ISOLATION OF THREE NATIVE FORMS OF MYELOPEROXIDASE FROM HUMAN POLYMORPHONUCLEAR LEUKOCYTES

Stephen O. PEMBER, Stelia M. FUHRER-KRÜSI+, Katherine C. BARNES and Joseph M. KINKADE, jr*
Department of Biochemistry, Emory University School of Medicine, Atlanta, GA 30322, USA

Received 18 December 1981

1. Introduction

Myeloperoxidase (MPO) is an unusual and complex heme-containing glycoprotein [1,2] which plays an important functional role in the oxidative metabolism of PMN from many sources [3]. The isolation and purification of this enzyme [1] has been described and fluorochrome-labeled antibodies made against highly purified human MPO have been useful in studies aimed at elucidating the role of MPO-containing azurophilic granules in phagocytosis by PMN [4]. However, there appear to be multiple forms of MPO which have different properties [5–8]. Polyacrylamide gel electrophoresis data in [5,7] support the idea that human MPO consists of a number of isoenzymes. Often, the genetic origin of the forms was not clear because PMN had been pooled from many donors.

PMN are an unusually rich source of proteolytic enzymes [9]; multiple forms of MPO may be degradative artifacts of the isolation procedure. MPO appears to be resistant to trypsin as shown by similarities in gel electrophoresis patterns before and after enzyme treatment [10]. However, according to [11] many commonly used procedures for preventing proteolysis, such as use of PMSF and divalent cation chelators, are not effective in the isolation of intact proteins from human PMN; only the use of DFP prevented proteolysis [11].

Here, we present evidence that MPO from a single human donor can be resolved into 3 distinct chromatographic species which are native to PMN, which have the same M_r-values, but which exhibit different sensitivities to inhibition by AT. This is the first description of a chromatographic procedure for the preparative and reproducible isolation of these different forms of MPO from normal, human PMN. Further studies on the biochemical and functional characterization of these enzymes should now be possible.

Abbreviations: MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte; PMSF, phenylmethylsulfonylfluoride; DFP, diisopropylfluorophosphate; AT, 3-amino-1,2,4-triazole; TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone; CETAB, cetyltrimethylammonium bromide; M_r, relative molecular mass

+ Present address: Department of Biochemistry, Princeton University, Princeton, NJ 08544, USA

* To whom reprint requests should be addressed

Portions of this work are part of a dissertation submitted by S. O. P. to the Graduate School of Emory University in partial fulfillment of the requirements for the PhD degree

2. Materials and methods

PMSF, TPCK, DFP, guaiacol and AT were obtained from Sigma (St Louis MO). Ficoll-Hypaque (lymphocyte separation medium) was obtained from Litton Bionetics (Kensington MD). CETAB was from Eastman Kodak (Rochester NY). All other chemicals were of reagent grade purity.

2.1. *Cells*

Human PMN were obtained from healthy male and female donors undergoing leukapheresis or by routine collection of peripheral blood in heparinized tubes. PMN were further purified by hypotonic lysis of residual red blood cells [12] followed by sedimentation through Ficoll-Hypaque [13]. Peripheral red blood cells were sedimented using dextran [13], and PMN were further purified as above. The human acute promyelocytic leukemia cell line (HL-60) was

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

obtained from Dr R. C. Gallo (National Cancer Institute) and was maintained in liquid suspension culture as in [14].

2.2. *Extraction procedures*

To prepare granules, PMN were suspended at 2.5 × 10^7 cells/ml in ice-cold 0.34 M sucrose containing 10 μg/ml each of PMSF and TPCK and 1 mM EDTA. In some preparations, PMN were also treated with DFP as in [11]. Homogenates were made using a motor-driven Teflon–glass homogenizer (900 rev./min, 5 min) followed by centrifugation at 500 × *g* for 5 min at 4°C. The supernatants were collected and the pellets containing unbroken cells, nuclei and membranes were rehomogenized, centrifuged and the supernatants pooled. This process was continued (usually 1–2 times) until the pellets lost most of their green color. The pooled supernatants were centrifuged at 27 000 × *g* for 15 min (4°C) to pellet granules which were used fresh or stored frozen at −20°C.

