Peroxidase and peroxidase–oxidase activities of isolated human myeloperoxidases

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Isolated neutrophils from healthy donors were used for the isolation of four highly purified forms of myeloperoxidase as determined by spectral (A_{430}/A_{280} ratio 0.80–0.87) and enzyme-activity measurements. Although the myeloperoxidases exhibited different elution profiles on cation-exchange chromatography, gel filtration indicated similar relative molecular masses. When these forms were assayed for peroxidase and peroxidase–oxidase activities with several substrates, they all exhibited virtually the same specific activities. These results suggest that possible functional differences between the enzymes may be related to differences in their sites of action rather than to differences in enzyme activity. Myeloperoxidase from a patient with chronic myeloid leukaemia also revealed a similar heterogeneity on cation-exchange chromatography. However, this myeloperoxidase contained in addition one form with a lower and one form with a higher relative molecular mass, as indicated by gel-filtration chromatography.

INTRODUCTION

Myeloperoxidase (MPO, EC 1.11.1.7) is a main constituent of azurophilic granules in neutrophils. Its function(s) is (are) still uncertain, but when combined with a halide and H_2O_2 it forms a highly toxic system, which can kill micro-organisms, injure host cells and inactivate humoral factors (reviewed by Klebanoff & Clark, 1978; Clark, 1983).

MPO was first isolated by Agner (1941), and subsequently the enzyme was purified in crystalline form (Agner, 1958; Harrison *et al.*, 1977). Felberg & Schultz (1972) demonstrated, by polyacrylamide-gel electrophoresis, the heterogeneity of MPO isolated from leucocytes from pooled blood of several donors. Later, Strauven *et al.* (1978) obtained four isoenzymes from single donors, and the relative amounts seemed to vary with the age of the donors. More recently, three forms were isolated from HL-60 cells (Yamada *et al.*, 1981a) and from healthy donors (Pember *et al.*, 1982) by cation-exchange chromatography. One of the forms required cetyltrimethylammonium bromide (Cetab) for extraction, and a variable distribution was seen in the high-density and low-density azurophilic granules as well as differences in degranulation (Kinkade *et al.*, 1983; Pember & Kinkade, 1983; Olsen & Little, 1984). Differences were also found in enzyme activity and inhibition-sensitivity towards 3-amino-1,2,4-triazole (Pember *et al.*, 1983). However, since the absorption ratios (A_{430}/A_{280}) and specific enzyme activities indicated enzyme impurity, the interpretation of these differences was in doubt.

We have now isolated four virtually pure forms of MPO in good yield from healthy donors and compared their peroxidase and peroxidase–oxidase (i.e. O_2 -consuming oxidation of compounds catalysed by peroxidase without H_2O_2 addition; Yamazaki & Yokota, 1973) activities with a few peroxidase and peroxidase–oxidase substrates. We have also studied and compared with these the heterogeneity of chronic-myeloid-leukaemia MPO isolated by Olsson *et al.* (1972).

EXPERIMENTAL

Materials

Fresh buffy coats and plasma from blood of healthy donors with CPD/adenine (Travenol Laboratories, Castlebar, Ireland) as anticoagulant were kindly provided by Dr. G. Liedén (Linköping, Sweden). Pooled plasma was further freed of platelets by centrifugation and diluted with 0.9% NaCl as described in the text. MPO from a patient with chronic myeloid leukaemia was kindly donated by Professor I. Olsson (Olsson *et al.*, 1972). Zymosan A, superoxide dismutase (3250 units/mg as assayed by the manufacturer) and bovine albumin were bought from Sigma Chemical Co. (St. Louis, MO, U.S.A.), $Na^{125}I$ was from The Radiochemical Centre (Amersham, Bucks., U.K.), molecular-mass markers were from Pharmacia Fine Chemicals (Uppsala, Sweden), 3-amino-1,2,4-triazole was from Fluka (Buchs, Switzerland) and $Na_2S_2O_4$ was from Merck (Darmstadt, Germany). Sources of other chemicals and biochemicals are indicated in the text below.

Isolation of MPO forms

Isolation of neutrophils. Neutrophils were isolated essentially as described by Bøyum (1976) at room temperature except where otherwise indicated. Pooled buffy coats from 18 donors (650–700 ml) were treated with Dextran T500 (Pharmacia Fine Chemicals) to give a final concentration of 0.75%, and after sedimentation the upper leucocyte-rich layer was aspirated off and centrifuged. The cell pellet was washed with plasma (diluted 1:3 with 0.9% NaCl) and the cells were suspended in plasma (diluted 1:2). The neutrophils were further purified by centrifugation on cushions containing 10% (w/v) sodium metrizoate (Nyegaard and Co., Oslo, Norway)/6% (w/v) Ficoll 400 (Pharmacia Fine Chemicals). The pellets were washed with plasma (diluted 1:7) and then suspended in plasma, and the suspension was counted for cell content and stored at 4 °C overnight.

