence of the proform of the mutant protein and confirms that the Y173C mutation adversely affected the stability and subsequent proteolytic maturation of promMPO.

In addition to the aberrant secretion and proteolytic processing seen in MPO<sub>Y173C</sub> transfectants, the mutant protein appeared to decay more rapidly than did normal MPO. After 24 h of chase, cells expressing MPO<sub>Y173C</sub> had a fivefold reduction (19.4±7.1% of WT) in MPO-related protein intracellularly, relative to cells expressing WT-MPO, and did not secrete MPO-related protein. Based on these and previous observations (22), we conclude that promMPO, once formed in the ER, has two potential fates: (a) to undergo proteolytic processing to the mature form of MPO destined for the azurophilic granule, or (b) to enter the secretory pathway with the associated carbohydrate modification during passage through the Golgi

**Table I.** Peroxidase Activities of WT-MPO and MPO<sub>Y173C</sub>

Cell line	(-) Hemin	(+) 2 µg/ml hemin
pREPMPO	1.0±0.11 (6)	2.81±0.11 (4)
pREPMPO <sub>Y173C</sub>	0.68±0.15 (4)	1.18±0.06 (6)
K562 (control)	0.36±0.11 (3)	0.39±0.05 (3)

Peroxidase activity was quantitated as described in Methods. The pREPMPO (WT-MPO) transfectant was given a value of 1.0 and all results are expressed relative to that value. Results are expressed as the mean±SD of *n* trials from three separate experiments. (The number of trials [*n*] is in parentheses next to each data set.)



**Figure 3.** Biosynthetic processing of WT MPO and MPO<sub>Y173C</sub>. (A) Transfected K562 cells expressing either WT MPO or MPO<sub>Y173C</sub> were pulse-labeled with [<sup>35</sup>S]methionine and then chased with cold methionine for the indicated time periods. After the chase period, cells and culture media from each transfectant were immunoprecipitated with αMPO and the resulting immunoprecipitate resolved by SDS-PAGE and autoradiography. (B) WT-MPO and MPO<sub>Y173C</sub> transfectants were pulse-labeled with [<sup>35</sup>S]methionine, chased for 24 h, and the resulting αMPO immunoprecipitations were digested with either endo H or N-GF. (C) The cDNA for WT MPO or MPO<sub>Y173C</sub> was transcribed/translated in vitro using a rabbit reticulocyte lysate assay as described previously (36). In the absence (–) of ER microsomes, the WT and/or 75–80 kD mutant core protein was observed to be translated equally. Glycosylated forms of the initial transcripts were generated when translation was initiated in the presence (+) of ER microsomes as indicated, and those of the WT or mutant were observed to be of identical size (~90-kD). (D) To identify heme-containing MPO-related precursors, transfected cells were pulse-labeled with [<sup>35</sup>S]methionine and chased for 24 h (left) or were continuously pulse-labeled for 24 h with [<sup>14</sup>C]δ ALA and then immunoprecipitated with αMPO. Results are representative of three to five separate experiments.

apparatus. Data from the pulse-chase biosynthetic labeling of MPO<sub>Y173C</sub> suggest proMPO<sub>Y173C</sub> was unable to follow either of these routes and was, instead, eventually degraded.