Granules were solubilized by a slight modification of the method in [15]. Granules were suspended in ice-cold 0.2 M sodium acetate buffer containing 1 M NaCl, 10 μg/ml each of PMSF, TPCK and 1 mM EDTA (pH 4.7) and homogenized as above for 2–4 min. Solubilized MPO was recovered in the supernatant after centrifugation at 27 000 × *g* for 10 min (4°C). The procedure was repeated until MPO was essentially undetectable in the supernatants (1–2 extractions). The MPO containing extract was dialyzed against 25 mM sodium acetate buffer containing 0.1 M NaCl (pH 4.7) and the precipitate which formed was removed by centrifugation at 27 000 × *g* for 15 min. MPO remaining in the pellet was removed by dispersing the pellet in 25 mM sodium acetate buffer containing 0.5 M NaCl (pH 4.7) and recentrifuged as above. MPO was also solubilized from granules prepared and extracted as in [16] (kindly supplied by Professor J. K. Spitznagel, Dept. Microbiology and Immunology). Alternatively, MPO was extracted from whole PMN by suspending the cells (2 × 10^7/ml) in an ice-cold lysing medium (0.3% CETAB in 10 mM sodium phosphate buffer (pH 7.0)) for 30–45 min [17]. Detergent lysates were then centrifuged at 27 000 × *g* for 15 min and solubilized MPO was recovered in the supernatant.

2.3. *Chromatography and immunodiffusion*

All chromatography was performed at 4°C. CETAB lysates of whole PMN were made 0.2 M NaCl and applied directly to a column of carboxymethyl-cellulose (CM-52, 1.6 × 23 cm) equilibrated in 25 mM sodium acetate buffer containing 0.2 M NaCl (pH 4.7). Alternatively, extracts were first dialyzed against the equilibration buffer prior to application to the column. Protein was followed at 280 nm using a flow-through absorbance monitor. After protein in column effluents had returned to baseline, a linear 800 ml salt gradient was used to elute the enzyme (0.2–1.0 M NaCl). A flowrate of 10 ml/h was maintained. In some experiments, granule extracts containing relatively small amounts of activity (50–200 units) were chromatographed on a smaller column (0.9 × 20 cm, 250 ml salt gradient) using a flow rate of 5 ml/h. Identical elution profiles were obtained with columns of either size.

Gel filtration chromatography using Sephadex G-150 (superfine) was done as in [2] except that the column dimensions were 16 × 90 cm and the experiment was performed at 4°C. For determination of M_r-values, the column was calibrated with the following standards (M_r): blue dextran (V_o); cytochrome *c* (12 400); lysozyme (14 400); horseradish peroxidase (44 050); conalbumin (86 180).

Double immunodiffusion was performed in 1% agarose, 10 mM sodium phosphate buffer containing 1.0 M NaCl (pH 7.0) overnight in a humid chamber. Proteins not precipitated were washed out with 0.15 M NaCl. The gels were then stained for protein (amido black) or enzyme activity (diaminobenzidine).

2.4. *Polyacrylamide gel electrophoresis*

Native slab gel electrophoresis (7.5% acrylamide) was done as in [23] without the use of a stacking gel. Gels were pre-electrophoresed for 45 min and were stained for protein (12 h) using Coomassie brilliant blue R-250 (1.8 g/l) in 25% methanol, 10% acetic acid. Destaining was carried out in 25% ethanol, 10% acetic acid. Peroxidase activity was visualized by incubating the gel (25°C) in a solution of diaminobenzidine (0.4 mg/ml) in 10 mM sodium phosphate buffer (pH 7.0) containing 1 mM H_2O_2. R_F-Values were calculated as the ratio of the distance the protein migrated to that of the dye front (methyl green).

2.5. *Assay of MPO*

MPO was assayed by measuring the formation of tetraguaiacol at 470 nm as in [8]. One unit was defined as that amount of enzyme catalyzing a change of 1.0 absorbance unit/min.

