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# DIFFERENTIAL DISTRIBUTION OF DISTINCT FORMS OF MYELOPEROXIDASE IN DIFFERENT AZUROPHILIC GRANULE SUBPOPULATIONS FROM HUMAN NEUTROPHILS

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SUMMARY: Myeloperoxidase (MPO), a characteristic enzyme of human polymorphonuclear neutrophils (PMN), is localized in specialized lysosomal or azurophilic granules, and can be resolved into three distinct forms (I, II, III) by ion-exchange chromatography. Granules were isolated from single donor PMN and fractionated with centrifugation into two different azurophilic subpopulations (high and low density) by banding in a continuous sucrose density gradient. Ion-exchange chromatography of granule extracts indicated that the lower density granules contained mainly MPO forms II and III while the higher density granules appeared to contain all three forms, but in much reduced amounts. Sodium dodecylsulfate polyacrylamide gel electrophoresis showed that the mobilities of the heavy subunits of MPO appeared to be inversely related to the density of the granule population from which they were extracted. These observations suggest that the different forms of MPO may have distinct functional roles and/or are a possible reflection of maturational differences among the granule subpopulations.

Based on a variety of ultrastructural, cytochemical and biochemical evidence, it is generally accepted that human polymorphonuclear neutrophils (PMN)[1] contain two major types of cytoplasmic granules, azurophilic or primary (AG) and specific or secondary (SG) (1,2). These two populations differ in morphology, are associated with different stages in the cytodifferentiation of PMN, and bud off different faces of the Golgi complex during granulogenesis (1,2). Both types of granules exhibit major differences in content (1-7), and the glycoproteins MPO and lactoferrin are commonly used markers for the AG and SG, respectively (4). From a functional point of view, AG and SG exhibit different kinetics of degranulation (8) as well as accessibility for mobilization and exocytosis (9-11). However, numerous studies

1. ABBREVIATIONS: PMN, polymorphonuclear leukocyte; AG, azurophilic granule; SG, specific granule; MPO, myeloperoxidase; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis; CETAB, cetyltrimethylammonium bromide; FMLP, N-formyl-methioninyl-leucyl-phenylalanine.

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indicate that PMN granules are more heterogeneous than often supposed (12). For example, studies of human PMN granules obtained by sucrose density gradient centrifugation procedures have clearly demonstrated the existence of three granule populations, two of which are MPO-containing AG (4,5,9,13). In addition, electron microscopic studies using the 3,3'-diaminobenzidine cytochemical peroxidase reaction lend further support to the existence of at least two different subpopulations of peroxidase-positive granules (5,14).

We have recently demonstrated that normal human PMN contain three different chromatographic forms of MPO (I,II,III) (15) which exhibit differences in enzymatic activity, subunit structure, and extractability, form I requiring the use of the cationic detergent CETAB to effect its solubilization (16). Furthermore, our observation that exposure of purified human PMN to the synthetic chemotactic peptide FMLP in the presence of cytochalasin B resulted in the specific exocytosis of forms II and III appeared to provide a functional correlation (17). These biochemical observations, when taken together with the morphological and cell fractionation evidence, suggested to us that distinct forms of MPO might be differentially associated with distinct subpopulations of AG from human PMN. In this report we present evidence to support this idea.

## MATERIALS AND METHODS

Materials: Guaiacol was from Sigma Chemical Co. and CETAB was from Eastman Kodak. Carboxymethyl cellulose (CM-52) was obtained from Whatman, Ltd. All reagents for PAGE were from Bio-Rad Laboratories.

Methods: Replicate experiments utilized cells obtained from a single donor. PMN (>93% total cells) were obtained by continuous flow leukapheresis from normal healthy adult male and female donors, and erythrocytes were removed by hypotonic lysis (17,18). Granules from about 5 x $10^9$ purified PMN were prepared by homogenization of the cells in 0.34 M sucrose as described previously (16), and were fractionated by centrifugation for 2 hr at 21,000 rpm in a Beckman Ti 14 rotor using a linear sucrose gradient (30-53%) as described earlier (4). AG were identified by the presence of MPO using both biochemical (see below) and immunochemical methods and SG were identified by the presence of lactoferrin using a rabbit anti-lactoferrin antibody (15,17,19). These data confirmed that the granule fractionation yielded results comparable to previously published reports (4-6).