Residual erythrocytes were lysed by treatment with

3 vol. of cold water for 30 s, and iso-osmoticity was restored by addition of 0.3 vol. of 9.9% (w/v) NaCl. After centrifugation, the neutrophils were washed with plasma (diluted 1:7) and suspended in plasma.

All subsequent procedures were performed at 4 °C.

Isolation of granules. The neutrophil suspension was centrifuged at 150 *g* for 10 min and washed twice with 20 ml of 137 mM-NaCl/2.7 mM-KCl/5 mM-EDTA/8.1 mM-Na₂HPO₄/1.5 mM-KH₂PO₄ buffer, pH 7.3, and twice with 20 ml of 0.34 M-sucrose. After suspension in 80 ml of the latter solution, homogenization (30 strokes) was carried out in a chilled Dounce homogenizer with a tight pestle (Kontes Glass Co., Vineland, NJ, U.S.A.). The homogenate was diluted with 100 ml of 0.34 M-sucrose ('cell homogenate' in Table 1) and centrifuged at 200 *g* for 20 min to sediment unbroken nuclei and cells. The supernatant was further centrifuged in a Beckman SW27 rotor at 14 000 rev./min (25 000 *g*) for 20 min (Rest & Spitznagel, 1977). The pellets (granules) were collected and frozen at -20 °C until processed further.

Cetab extraction and (NH₄)₂SO₄ precipitation. Granules were homogenized in 80 ml of 0.1 M-potassium phosphate buffer, pH 7.5 (0.4 M-KH₂PO₄/0.6 M-K₂HPO₄ adjusted to pH 7.5 with KOH and diluted to 0.1 M). Then 0.5 vol. of 0.1 M-potassium phosphate buffer, pH 7.5, containing 1% Cetab (Sigma Chemical Co.) was added and the mixture was stirred for 1 h. The supernatant obtained after centrifugation at 13 000 *g* for 20 min was collected ('Cetab extract'), adjusted to 40% saturation with solid (NH₄)₂SO₄, stirred for 1 h and centrifuged as above. The supernatant was treated with solid (NH₄)₂SO₄ to give 55% saturation and the supernatant collected as above. MPO was precipitated at 70% saturation with (NH₄)₂SO₄, and the precipitate was collected by centrifugation at 17 000 *g* for 20 min, suspended in 10 ml of 0.1 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab and stored frozen at -20 °C ('ammonium sulphate precipitate').

Cation-exchange and gel-filtration chromatography. The enzyme was further purified by column chromatography on CM-Sephacryl CL-6B and Sephacryl S-200 (Pharmacia Fine Chemicals) at 4 °C essentially as described by Yamada *et al.* (1981a). The cation-exchange chromatography column (2.5 cm × 23.1 cm) was equilibrated with 0.1 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab and loaded with enzyme in 50 ml of the same buffer. After the column had been washed with 135 ml of the same buffer, the peroxidases were eluted (18.7 ml/h) with linear gradients of 0.1–0.28 M- (630 ml) and 0.28–1 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab (270 ml). Pooled fractions were adjusted to 70% saturation with solid (NH₄)₂SO₄, stirred for 1 h and centrifuged at 17 000 *g* for 20 min. Pellets were dissolved in 0.2 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab.

A Sephacryl S-200 column (2.6 cm × 89.2 cm) was equilibrated with 0.2 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab. Elution was carried out in the same buffer, and pooled fractions were precipitated as above, dissolved in distilled water and stored at -20 °C.

MPO from a patient with chronic myeloid leukaemia was chromatographed as described above.

Large-scale isolation of MPO. Buffy coats from 257 healthy donors were processed essentially as described above. Cation-exchange and gel-filtration chromatography were performed on 2.6 cm × 78.9 cm and 2.6 cm × 186.5 cm columns respectively. The MPO forms were dialysed against distilled water.