3. Results

3.1. *Isolation and fractionation of MPO*

When a CETAB extract of whole PMN was subjected to ion-exchange chromatography on carboxymethyl-cellulose, the typical elution profile in fig.1A was obtained. Three peaks of MPO activity, designated I, II and III, were routinely resolved, eluting at ~0.36 M, 0.42 M and 0.48 M NaCl, respectively.

To investigate whether these 3 peaks of activity represented different native forms of MPO or were perhaps artifacts resulting from proteolytic degradation during isolation, the enzyme was isolated using several different procedures. These included:

(i) Direct extraction of whole PMN at pH 7.0 in the presence of the cationic detergent CETAB as in [17];
(ii) Extraction of PMN granules at pH 7.0 in the presence of CETAB;
(iii) Extraction of PMN granules with 0.2 M sodium acetate buffer (pH 4.0) as in [16];
(iv) Extraction of PMN granules with 0.2 M sodium acetate buffer containing 1 M NaCl (pH 4.7) a slight modification of the procedure in [15].

In all cases, MPO peaks II and III were observed following ion-exchange chromatography such as described in fig.1, while the amount of peak I was variable, depending on the extraction procedure employed (see section 3.2).

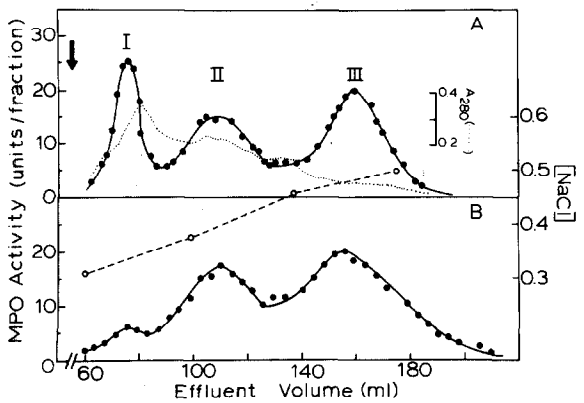

Fig.1. Ion-exchange chromatography of normal human MPO from CETAB extracted PMN (A) and salt extracted PMN granules (B). Extracts were prepared as in section 2. The carboxymethyl-cellulose (CM-52, 1.6 × 23 cm) was equilibrated with 0.025 M sodium acetate buffer containing 0.2 M NaCl (pH 4.7). After applying the sample (800–1000 units MPO), the column was washed with equilibration buffer until the A_{280} returned to baseline at which time the linear salt gradient was started (0.2–1.0 M, 800 ml total vol.). The flow rate was 10 ml/h and 2 ml fractions were collected. (•——•) MPO activity; (. . .) A_{280}; (o– – –o) NaCl gradient. Arrow indicates start of gradient.

Use of whole cell extracts often resulted in poorer resolution of the 3 peaks of MPO activity as compared to granule preparations. This was especially true when equivalent amounts of protein were loaded onto the ion-exchange column. Most likely, this was due to association of the highly cationic MPO [18] with other anionic species present in whole cell lysates. For example, it was necessary to include 1 M NaCl in agar double immunodiffusion gels of MPO to counter such non-specific interactions [12].

All 3 peaks of MPO activity eluted at their characteristic salt concentrations when subjected to rechromatography under identical conditions on the same ion-exchange column. In addition, rechromatography of the poorly resolved peaks resulting from fractionation of certain whole cell lysates yielded peaks I–III. Furthermore, these same 3 peaks were observed whether or not a mixed or homogenous buffer system was employed, i.e., loading of the sample in sodium phosphate buffer (pH 7.0) onto the column equilibrated with sodium acetate buffer (pH 4.7) gave the same elution profile as when the sample was first dialyzed overnight against the pH 4.7 equilibration buffer. These results indicated that the 3 peaks of MPO activity were not due to an artifact of the chromatographic procedure.