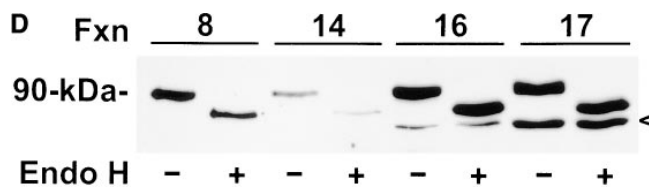
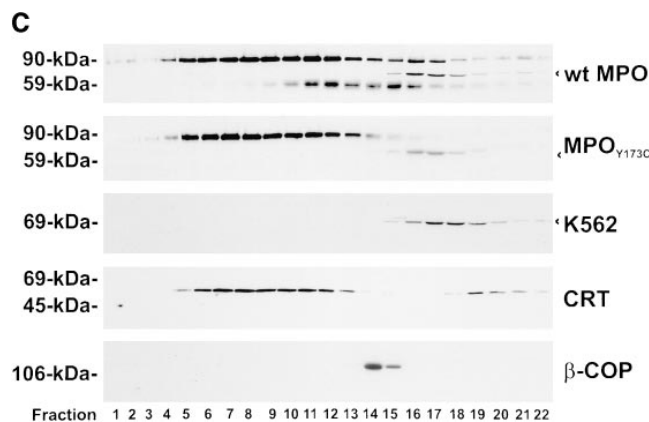
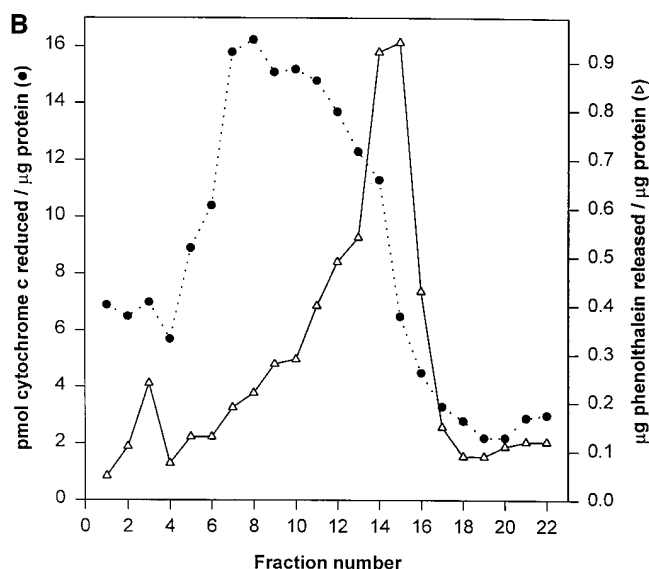
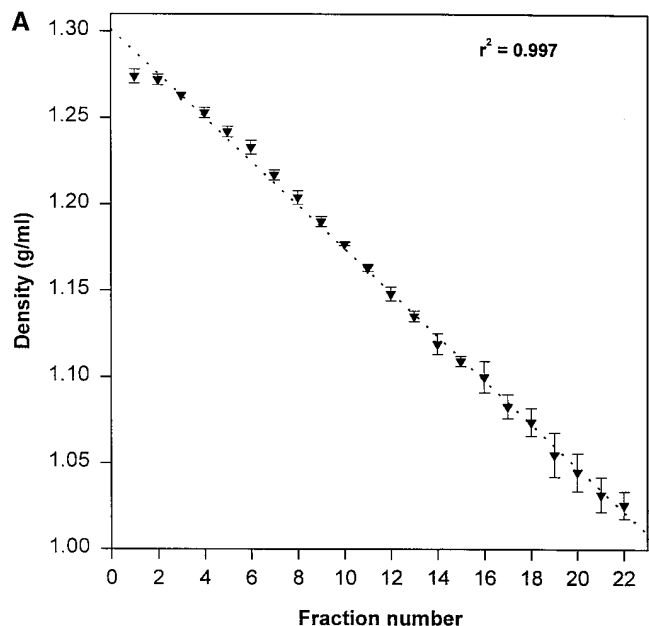
**Subcellular localization of MPO and MPO<sub>Y173C</sub> in transfected K562 cells.** Since proMPO<sub>Y173C</sub> was neither processed to mature protein nor secreted, we sought to identify its subcellular location using sucrose density gradient centrifugation. Fractions were collected and organelles identified by the presence of specific marker proteins, assessed enzymatically or immunochemically. ER (cytochrome *c* reductase), lysosomes (β-glucuronidase), and Golgi (β-COP) were identified as were the distributions of MPO and CRT (Fig. 4, A–C). The subcellular distributions of the marker enzymes and WT-MPO derived from the transfected K562 cells were similar to those in PLB-985 cells, a cultured human promyelocytic cell line (23) (data not shown). Thus, the distribution of subcellular organelles in these sucrose density gradients was the same for cells either transfected with MPO cDNA or expressing endogenous MPO.