Quantitative determinations of total protein and MPO. These were carried out by the method of Lowry *et al.* (1951), with albumin as standard ('Cetab extract' and 'ammonium sulphate precipitate'), or by utilizing the following formulae (Andersen *et al.*, 1982):

$$\begin{aligned}\text{Concn. of total protein (mg/ml)} &= A_{280} - 0.365 \times A_{430} \\ \text{Concn. of MPO (mg/ml)} &= 0.811 \times A_{430}\end{aligned}$$

In the latter formula a constant ratio of haem to protein is assumed.

Enzyme assays

All light-absorption measurements were carried out with a Beckman DU-7HS spectrophotometer equipped with a kinetics accessory.

Assay with 4-aminoantipyrine. MPO activity was measured spectrophotometrically at 25 °C with 4-aminoantipyrine (Sigma Chemical Co.) as hydrogen donor, essentially as described in the Worthington Enzyme Manual (1977) (Worthington Biochemical Corp., Freehold, NJ, U.S.A.). Final concentrations were 1 mM-4-aminoantipyrine, 0.85 mM-H₂O₂ (Perhydrol; Merck), 0.01% Cetab, 100 mM-potassium phosphate buffer, pH 7.0, 68 mM-phenol and about 0.1 µg of MPO/ml. The initial increase in absorbance at 510 nm was measured, and enzyme activity, expressed as mol of H₂O₂ decomposed/s (= 1 kat), was calculated (Worthington Enzyme Manual). H₂O₂ concentration was determined by using the molar absorption coefficient $\epsilon_{240} = 40.0 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Nelson & Kiesow, 1972). No activity was observed when MPO or H₂O₂ was omitted.

Assay with guaiacol. The initial rate of tetraguaiacol formation was recorded at 470 nm at 25 °C. The final concentrations were 20 mM-guaiacol (Sigma Chemical Co.), 300 µM-H₂O₂, 0.01% Cetab, 100 mM-potassium phosphate buffer, pH 7.0, and MPO. Enzyme activity was expressed as mol of H₂O₂ utilized/s (= 1 kat) by using the molar absorption coefficient $26.6 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ (Chance & Maehly, 1955). No activity was observed when MPO or H₂O₂ was omitted.

Assay of taurine chlorination. The method used was a slight modification of that described by Zgliczyński *et al.* (1971) and Stelmaszyńska & Zgliczyński (1974). The final concentrations were 10 mM-aurine (Fluka), 200 mM-NaCl, 0.1–1.5 mM-H₂O₂, 0.015% Cetab, 50 mM-sodium citrate/phosphate buffer, pH 5.25, and MPO. The reaction mixture was equilibrated at 37 °C and the reaction was started by addition of enzyme. The difference in absorbance at 250 nm (taurine monochloramine formation) after 1 and 10 min was used to calculate enzyme activity. The presence of both MPO and H₂O₂ was necessary for chlorination.

Assay of zymosan iodination. The procedure described by Klebanoff & Clark (1977) was used except that MPO and 11–33 µM-H₂O₂ were substituted for granulocytes

and serum. The reaction was started by addition of H_2O_2 , and the incubation time was 10 min. No iodination was observed when MPO or H_2O_2 was omitted.

Assay of cysteamine oxidation. MPO-dependent cysteamine oxidation (without H_2O_2 addition) to cystamine can be followed by scanning the u.v.-absorption spectrum at 37 °C (B. E. Svensson & S. Lindvall, unpublished work). A 10 μl portion of daily prepared and chilled 40 mM-cysteamine hydrochloride (Fluka) solution was added to the reaction mixture (1.0 ml) containing 200 μM -diethylenetriaminepenta-acetic acid (Sigma Chemical Co.), 50 mM-potassium phosphate buffer, pH 7.0, and MPO. The difference in molar absorption coefficient between cysteamine in its oxidized form (cystamine dihydrochloride; Fluka) and reduced form in the reaction mixture was determined to be $91 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 270 nm, which was used for the calculation of the initial rate of cysteamine oxidation. Oxidation of cysteamine did not occur on omission of MPO.