3.2. *Variation in the relative proportion of activities*

Although different isolation procedures consistently revealed the presence of 3 MPO activities, the relative proportions varied, especially the amount of peak I. The chromatographic profile in fig.1B was obtained when a sample of salt extracted PMN granules, prepared as in [16] (kindly supplied by Professor Spitznagel) was chromatographed using our standard conditions. It can be seen that all 3 peaks of MPO activity were present, but that peak I was considerably reduced in amount. Further studies have shown that complete extraction of peak I required the use of CETAB, while peaks II and III were completely extracted by salt solutions in the absence of a detergent. Moreover, when PMN are pretreated with cytochalasin B and subsequently challenged with a chemotactic factor, there is a preferential degranulation of only MPO peaks II and III. These results, including the purification and properties of peaks I–III, will be fully described elsewhere [19].

3.3. *Use of protease inhibitors*

Many of the proteolytic enzymes associated with PMN such as elastase-like enzymes, collagenase and cathepsin-G have optimum activities around neutral pH [9]. Use of acid extraction procedures is designed to minimize degradation by these neutral proteases. However, the fact that MPO peaks I–III were observed whether isolation utilized whole cells or granules extracted at pH 4.0 or pH 7.0 indicated that these different activities did not result from random proteolytic degradation. To further test this idea, MPO was isolated in the combined presence of 3 protease inhibitors which have been utilized with PMN: EDTA (1 mM) and PMSF and TPCK (10 μg/ml each) [11]. The same results as in fig.1 were obtained whether or not these inhibitors were present during the isolation procedure. In addition, preincubation of the cells with 5 mM DFP [11] prior to extraction or preparation of the granules did not reduce the complexity of the chromatographic profile of MPO activities.

3.4. M_r-*Values and native gel electrophoresis*

The app. M_r of each of the 3 different peaks of MPO activity was determined from its respective elution volume using a calibrated Sephadex G-150 column. No significant differences were observed between any of the 3 MPO activities, and the calculated M_r of 89 000 agreed well with the M_r of 88 000 for highly purified human MPO [2]. When non-denaturing polyacrylamide gel electrophoresis was performed on each of the different MPO activities, no significant differences were observed in their mobilities. There was an exact correspondence between the single band visualized by the activity stain in each of the MPO samples. Furthermore, the mobility of these different MPO species (R_F 0.25, 7.5% acrylamide) was comparable to that reported for MPO purified from human PMN ([2], R_F 0.24, 6% acrylamide).

3.5. *Differential sensitivity to AT*

MPO derived from elicited murine peritoneal exudate cells could be resolved into 2 chromatographic forms exhibiting different sensitivity to inhibition by AT [8]. Thus, it was of interest to examine the sensitivity of the 3 forms of human MPO activity to this inhibitor. The data in table 1 show that the activity associated with peak I was most sensitive to inhibition while the activity associated with peak III was the least sensitive. Moreover, the differences in inhibition were most pronounced at lower concentrations of AT ($\leq$ 10 mM).

Table 1
Inhibition (%) of peroxidase activity by 3-amino-1,2,4-triazole (AT)

Chromatographic form[a]	[AT] (mM)[b] 1	2	5	10	20	40
I	23	33	47	55	63	81
II	3	10	41	50	63	80
III	2	8	24	34	57	79

[a] Equivalent amounts of peroxidase activity were compared for each chromatographic form (0.31–0.38 guaiacol units) (see fig.1)

[b] AT was mixed directly with the reaction mixture prior to the start of the reaction

Values represent the mean of duplicate determinations and are expressed as % of the untreated control; range of the values was ≤5% of the mean

3.6. *Reaction with antibody*

The immunological cross-reactivity of the different forms of MPO from normal human PMN was tested using a rabbit antibody (generously provided by Professor Spitznagel) prepared against highly purified human MPO [16]. Fig.2 shows that all 3 peaks