MPO was solubilized by lysing the granules in 10 mM sodium phosphate buffer, pH 7.0 containing CETAB (0.3%) (15,16) and enzymatic activity was measured spectrophotometrically using guaiacol as the electron donor (16). The three different forms of MPO (I,II,III) were identified by ion-exchange chromatography on carboxymethyl cellulose (15) and by PAGE in the presence of

SDS (16). All three forms reacted with a line of identity using a polyclonal rabbit antibody (15).

## RESULTS

Figure 1 shows a typical sucrose density gradient profile of fractionated PMN granules. It can be seen that MPO activity was associated with two different azurophilic granule subpopulations, high (H) and low (L) density, respectively. Most of the MPO activity was found to be associated with the low density granules, and material in both fractions reacted specifically with rabbit anti-MPO antibody but not with anti-lactoferrin antibody. In contrast, none of the material in granule fraction SG (Fig. 1) reacted with the anti-MPO antibody but did react with rabbit anti-lactoferrin antibody. These data, taken together with the MPO activity measurements and PAGE studies (see below) clearly distinguished the two AG subpopulations from the SG population.

Individual fractions from the high and low density MPO-containing peaks (see Fig. 1) were lysed in the presence of CETAB and the lysates were subjected

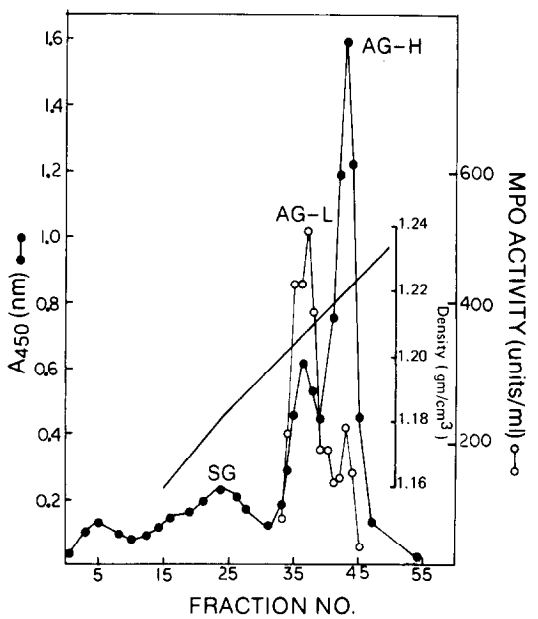


Fig. 1. Sucrose density gradient fractionation of human PMN granules. The granule profile (•—•) was determined by measuring turbidity at 450 nm. L and H refer to low and high density AG granules, respectively, which were identified by the biochemical (o—o) and immunochemical presence of MPO. Sucrose gradient (——). See Materials and Methods for details.