Assay of Nitro Blue Tetrazolium reduction. Cysteine and 2,4-dichlorophenol can reduce Nitro Blue Tetrazolium in an MPO-dependent reaction (B. E. Svensson & S. Lindvall, unpublished work). This reduction is inhibited by native superoxide dismutase, which indicates that the reduction measures superoxide radical anion formation. Reaction mixtures, at 37 °C, contained 25 μM -Nitro Blue Tetrazolium (Sigma Chemical Co.), 200 μM -diethylenetriaminepenta-acetic acid, 20 μM -2,4-dichlorophenol (Aldrich, Beerse, Belgium), 50 mM-potassium phosphate buffer, pH 7.0, 0.5 μg of superoxide dismutase/ml (where indicated) and MPO in 1.0 ml. Upon addition of 10 μl of daily prepared and chilled 40 mM-L-cysteine (Fluka) a continuous increase in absorbance at 560 nm is observed. The absorption increase after 4 min was used. At the MPO concentration used in this study (0.51 $\mu\text{g}/\text{ml}$) this absorption increase is

approximately proportional to the enzyme concentration used. Omission of MPO, cysteine or 2,4-dichlorophenol abolished Nitro Blue Tetrazolium reduction. Inactivation of MPO was carried out by heating at 95 °C for 40 min, and superoxide dismutase was inactivated by heating at 120 °C for 20 min in an autoclave in sealed ampoules.

RESULTS

Isolation of MPO

A typical MPO purification is shown in Table 1. Neutrophils were isolated from fresh buffy coats within 12 h, since prolonged storage of the buffy coats before isolation greatly diminished the yield and purity of neutrophils. The cell suspensions used contained 96–97% neutrophils, 2–3% eosinophils, 1% lymphocytes and less than 1% erythrocytes. Granule isolation resulted in a loss of about 35% of the peroxidase activity as measured by the 4-aminoantipyrine assay, but all peroxidase was recovered from the granules with one Cetab extraction as measured by enzyme activity. Differential $(\text{NH}_4)_2\text{SO}_4$ precipitation between 55% and 70% saturation resulted in a 4-fold purification and about 90% yield. Some peroxidase activity was precipitated by 55%-saturated $(\text{NH}_4)_2\text{SO}_4$. However, ion-exchange chromatography and light-absorption spectra indicated a larger proportion of eosinophil peroxidase in this fraction.

Cation-exchange chromatography of the ammonium sulphate precipitate gave five peaks with absorption at 430 nm (Fig. 1). The light-absorption spectra of the last peak revealed a major absorbance maximum at 413 nm and smaller ones at 500, 541, 582 and 638 nm. Upon reduction with $\text{Na}_2\text{S}_2\text{O}_4$ absorption maxima at 449, 565 and 596 nm were obtained (Fig. 4b). This is consistent with the eosinophil peroxidase pattern (Wever *et al.*, 1981; Jörg *et al.*, 1982; Olsen & Little, 1983; Bolscher *et al.*, 1984). In spite of the differences in elution from the cation-exchanger, the elution profiles of the four MPO

Table 1. Purification of MPO forms I–IV from 7.6×10^9 neutrophils

Neutrophils isolated from pooled buffy coats of 18 healthy donors were used for the isolation (described in the Experimental section). Protein and 4-aminoantipyrine enzyme activity values shown are means of duplicate or triplicate measurements (the ranges of values were < 5% of the means).

	Total protein (mg)	Enzyme activity (μkat)	Specific activity ($\mu\text{kat}/\text{mg}$ of total protein)	Yield of enzyme (%)	A_{430}/A_{280} ratio
Cell homogenate	—	21.9	—	100	—
Cetab extract	193	15.5	0.08	71	—
Ammonium sulphate precipitate	41.2	13.4	0.33	61	0.18
CM-Sepharose CL-6B chromatography					
I	0.82	0.50	0.61	2	0.59
II	5.42	3.15	0.58	15	0.41
III	2.16	0.87	0.40	4	0.39
IV	4.22	4.31	1.02	20	0.58
Sephacryl S-200 chromatography					
I	0.26	0.30	1.13	1	0.74
II	1.25	1.86	1.49	8	0.82
III	0.37	0.41	1.12	2	0.74
IV	2.13	3.76	1.77	17	0.85

Fig. 1. Separation of four MPO forms and eosinophil peroxidase by cation-exchange chromatography

Partly purified enzyme (ammonium sulphate precipitate) from buffy coats of 257 donors in 150 ml of 0.1 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab was applied on a CM-Sepharose CL-6B column (2.6 cm $\times$ 78.9 cm), and enzymes were eluted (12.6 ml/h) with gradients of 0.1–0.28 M- and 0.28–1 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab (—). ———, A_{280} ; —, A_{430} . MPO forms I–IV and eosinophil peroxidase (EPO) are indicated.

forms were identical when further purified by gel filtration (Figs. 2a–2d). The overall recovery of MPO from the cell homogenate was about 30% as measured by enzyme activity, which corresponds to about 0.5 μ g of active MPO/ 10^6 neutrophils.