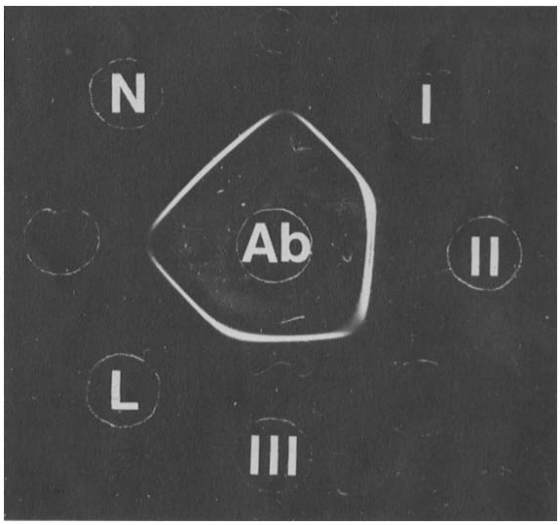

Fig.2. A double immunodiffusion gel of the different chromatographic forms of normal human MPO and MPO from human leukemic HL-60 cells (details in section 2); Ab, rabbit anti-human MPO; N, normal PMN lysate; L, HL-60 cell lysate; I, II, III refer to the MPO peaks described in fig.1.

reacted with one another to give a single line of identity in Ouchterlony double diffusion gels. In addition, unfractionated MPO in normal whole PMN lysates and from a line of human acute promyelocytic leukemia cells (HL-60), as well as the purified MPO antigen (not shown), all reacted with the antibody to give a single line of identity.

4. Discussion

These data support the conclusion that normal human PMN contain 3 distinct chromatographic forms of MPO. The use of different isolation procedures, including whole cells, granules, extractions at different pH values, with and without detergents and/or protease inhibitors, all gave the same 3 peaks of MPO activity. The fact that MPO peak I apparently required the use of detergent to effect its extraction, while peaks II and III were extracted without the use of detergent suggests that there may be differences in the compartmentalization of the different forms of this enzyme within the PMN, perhaps in different granules [19]. These 3 different forms of MPO appear to be native to the PMN rather than artifacts of the isolation procedure.

When homogenous preparations of MPO, prepared as in [15,16], were subjected to chromatography as described here, multiple peaks were observed. In addition, there were no significant differences in the M_r-values, of the 3 MPO activities (89 000); this being essentially identical to the anomalously low M_r of 88 000 reported for purified human MPO [2] when measured using the same gel filtration conditions. Moreover, the 3 different MPO activities had essentially the same mobility when subjected to native polyacrylamide gel electrophoresis; migration was similar to that for purified human MPO run under similar conditions [2]. That these 3 forms were also observed in cells from a single donor indicated that such heterogeneity was not the result of genetic diversity arising from multiple donors.

HL-60 cells, a line of human acute promyelocytic leukemia cells, contain multiple forms of MPO [20, 21]. One of the forms was smaller in M_r, and there were differences in the relative proportions of the large and small forms in more mature, differentiated cells as compared to the relatively undifferentiated, immature cells [20]. Normal, mature peripheral blood PMN contained only the large form of MPO. MPO binds to concanavalin A–Sepharose [21,22] and differential elution of one of the large forms of MPO suggested that the heterogeneity was partly due to differences in sugar moieties [21]. Although the small and large forms of MPO from HL-60 cells could not be distinguished by Ouchterlony double immunodiffusion, a difference was observed using a microcomplement fixation test [21]. Our data are consistent with these observations, and indicated that the multiple forms of MPO from normal human peripheral blood PMN were immunologically cross-reactive with the MPO isolated from the leukemic HL-60 cells.

These results demonstrate that, as with different forms of MPO in the mouse [8], there were differences in the sensitivity of the human MPOs to inhibition by AT. A similar difference has been reported between 2 electrophoretically different forms of human MPO [7], and differences in the ability to oxidize halide ions was observed between chromatographically distinct murine forms of MPO [8]. This suggests that there may be differences in the catalytic centers of these forms. MPO contains 2 heavy-light protomers joined along their long axes by a single disulfide bond between the heavy subunits [2,18]. There are distinct differences in the patterns of purified MPO from peaks I–III when run on SDS–polyacrylamide gel electrophoresis under reducing conditions [19]. Moreover, there are significant differences in the amino acid composition between the different forms of MPO [19]; e.g., there is a progressive increase in the amount of arginine (I < II < III) while the ratio of aspartic acid to glutamic acid differs in a characteristic manner (III > I > II) [19]. Further work on the chemical and enzymatic characterization of each of the different forms of MPO and their respective subunits should provide additional insight into the structure–function relationships of this complex enzyme system.