MPO-related species were identified immunochemically and included the 90-kD precursors and the 59-kD heavy subunit of mature MPO. In addition, the antiserum recognized a 69-kD protein (Fig. 4 C, *indicator*) that was detected even in K562 cells that had not been transfected with cDNA for MPO. Since K562 cells express neither mRNA nor protein for MPO (24) this 69-kD protein is not related to MPO, although its identity is unknown.

As observed in pulse-chase experiments, cells transfected with WT-MPO generated the 59-kD subunit and this species sedimented predominantly in fractions 10–16, coincident with the lysosomal marker β-glucuronidase (Fig. 4, B and C). In contrast, cells transfected with MPO<sub>Y173C</sub> did not synthesize the 59-kD heavy subunit of mature MPO (Fig. 4 C). The majority of both normal and mutant 90-kD precursor MPO sedimented in fractions 5–12, correlating well with the distribution of the ER markers CRT and cytochrome *c* reductase (Fig. 4, B and C).

The subcellular fractionation of WT-MPO differed from that of the mutant protein in the distribution of the 90-kD MPO precursor. Fractions 16–17 of the WT-MPO gradient contained the 90-kD precursor, whereas there was no MPO-related protein in corresponding fractions from the MPO<sub>Y173C</sub> gradient (Fig. 4, B and C). Since MPO<sub>Y173C</sub> transfectants did not secrete the 90-kD precursor during biosynthesis (Fig. 3 A), we reasoned that the 90-kD precursor in fractions 16 and 17 of WT-MPO might represent exocytotic vesicles bound for secretion. However, none of the 90-kD MPO from fractions 16 or 17 were resistant to digestion with endo H (Fig. 4 D), in contrast to the partially endo H-resistant state of the secreted precursor (Fig. 3 B). These fractions may represent a post-ER, prelysosomal compartment, although studies to determine the subcellular location of these fractions are ongoing. Nonetheless, these data suggest that MPO<sub>Y173C</sub> failed to enter targeting pathways destined for the Golgi complex or the lysosome and instead remained associated with the ER.

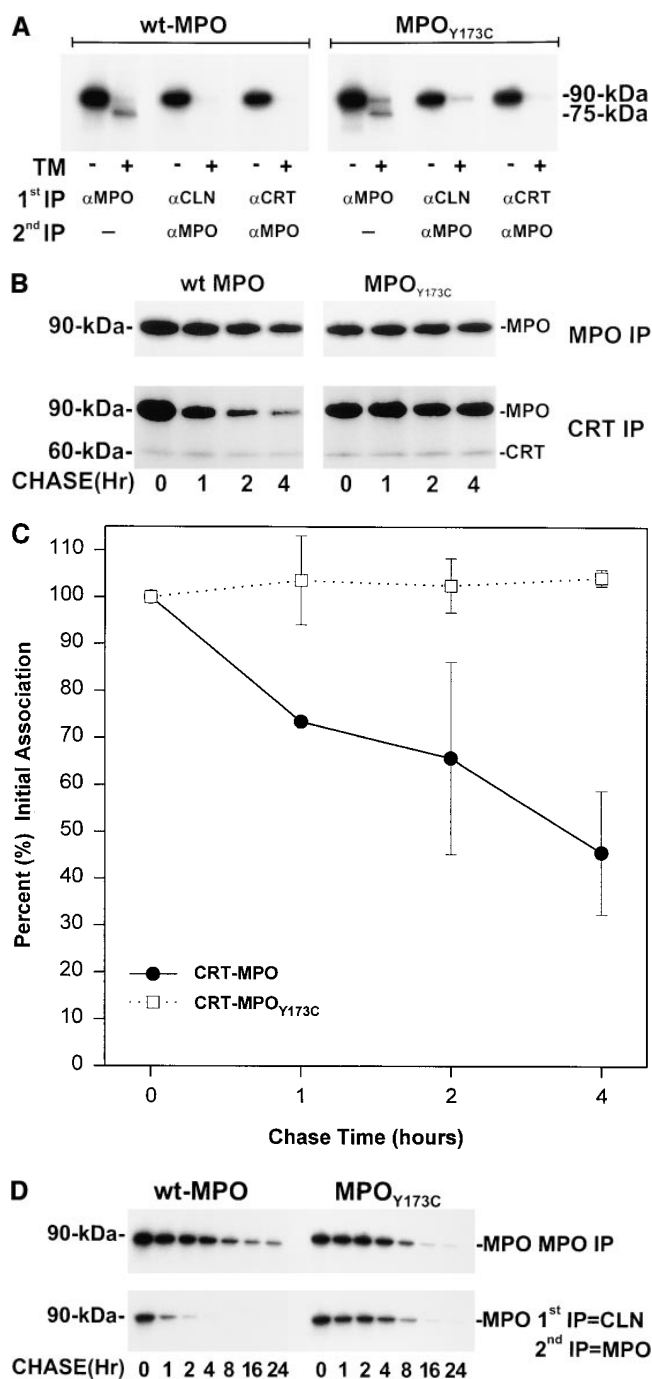
**Association of MPO<sub>Y173C</sub> with CLN and CRT is abnormal.** We have demonstrated previously that CRT functions as a molecular chaperone during MPO biosynthesis and associates specifically with apoproMPO (9). In addition, we have shown recently that CLN associates both with apoproMPO and proMPO (10). Glycosylation of normal MPO precursors is necessary for interaction with CRT or with CLN, as nonglyco-