Upon rechromatography on CM-Sepharose CL-6B the four MPO forms were eluted at the same relative positions (Fig. 3). Although the initial cation-exchange chromatography did not completely separate form III from form IV, the elution peaks of both forms appeared virtually symmetrical upon rechromatography. This might indicate, in turn, that each form is heterogeneous. It is also notable that no interconversion of the forms was observed.

MPO oxidation states

All forms of the enzyme had the characteristic light-absorption spectra of ferric MPO (Agner, 1963; Bakkenist *et al.*, 1978), i.e. absorption maxima at 429, 570 and 690 nm with shoulders at 370 and 620 nm (Fig. 4a). Furthermore, treatment of the ferric forms with 200 μ M- H_2O_2 resulted in absorption maxima at 455 and 628 nm, and further H_2O_2 addition gave maxima at 451 and 626 nm. Reduction with $Na_2S_2O_4$ resulted in absorption maxima at 473 and 638 nm. All four MPO forms gave the same spectral changes, and these changes are consistent with Compound II, Compound III and ferro-MPO formation (Odajima & Yamazaki, 1970).

Peroxidase and peroxidase-oxidase activities of the MPO forms

Since MPO exhibits substrate inhibition with respect to H_2O_2 (Agner, 1963; Andrews & Krinsky, 1982), the optimal H_2O_2 concentrations were determined for guaiacol oxidation, taurine chlorination and zymosan iodination (Fig. 5). In each assay system the MPO forms showed the same dependence on H_2O_2 concentration. At optimal H_2O_2 concentrations the MPO forms showed the same relative specific activity in each assay system, which

roughly correlated with their purity as measured by their A_{430}/A_{280} ratios.

Upon $(NH_4)_2SO_4$ precipitation and dialysis of the enzymes, some inactivation was observed, as measured by enzyme activity and absorption-ratio measurements. Therefore we made the quantitative comparison of the specific activities of the various enzyme forms on samples collected directly from the cation-exchange chromatography shown in Fig. 3. These enzymes had A_{430}/A_{280} ratios of 0.80–0.87. All MPO forms showed virtually the same specific activity in the peroxidase assays with 4-aminoantipyridine and guaiacol as substrates (Table 2). With the latter substrate all forms were

Fig. 2. Gel-filtration chromatography of MPO forms separated on cation-exchange chromatography

MPO forms in 5 ml of 0.2 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab were individually loaded on the same Sephacryl S-200 column (2.6 cm $\times$ 89.2 cm; V_0 131 ml) and eluted with the same buffer at 9.8 ml/h. (a)–(d) Normal MPO forms I–IV. (e) Chronic-myeloid-leukaemia MPO form I. Form II yielded a similar elution profile. (f) Chronic-myeloid-leukaemia MPO form III. Form IV yielded a similar elution profile. The elution volumes of the M_r markers chymotrypsinogen A (M_r 25000) and ribonuclease A (M_r 13700) are indicated by arrows.

Fig. 3. Rechromatography of MPO forms on a cation-exchange column

Purified normal MPO forms I-IV were chromatographed individually on the same CM-Sepharose CL-6B column (1.0 cm $\times$ 35.4 cm). Elutions (1.44 ml/h) were made with gradients of 0.1–0.3 M-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab (—), (a) A_{430} ; (b) A_{280} .

Fig. 4. Visible-light-absorption spectra of oxidation states of normal MPO forms and eosinophil peroxidase

(a) Absorption spectra of rechromatographed MPO forms I-IV (see Fig. 3) were recorded in a Beckman DU-7 HS spectrophotometer at 1200 nm/min. Each ferric MPO form in 50 mM-potassium phosphate buffer, pH 7.5, containing 0.02% Cetab was successively treated with 200 μ M- H_2O_2 (Compound II), 2.2 mM- H_2O_2 (Compound III) and a few grains of $Na_2S_2O_4$ (ferro-MPO). All four MPO forms induced identical spectral changes. (b) Eosinophil peroxidase (EPO, Fig. 1). The reduced spectrum was obtained after treatment with solid $Na_2S_2O_4$.

inhibited to the same extent by 3-amino-1,2,4-triazole, i.e. 17–18% by 2 mM and 30–33% by 5 mM of the compound (results not shown).

