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Lessons from MPO Deficiency about Functionally Important Structural Features

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SUMMARY: Genetic abnormalities often serve as the catalyst to stimulate critical insights into normal biology. In many cases, examining such experiments of Nature explicates not only the abnormal but also serves to illustrate underlying normal principles. Within the context of myeloperoxidase biosynthesis, we have examined the impact of specific missense mutations that cause inherited myeloperoxidase deficiency. Such studies have provided novel insights not otherwise possible. We have described three such mutations and are completing the analysis of a fourth.

Polymorphonuclear neutrophils (PMNs) represent the cornerstone of cell-mediated antimicrobial activity in the human innate immune system. To successfully eradicate infection, PMNs must ingest, kill, and degrade invading microbes. Central in our conceptualization of PMN-mediated antimicrobial activity have been events dependent on the generation of hydrogen peroxide (H_2O_2) and amplified by the PMN granule protein myeloperoxidase (MPO) (1). Recent studies have challenged the importance of the MPO- H_2O_2 system in antimicrobial action, suggesting instead that a potassium-dependent activation of granule proteases is the intraphagosomal event lethal to ingested bacteria (2). Although this alternative hypothesis has stimulated a reconsideration of the precise mechanisms by which PMNs kill ingested bacteria, most evidence, direct and indirect, places the MPO-H_2O_2-halide system in a prominent position in innate host defense.

Our first identification of individuals with the absence of MPO activity from their circulating PMNs prompted studies to determine whether the myeloid precursors in these individuals synthesized aberrant protein or if they lacked the capacity to synthesize any MPO-related proteins. The need to understand the biosynthesis of normal MPO was the stimulus for more than two decades of work on myeloid cell synthesis of MPO and related proteins. MPO is encoded by a single gene and the 80-kDa MPO primary translation product undergoes cotranslational cleavage of the signal peptide, N-linked glycosylation, and limited deglucosylation of high mannose oligosaccharide sidechains to generate the 90-kDa, enzymatically inactive precursor apoproMPO. Within the ER apoproMPO interacts transiently and reversibly with the molecular chaperones calreticulin (CRT) and calnexin (CLN) with subsequent incorporation of heme to generate the enzymatically active 90-kDa proMPO. It is not known whether the association with CRT or CLN is essential for heme acquisition and the maturation of apoproMPO to proMPO. Also unknown is the existence and role of additional chaperones that might facilitate heme insertion into the apoprotein. We have assessed the role in MPO biosynthesis of other known molecular chaperones. Whereas ERp57 (aka glucose regulated protein 58) generally associates with CRT and the complex functions as an ER chaperone, we find very little participation of ERp57 in myeloid cell protein synthesis. ERp57 associates only transiently with apoproMPO very early in biosynthesis and there is no CRT-ERp57 complex formation.

Given its peroxidase activity, it is obvious that heme incorporation is required for its enzymatic function. However heme insertion has structural implications independent of its impact on catalytic activity. Heme synthesis inhibition blocks proteolytic maturation as well as export of MPO precursor from the ER, indicating that its acquisition of heme induces conformational changes necessary for successful maturation and intracellular targeting. Although 10% of proMPO enters the secretory pathway and is recovered from the culture media as a monomer (3), most proMPO undergoes dimerization, modification of oligosaccharide sidechains, and proteolytic processing *en route* to the azurophilic granule. The functional consequences of dimer formation are not known nor are the bases for the differential glycosylation of cell-associated and secreted forms MPO.

Characterizing the consequences of the genetic abnormalities arising in the population to elucidate principles of normal biology represents one of the guiding principles of biomedical research. Advances in understanding the molecular bases of cystic fibrosis and chronic granulomatous disease serve as excellent examples of this principle. Examining such "experiments of Nature" has provided us insights into MPO biosynthesis that we might not have achieved otherwise. We have examined the impact of specific missense mutations that cause inherited MPO deficiency on its biosynthesis, reasoning that such studies will reveal structural and organizational principles not apparent from the structure of the mature protein. We have described three such mutations and are completing the analysis of a fourth.

The first mutation we identified was R569W, wherein the arginine at codon 569 was replaced with a tryptophan (4). This particular mutation occurs commonly among the patients we examined and the inheritance pattern was complex, with most individuals being compound heterozygotes, although not all the specific mutations were identified. The R569W mutation results in a maturational arrest in MPO biosynthesis at the stage of apoproMPO. That is, apoproMPO is synthesized in a quantitatively normal fashion but fails to acquire heme. As a result, proMPO is not formed and there is not progression down the pathway towards proteolytic processing and granule targeting. The degradation of the mutant apoproMPO is not blocked by proteasomal inhibition, indicating either that its ER degradation is not mediated by the proteasome or that the specific proteases responsible are not susceptible to the inhibitors used.