sylated forms synthesized in the presence of TM do not associate with either chaperone (9, 10). To determine whether these chaperones participate in the ER retention of MPO<sub>Y173C</sub>, we compared the association of CRT and CLN with WT-MPO and MPO<sub>Y173C</sub> (Fig. 5, A–E). To determine if glycosylation of both WT-MPO and MPO<sub>Y173C</sub> was required for association with CRT and/or CLN, we compared association of the chaperones with nonglycosylated WT and mutant MPO proteins synthesized in the presence of TM. Nonglycosylated precursors (75–80 kDa) of neither WT-MPO nor MPO<sub>Y173C</sub> associated with CRT or CLN, confirming our previous results from studies of the promyelocytic cell line, PLB 985 (9, 10) (Fig. 5 A). WT-MPO in transfected K562 cells associated with CRT transiently and the apopMPO–CRT complex was partially dissociated by 4 h (Fig. 5, B and C). In contrast, the fraction of apopMPO<sub>Y173C</sub> associated with CRT immediately after the pulse-labeling was reduced (~39.0%) in comparison to that of control cells and remained constant for up to 4 h of chase (Fig. 5, B and C). These data demonstrate that the MPO<sub>Y173C</sub>–CRT complex remained intact for prolonged periods, consistent with the interpretation that CRT retained the mutant protein within the ER, presumably for eventual degradation.

Similar results were obtained when the association of MPO<sub>Y173C</sub> with CLN was studied (Fig. 5, D and E). WT-MPO transiently associated with CLN, leaving only 10.2±1.9% of the initial complex after 4 h of chase (Fig. 5, C and D). There was less precursor MPO<sub>Y173C</sub> (~37.2% less) associated with CLN immediately after pulse-labeling. In addition, CLN associated with the MPO<sub>Y173C</sub> precursor for a prolonged period during the chase (Fig. 5, D and E). In contrast to the dissociation of CLN-MPO complexes observed with WT-MPO transfectants, at 4 h of chase, 81.0±0.4% of the initial CLN–MPO<sub>Y173C</sub> complex remained intact. Even at 24 h of chase there was still 40.1±7.9% of the initial complex remaining, compared to 2.8±1.3% of the complex remaining with WT-MPO (Fig. 5, D and E). Although MPO<sub>Y173C</sub> was significantly degraded at 24 h of chase, much of the remaining mutant MPO species appeared complexed to CLN (Fig. 5, D and E). Collectively, these data indicate that the Y173C mutation adversely