Acknowledgements

We thank Drs E. F. Winton and W. R. Vogler for providing PMN from donors undergoing leukapheresis, Dr R. C. Gallo for providing the HL-60 cell line, and Drs D. Jones and R. Shapira for helpful discussions. S. M. F. was supported by a postdoctoral fellowship from the Swiss National Foundation. This work was supported in part by USPHS grants CA 22294 (National Cancer Institute, NIH) and RR 5364 (Biomedical Research Support).

References

[1] Schultz, J. (1980) in: The Reticuloendothelial System (Sbarra, A. J. and Strauss, R. R. eds) vol. 2, pp. 231–254, Plenum, New York.

[2] Andrews, P. C. and Krinsky, N. I. (1981) J. Biol. Chem. 256, 4211–4218.

[3] Klebanoff, S. J. (1980) in: The Reticuloendothelial System, (Sbarra, A. J. and Strauss, R. R. eds) vol. 2, pp. 279–308, Plenum, New York.

[4] Pryzwansky, K. B., Steiner, A. L., Spitznagel, J. K. and Kapoor, C. L. (1981) Science 211, 407–410.

[5] Felberg, N. T. and Schultz, J. (1972) Arch. Biochem. Biophys. 148, 407–413.

[6] Bakkenist, A. R. J., Wever, R., Vulsma, T., Plat, H. and Van Gelder, B. F. (1978) Biochim. Biophys. Acta 524, 45–54.

[7] Strauven, T. A., Armstrong, D., James, G. T. and Austin, J. H. (1978) Age 1, 111–117.

[8] Pember, S. O., Kellar, K. L., Winton, E. F. and Kinkade, J. M. jr (1981) J. Reticuloendothel. Soc. 29, 451–458.

[9] Spitznagel, J. K., Dalldorf, F. G., Leffell, M. S., Folds, J. D., Welsh, I. R. H., Cooney, M. H. and Martin, L. E. (1974) Lab. Invest. 30, 774–785.

[10] Felberg, N. T., Putterman, G. J. and Schultz, J. (1969) Biochem. Biophys. Res. Commun. 37, 213–218.

[11] Amrein, P. C. and Stossel, T. P. (1980) Blood 56, 442–447.

[12] Himmelhoch, S. R., Evans, W. H., Mage, M. G. and Peterson, E. A. (1969) Biochemistry 8, 914–920.

[13] Boyum, A. (1974) Tiss. Antig. 4, 269–274.

[14] Collins, S. J., Ruscetti, F. W., Gallagher, R. E. and Gallo, R. C. (1978) Proc. Natl. Acad. Sci. USA 75, 2458–2462.

[15] Matheson, N. R., Wong, P. S. and Travis, J. (1981) Biochemistry 20, 325–330.

[16] Olsson, I., Olofsson, T. and Odeberg, H. (1972) Scand. J. Haematol. 9, 483–491.

[17] Desser, R. K., Himmelhoch, R. S., Evans, W. M., Januska, M., Mage, M. and Shelton, E. (1972) Arch. Biochem. Biophys. 148, 452–465.

[18] Harrison, J. E., Pabalan, S. and Schultz, J. (1977) Biochim. Biophys. Acta 493, 247–259.

[19] Pember, S. O., Shapira, R. and Kinkade, J. M. jr (1982) in preparation.

[20] Yamada, M., Mori, M. and Sugimura, T. (1981) Biochem. Biophys. Res. Commun. 98, 219–226.

[21] Yamada, M., Mori, M. and Sugimura, T. (1981) Biochemistry 20, 766–771.

[22] Merrill, D. P. (1980) Prep. Biochem. 10, 133–150.

[23] Williams, D. E. and Reisfeld, R. A. (1964) Ann. NY Acad. Sci. 121, 373–381.