More recently we identified a novel mutation in a family in Iowa. This missense mutation, Y173C, results in the potential for novel disulfide bonds in the light subunit at a site between two cysteines known from the crystal structure data to be linked in a disulfide bond (5). The Y173C mutation has a very different impact on MPO biosynthesis than does R569W. In contrast to the situation with R569W, apoproMPO Y173C acquired heme to form proMPO, but very little proMPO could be recovered. Given that proMPO Y173C fails to undergo proteolytic processing to form mature MPO subunits, the proMPO in Y173C must be very unstable. Consistent with that interpretation are observations of the interactions of precursors of Y173C with molecular chaperones. ProY173C is retained in the ER by association with CLN and eventually is degraded in the proteasome (12). This observation as well as analysis of limited proteolytic digestion of constructs synthesized on microsomes suggest that this mutation results in abnormal folding of apoproMPO with untoward consequences on heme acquisition, ER export, and proteolytic maturation into mature protein.

Drs. Romano and colleagues described an Italian patient with complete MPO deficiency who has as its cause a missense mutation replacing a methionine at residue 251 with threonine, M251T (6). In our hands stable transfectants expressing M251T synthesize MPO precursors that were inefficiently processed into mature MPO in granules but entered the secretory pathway in a normal fashion. The biosynthetic precursors of M251T interact transiently in the ER both with CRT and CLN, although retention was relatively prolonged in

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comparison to that seen with wild type MPO. Similar to the events in Y173C, the apoproM251T acquired heme inefficiently, at levels ~ 10% of controls and expressed profoundly reduced peroxidase activity. These data suggest that M251T transfectants incorporate heme either inefficiently or with low affinity. Definition of the functional impact of M251T may be especially informative because of its proximity to D260, the residue in the light subunit covalently bound to the heme group.

Solution of the crystal structure of human MPO at 1.8 Å (7) has resolved many questions about the structure around the heme of MPO, once the subject of considerable debate. The heme pocket is in a crevice ~ 15 - 20 Å deep, formed by a core of helices. Solvent is accessible via an open channel at the catalytic site, on the distal side of the heme. Significant data implicate five residues in MPO in heme binding. Histidines at residues 261 and at 502 represent the distal and proximal ligands, respectively. As noted above, the M251T mutation, because of its proximity to the distal histidine covalently bound to heme, may be especially instructive. In addition to the histidines, the heme is covalently bound to MPO through a methionyl sulfonium linkage with M409 and through ester linkages to E408 and D260. Although crystal structures of other members of the animal peroxidase family have not yet been solved, models based on domain organization homologous with that of MPO suggest that this format may apply to all proteins with the exception of the sulfonium linkage. This covalent bond is seen only in MPO and may explain both its unique enzymatic capacity to oxidize chloride to the +1 state, forminig HOCl, and its peculiar spectral properties.

We are now completing a detailed molecular characterization of a missense mutation in MPO causing MPO deficiency in a patient identified by Dr. Kazuo Suzuki and colleagues in Japan (8). Pertinent to the discussion above, the mutation, G501S, occurs immediately adjacent to H502, the proximal ligand to the heme. Since glycine at this position is conserved among all members of the animal peroxidase family, we anticipated that such a mutation in the heme pocket would not be tolerated. We predicted that G501S precursors would remain in the ER and be degraded in the cytosolic proteasome. Stably transfected K562 cells expressing G501S synthesize and secrete the 90-kDa MPO precursor in normal fashion but the precursor fails to undergo proteolytic processing to mature MPO subunits. The G501S precursor associates transiently with CRT and CLN with normal kinetics, undergoes normal oligosaccharide modification, and, contrary to our prediction, is not degraded in the proteasome. G501S cell lysates lack peroxidase activity when judged by the native gel analysis. Enzymatic inactivity coupled with the failure of proteolytic processing to occur, a process dependent on formation of proMPO, suggested that the G501S apoproMPO was unable to acquire heme. However, when heme acquisition was assessed by biosynthetically radiolabeling with the heme precursor [^{14}C]-δ-aminolevulinic acid, the G501S MPO precursor incorporates heme. When corrected for the relative amount of MPO precursor synthesized, the level of the heme-containing G501S precursor made is ~ 38% of that for normal MPO. These data suggest that the maturation arrest in G501S is at the level of proMPO. Stable transfectants exhibit peroxidase activity when evaluated in a hydrogen peroxide consumption as a more sensitive assay for MPO activity. In this sensitive system, cell lysates of G501S expressing K562 cells exhibit activity that was greater than background (i.e. non transfected K562 cells) but less than cells with endogenous MPO (i.e. PLB-985 cells) or K562 cells stably transfected with wild type MPO. Additional studies using recombinant protein recovered from transfected cells will assess the spectral properties and chlorinating activity of the G501S, thereby providing important new information about structural features in the heme environs that mediate functional consequences.

These studies examining the molecular consequences of naturally occurring mutations on the structure and function of MPO reinforce our conviction that defining the functional impact of specific genotypes of MPO deficiency represents an informative strategy to probe the structural determinants of normal MPO.

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