**Figure 4.** Subcellular fractionation of WT-MPO and MPO<sub>Y173C</sub>. Lysates of K562 cells expressing WT-MPO and MPO<sub>Y173C</sub> were separated by centrifugation on 10–60% sucrose gradients as described in Methods. A and B indicate density of fractions from the gradient (closed triangles), β-glucuronidase (open triangles) (lysosomal marker), and cyanide-insensitive cytochrome c reductase activity (circles) (ER marker), respectively. (C) Gradient fractions were resolved by 10% SDS-PAGE and then probed with αMPO (WT MPO, MPO<sub>Y173C</sub>, and K562) or αCRT and anti-β-COP, as indicated. (D) 20 μl of the indicated WT-MPO gradient fractions was digested with endo H, separated by SDS-PAGE, and the electroblots probed with αMPO. Digestion with N-GF yielded MPO protein of identical size to that cut with endo H (data not shown). The indicator marks the presence of a protein nonspecifically recognized by the MPO antiserum that is present in K562 cells that were not transfected with cDNA encoding MPO. The linearity plot (A) is the mean±SD of two separate gradients. Results in B and C are from one experiment representative of at least three separate fractionation experiments, and those in D are from one experiment representative of two separate experiments (fractionations).

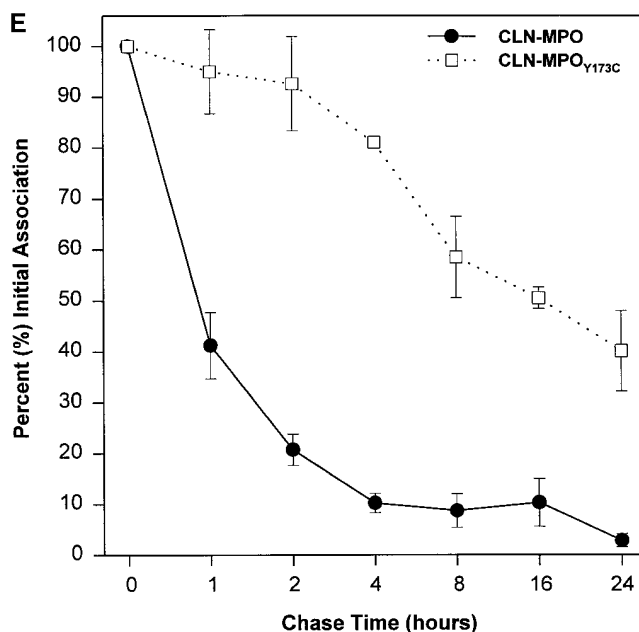


**Figure 5.** Association of WT-MPO and MPO<sub>Y173C</sub> with CRT and CLN. (A) WT-MPO and MPO<sub>Y173C</sub> transfectants were cultured in the presence of 5  $\mu$ g/ml of TM as indicated and then pulse-labeled with [<sup>35</sup>S]methionine and immunoprecipitated with  $\alpha$ MPO or sequentially with  $\alpha$ CRT or  $\alpha$ CLN followed by  $\alpha$ MPO. Autoradiograms of the sequential immunoprecipitations were exposed four to eight times longer than those of  $\alpha$ MPO to maximize detection of any coprecipitating 75–80 kD nonglycosylated MPO. Results are representative of two separate experiments. (B–E) K562 cells expressing WT MPO and MPO<sub>Y173C</sub> were pulse-labeled with [<sup>35</sup>S]methionine and then chased with cold methionine for the indicated time periods. Cell lysates were immunoprecipitated with either  $\alpha$ MPO or  $\alpha$ CRT (B, as indicated) and subjected to 5–20% SDS-PAGE and autoradiography. The amount of CRT coimmunoprecipitating with MPO (circles) or with MPO<sub>Y173C</sub> (squares) was quantitated by PhosphorImager analysis (C) and is the mean  $\pm$ SD of two to three separate experiments. Alterna-

affected both the association as well as the dissociation of MPO precursors with the ER-resident chaperones CRT and CLN. Given the absence of cell-associated mature or secreted protein in MPO<sub>Y173C</sub> (Fig. 3 A), we reasoned that prolonged association of the mutant protein with CRT and/or CLN may serve to retain the misfolded protein in the ER, which is incompetent for proteolytic processing and normal maturation.