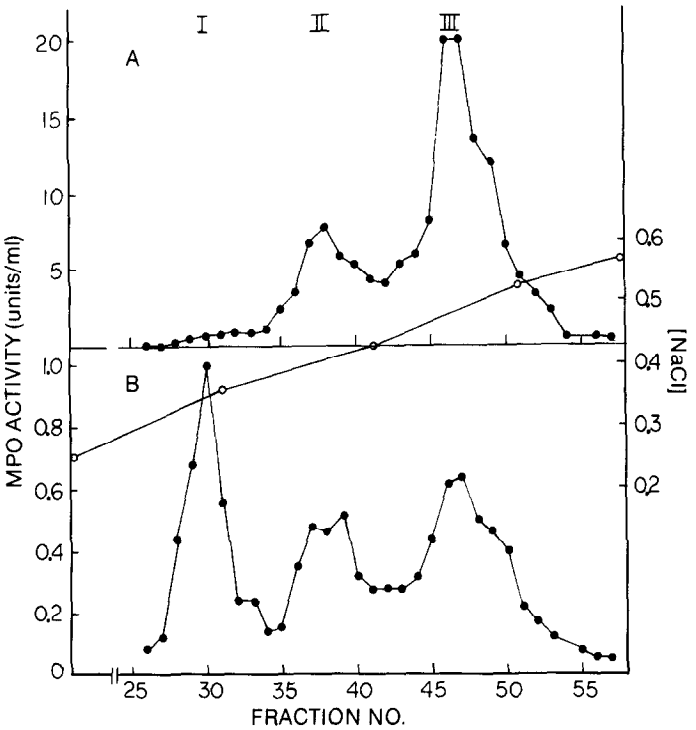


Fig.2. Carboxymethyl cellulose ion-exchange chromatography of the different forms of MPO contained in the AG-L(2A; fraction 35 of Fig. 1) and AG-H(2B; fractions 44 and 45 of Fig. 1) subpopulations of human PMN. The column (0.9 x 30 cm) was loaded with 200 units (2A) and 17 units (2B) of MPO activity in equilibration buffer (25 mM sodium acetate, 0.2 M NaCl, 0.02% CETAB, pH 4.7) and eluted using a linear NaCl gradient (0.2 M-1.0 M, 250 ml, 6 ml/hr) (16). Note that the scales for MPO activity (•—•) differ by a factor of 20 between 2A and 2B. NaCl gradient (o—o) for profiles in A and B.

to ion-exchange chromatography in order to determine the distribution of the different forms of MPO (16). The results of a representative experiment are presented in Figure 2. It can be seen that the low density granules contained primarily MPO forms II and III, with only a trace amount of form I (Fig. 2A) On the other hand, MPO form I, together with forms II and III, was extracted from the higher density granules (Fig 2B). At the present time, it is not clear whether forms II and III are true components of the higher density granules. Given that there is a disproportionate amount of MPO activity associated with the low density subpopulation (note the 20-fold difference in enzyme activity scales between Figs. 2A and 2B), the presence of forms II and III may be a reflection of overlap between the two AG subpopulations.

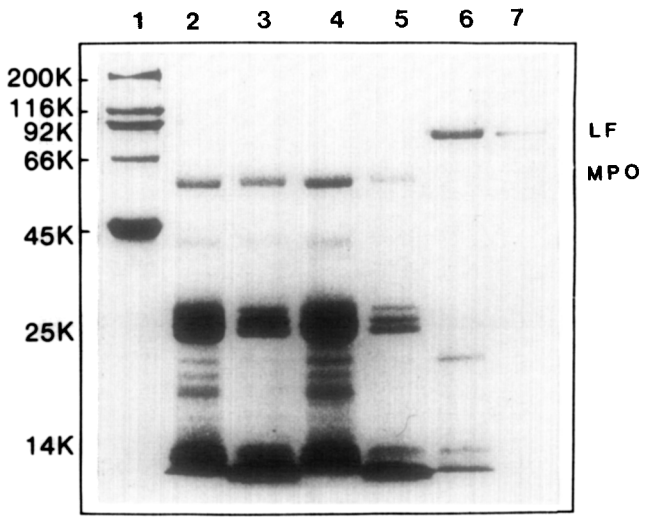


Fig. 3. Gradient (5-20%) PAGE of proteins contained in low and high density AG fractions of human PMN. Electrophoresis employed slab gels (1.5 x 130 x 130 mm), 0.1% SDS, 70 mM 2-mercaptoethanol and were stained using Coomassie Brilliant Blue R-250. Lane 1, molecular weight markers (myosin, 200,000; β-galactosidase, 116,000; phosphorylase b, 92,500; bovine serum albumin, 66,200; ovalbumin, 45,000) Lanes 2,4 and lanes 3,5 are AG-L and AG-H, respectively, and correspond to the following fractions/μg protein in Fig. 1: 37/132, 35/110 and 43/200, 44/128. Lane 6, lysate of specific granules (SG, Fig. 1, fraction 24, 110μg); Lane 7, purified human milk lactoferrin (LF, 10μg).