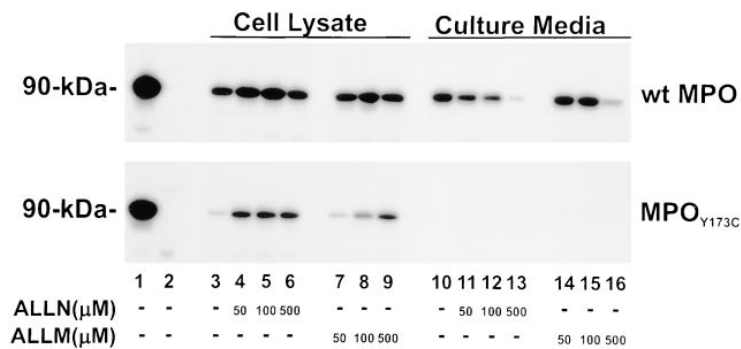
**ER degradation of MPO<sub>Y173C</sub> is linked to the activity of the cytosolic proteasome.** Since MPO<sub>Y173C</sub> was retained in the ER, we examined the possibility that its degradation might be mediated by the cytosolic proteasome. We tested the ability of two peptide aldehyde 20S proteasome inhibitors, *N*-acetyl-L-leucyl-L-leucyl-L-norleucyl (ALLN) and *N*-acetyl-L-leucyl-L-leucyl-L-methionyl (ALLM), to block degradation of MPO<sub>Y173C</sub>. Although both ALLN and ALLM are also cysteine protease inhibitors, ALLM is significantly less potent in inhibiting the proteasome when compared with the activity of ALLN (25).

Immediately after the pulse-labeling, nearly identical amounts of 90-kD MPO precursor were made in WT-MPO and MPO<sub>Y173C</sub> transfectants (Fig. 6, lane 1). However, as noted previously (Fig. 3 A), there was relatively less MPO precursor still present intracellularly at 24 h of chase in MPO<sub>Y173C</sub> than in WT-MPO cells (Fig. 6, lane 3, compare *top* and *bottom*). The addition of 50  $\mu$ M of ALLN to MPO<sub>Y173C</sub> transfectants significantly reduced degradation of the 90-kD precursor and increased the level of intracellular protein to a level comparable to that observed in WT-MPO transfectants (Fig. 6, compare lane 4, *bottom* with lane 3, *top*). However, treating the cells with up to 10 times more ALLN did not further inhibit degra-



tively, cell lysates were immunoprecipitated with either  $\alpha$ MPO or sequentially with  $\alpha$ CLN followed by  $\alpha$ MPO (D, as indicated) and subjected to 5–20% SDS-PAGE and autoradiography. The amount of CLN coimmunoprecipitating with MPO (circles) or with MPO<sub>Y173C</sub> (squares) was quantitated by PhosphorImager analysis (E) and results are expressed as the mean  $\pm$ SD of two to four separate experiments.





**Figure 6.** Degradation of MPO<sub>Y173C</sub> is blocked by the proteasome inhibitor ALLN. Transfected K562 cells expressing either WT MPO or MPO<sub>Y173C</sub> were pulse-labeled with [<sup>35</sup>S]methionine in the presence (+) or absence (−) of 50, 100, and 500 μM of ALLN or ALLM, as indicated, and then chased with cold methionine for 24 h. After the chase period, cells and culture media from each treatment were immunoprecipitated with αMPO. The resulting immunoprecipitate was re-solved by SDS-PAGE and the proteins were visualized using autoradiography. Lanes 1 and 2 represent MPO immunoprecipitated from cell lysates immediately after the pulse label or the resulting supernatant, respectively, in the absence of a chase. Results are from one experiment representative of two separate experiments.

degradation nor did it correct the observed defects in secretion or proteolytic maturation seen in MPO<sub>Y173C</sub> (Fig. 3, *A* and *B*, and Fig. 6). In contrast to the effects of ALLN, ALLM was less effective in inhibiting degradation of MPO<sub>Y173C</sub>; at 50 μM of ALLM, the degradation of mutant precursor was not inhibited. Only at very high concentrations (> 10× that of ALLN) was the reduction in degradation similar (Fig. 6, *bottom* compare lanes 4–6 with 7–9) to that observed with ALLN. The lack of secreted 90-kD species or processing to mature subunits in MPO<sub>Y173C</sub> transfectants treated with ALLN is consistent with our hypothesis that MPO<sub>Y173C</sub> species were retained in the ER by association of these species with CRT and CLN en route to their degradation in the proteasome.

## Discussion

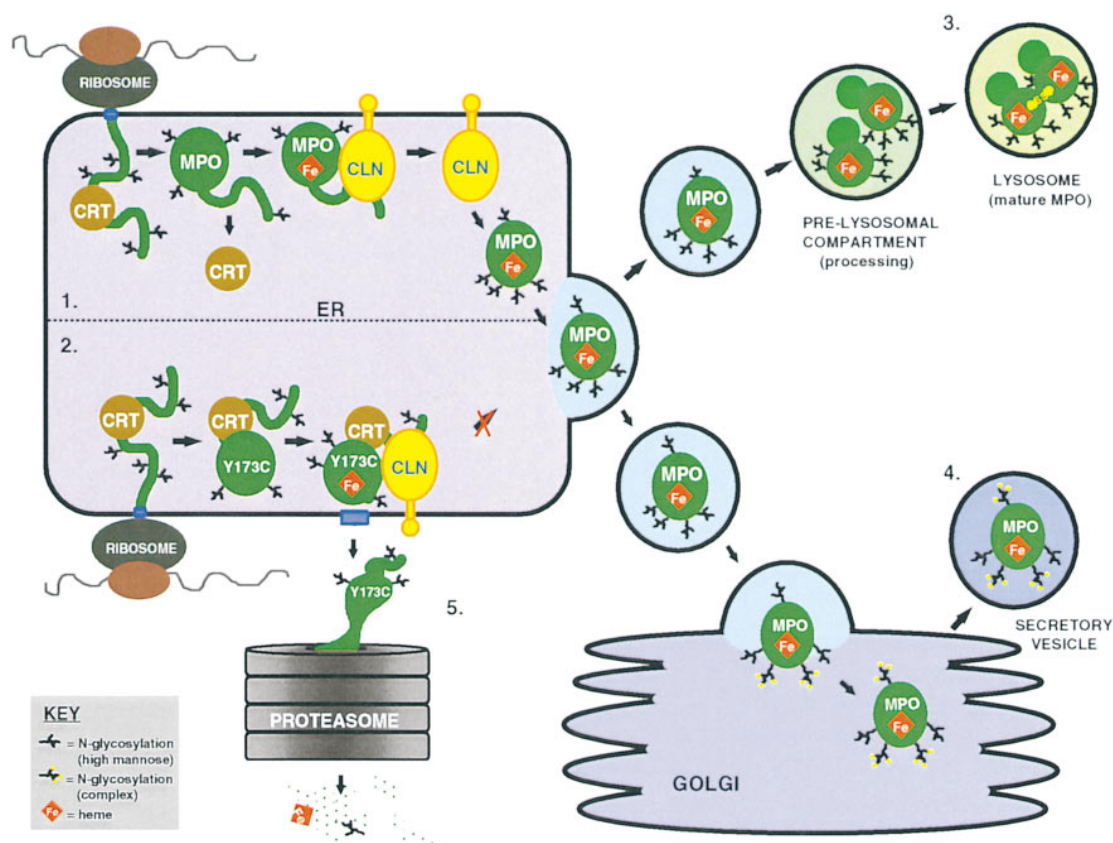
We have identified a novel genotype, Y173C, underlying hereditary MPO deficiency and described its impact on MPO synthesis. As a result of these studies, we have made several new observations pertinent to the biosynthesis of MPO specifically but that likely have implications for protein synthesis and ER quality control in general. Our findings are summarized schematically in a model that contrasts the fates of normal and Y173C MPO gene products (Fig. 7).

In the ER, the normal MPO transcript is cotranslationally modified by N-linked glycosylation and the resultant apoproMPO associates transiently with CRT and with CLN in succession (10). We have assigned this order of sequential association of apoproMPO with CRT and CLN based on our previous finding that CLN but not CRT associates with proMPO (10). Thus, it appears that chaperone association culminates in heme insertion and the conversion of apoproMPO into the enzymatically active precursor, proMPO. Once generated, proMPO can be directed toward the lysosome or into the secretory pathway. The former process involves budding from the ER and proteolytic processing in a prelysosomal, brefeldin-A-sensitive compartment (12). It is clear that only proMPO, and not apoproMPO, can undergo proteolytic processing into the subunits of mature MPO, since either inhibition of heme synthesis by succinyl acetone (12, 26, 27) or mutations, such as that described previously in exon 10 (11), which impair heme insertion block proteolytic maturation. ProMPO not destined for the lysosome enters the constitutive secretory pathway via the Golgi complex, wherein modification of the oligosaccharide sidechains results in glycoproteins partially resistant to digestion with endo H.