Additional support for differences in the granule distribution of the different forms of MPO was obtained by gradient PAGE of material solubilized from both the high and low density AG subpopulations. The results of a typical experiment are shown in Fig. 3. In general, the heavy subunits of MPO from lower density AG appeared to have a mobility somewhat greater than the heavy MPO subunits extracted from higher density AG (compare lanes 2,4 to lanes 3,5). Nevertheless, it was difficult to unequivocally relate individual bands on the gel to different chromatographic forms of MPO because of relatively small differences in mobilities and limited amounts of material which could be obtained from single donors. While the PAGE data were consistent with the chromatographic data presented in Fig 2, it was not clear whether form I was restricted exclusively to the higher density AG subpopulation. It is interesting to note that PAGE analysis confirmed the absence of lactoferrin in the MPO-positive AG but demonstrated its presence in the SG population (Fig. 3, lane 6).

## DISCUSSION

The conventional view regarding the physiological role of MPO has centered on its participation in the well established intracellular microbicidal system involving halogenation and oxidation reactions during phagocytosis (20). There is also evidence to suggest a possible involvement of MPO in the respiratory burst or subsequent interaction with the products of the burst (8). On the other hand, it has been known for some time that the MPO-$H_2O_2$-halide system may operate at extracellular as well as intracellular sites (20), and there is a growing body of evidence which strongly supports a number of important extracellular roles for this enzyme: inactivation of chemotactic factors (21-23) and human α-1-proteinase inhibitor (24), transformation of prostaglandins (25) and PMN-mediated lysis of both normal (26) and neoplastic (27) mammalian cells.

The cellular strategy of differentially packaging distinct forms of MPO into different granule subpopulations within the same cell suggests a functional correlate. One possible interpretation is that heterogeneity of AG reflects differences in the principal sites of action of the structurally and enzymatically different forms of MPO. Thus, selective exocytosis of MPO forms II/III from low density AG (17) might reflect extracellular modulation of both chemotactic and inflammatory responses. In this regard, it is interesting to note that the low density AG subpopulation does not appear to contain the bacteriolytic enzyme lysozyme (9). On the other hand, the high density AG subpopulation with its content of MPO form I and lysozyme might function primarily in an intracellular role in microbicidal killing via the halide peroxidation mechanism (20), and perhaps in the respiratory burst itself (8). A recent report describes several patients with total MPO deficiency as judged by histochemical staining procedures (28). Surprisingly, cells from these individuals were able to carry out the iodination of protein, a process thought to be mediated by MPO (28). The independent packaging and secretion of structurally and enzymatically different forms of MPO may provide a possible explanation for this phenomenon.

A second possible interpretation of our observations is that the two AG subpopulations might reflect different stages of granule maturation. In this case the larger molecular weight form I of MPO might represent a precursor which undergoes processing (carbohydrate and/or primary structure) to the smaller forms II and III. In this regard, it is of interest that MPO form I, in contrast to forms II and III, required the use of CETAB to extract it from the higher density AG subpopulation (16).

The present work provides the first information regarding the relationship of MPO structure to its packaging into granules. Recent studies have shown that a carbohydrate moiety, mannose-6-phosphate, acts as a recognition marker for the transport of acid hydrolases to lysosomes (29). In addition, there is evidence that the primary structure of the protein dictates the processing of more complex carbohydrate moieties which are originally transferred to the polypeptide chains (30). The existence of distinct forms of MPO which are packaged into different granules provides a unique model system for studying this general problem as well as the particular problems of abnormal granulogenesis associated with myeloid leukemia (31). Our data suggest a greater degree of complexity than generally realized in that AG heterogeneity may be a reflection of both maturational and functional diversity. Further studies on structure-activity relationships of the different forms of MPO using specific antibodies should provide the basis for ultrastructural and biochemical approaches to these fundamental questions (32).

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