Like the normal MPO gene product, Y173C underwent cotranslational N-linked glycosylation and association with CRT and with CLN in the ER. However the associations of the apoproMPO<sub>Y173C</sub> with CRT and with CLN were prolonged, presumably due to aberrant folding of apoproMPO<sub>Y173C</sub>. Although heme was inserted into the mutant protein, the resultant proMPO<sub>Y173C</sub> failed to proceed along the pathway of normal MPO biosynthesis. In fact, proMPO<sub>Y173C</sub> did not undergo proteolytic maturation into the lysosomal form of MPO nor did it enter the secretory pathway. Instead the mutant protein was degraded by a mechanism dependent on a functional cytosolic proteasome.

Several studies indicate that proteins previously thought to be degraded within the ER lumen are in fact transported in a retrograde fashion through the translocon and destroyed by the cytosolic proteolytic complex known as the proteasome (25, 28–31). Furthermore, recent studies of the cystic fibrosis transmembrane conductance regulator (CFTR) and a deletion mutant underlying cystic fibrosis (a phenylalanine deletion at codon 508 in the CFTR [ΔF508]) have shown that ER degradation is facilitated by prolonged association of ΔF508 with CLN (32) and that the mutant protein is subsequently proteolyzed by the ubiquitin/proteasome machinery (25, 28). Just as we found in our studies of the Y173C mutation of MPO, specific proteasome inhibitors inhibit degradation of the mutant CFTR precursor (25, 28). Thus, we believe that ER retention of proMPO<sub>Y173C</sub> by CRT and CLN serves an important role in the quality control of MPO biosynthesis by myeloid cells.

In contrast to CFTR, which is a transmembrane protein, MPO and its precursors are soluble proteins, localized in the lumen of the lysosome and ER, respectively. Because MPO lacks a membrane-spanning domain, its mechanism for targeting to the proteasome may be different from that for CFTR or other integral membrane proteins. Based on our data the range of proteins degraded by the proteasome can be extended to include those destined for the lysosome. In addition, these studies represent the first demonstration that the ER/proteasome degradation machinery functions in biosynthetically active human myeloid cells. It is probable that genotypes underlying other inherited myeloid disorders, such as X-linked chronic granulomatous disease (33, 34) and leukocyte adhesion deficiency syndrome (35), result from abnormal protein biosynthesis leading to ER/proteasome degradation. Characterization of such processes and determination of the mechanisms for ER retention and subsequent export await further study.



**Figure 7.** A model contrasting the biosynthesis and maturation of WT MPO and MPO<sub>Y173C</sub>. Both WT-MPO and MPO<sub>Y173C</sub> are cotranslationally modified via the addition of five N-linked glycosylations and then associate with CRT (1 and 2 in the ER). Since in WT MPO CRT associates only with apoproMPO, normal apoproMPO must be released from CRT before heme insertion. Since CLN associates with apoproMPO as well as proMPO, the association of MPO precursors with CLN most likely follows dissociation of CRT–apoproMPO (1 in the ER). Normal proMPO then leaves the ER where it is targeted to either lysosomes (3) or the Golgi complex for secretion (4). In contrast, the precursor forms of MPO<sub>Y173C</sub> associate in a prolonged fashion with both CRT and CLN (2). As a result, MPO<sub>Y173C</sub> precursors are retained in the ER lumen for subsequent export to the cytosolic proteasome and degradation (5). Since MPO lacks a membrane-spanning region, it must associate with another protein, which is either membrane-associated (e.g., CRT) or an integral membrane protein (e.g., CLN), to mediate transport to the translocon and retrograde export to the cytosolic proteasome.

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