MPO can also function in peroxidase-oxidase reactions with certain substrates (B. E. Svensson & S. Lindvall, unpublished work). When we used cysteamine as substrate, the thiol was oxidized at the same rate by the four enzyme forms (Table 2). Also, when 2,4-dichlorophenol and cysteine were used as substrates, the enzyme forms catalysed the reduction of Nitro Blue Tetrazolium to the same extent. Neither form caused any reduction in the presence of 0.5 μ g of superoxide dismutase/ml (Table 2). With the same conditions as in Table 2, heat-inactivated MPO form IV did not catalyse

Fig. 5. Dependence on H_2O_2 concentration of enzymic activities mediated by MPO forms

The purified MPO forms (see Table 1) were investigated by (a) guaiacol oxidation (41 ng of MPO/ml), (b) taurine chlorination (811 ng of MPO/ml) and (c) zymosan iodination (811 ng of MPO/ml) with different H_2O_2 concentrations as described in the Experimental section. The same line forms as in Fig. 3 were used for the MPO forms I-IV. Means of duplicate or triplicate measurements are shown [the ranges of values were < 5% (a) and < 10% (b and c) of the means].

Table 2. Comparison of peroxidase and peroxidase-oxidase activities of MPO forms I-IV

MPO forms obtained after rechromatography (Fig. 3) were used without further treatment. 4-Aminoantipyridine (162 ng of MPO/ml), guaiacol (81 ng of MPO/ml) and cysteamine (2.0 μ g of MPO/ml) oxidation and Nitro Blue Tetrazolium reduction (0.51 μ g of MPO/ml) were carried out as described in the Experimental section. The last-mentioned assay was also carried out in the presence of 0.5 μ g of superoxide dismutase (SOD)/ml. Mean values of triplicate measurements are given, with ranges in parentheses.

MPO form	A_{430}/A_{280} ratio	Peroxidase reactions		Peroxidase-oxidase reactions		
		4-Aminoantipyridine oxidation (μ kat/mg)	Guaiacol oxidation (μ kat/mg)	Cysteamine oxidation (nkat/mg)	Nitro Blue Tetrazolium reduction ($10^4 \times \Delta A_{560}/4$ min)	
					-SOD	+SOD
I	0.87	1.9 (1.8-1.9)	8.5 (8.4-8.6)	69 (65-74)	105 (103-107)	1 (0-2)
II	0.83	1.7 (1.7-1.7)	7.9 (7.7-8.0)	66 (64-67)	98 (96-101)	-1 (-2-0)
III	0.80	1.8 (1.8-1.9)	8.4 (8.3-8.6)	73 (70-76)	111 (104-116)	0 (-1-0)
IV	0.83	1.9 (1.8-1.9)	7.8 (7.7-7.9)	63 (61-63)	101 (99-104)	-1 (-4-0)

reduction [$\Delta A_{560}/4$ min $5 (4-6) \times 10^{-4}$] and heat-inactivated superoxide dismutase did not inhibit reduction catalysed by MPO form IV [$\Delta A_{560}/4$ min $102 (100-103) \times 10^{-4}$]. These results indicate that the reduction of the dye provides a measure of MPO-dependent superoxide radical anion generation.

Chronic-myeloid-leukaemia MPO

Cation-exchange chromatography of leukaemia MPO resulted in a similar elution pattern to that shown in Fig. 1. The A_{430}/A_{280} absorption ratios were 0.81-0.84 and the specific enzyme activities with 4-aminoantipyridine and cysteamine as substrates were about the same as those obtained with the normal enzyme forms. The specific activities of the four leukaemia forms were 2.0-2.1 μ kat/mg when the peroxidase substrate 4-aminoantipyridine was used under the same conditions as in Table 2. Similarly, the specific activities were 61-64 nkat/mg when tested with the peroxidase-oxidase substrate cysteamine. Gel filtration of the forms resulted in a main peak at the same position as the normal enzyme forms. However, the leukaemia MPO form I (Fig. 2e) and to a smaller extent form II yielded an additional peak. The M_r of this peak was estimated to be 90000-95000 by semi-logarithmic plotting with ribonuclease A (M_r 13700), chymotrypsinogen A (M_r 25000) and normal MPO (M_r 144000; Yamada *et al.*, 1981a,b) as standards. MPO forms III (Fig. 2f) and IV also yielded an additional peak with an estimated M_r of 230000-240000. Upon rechromatography of these abnormal forms, they were still eluted at their abnormal positions. When these abnormal MPO forms were analysed by light-absorption spectroscopy, the typical spectral characteristics of MPO shown in Fig. 4(a) were obtained. The specific enzyme activities of the forms after rechromatography on Sephacryl S-200 (A_{430}/A_{280} ratios 0.83 and 0.81 respectively) were 1.3 and 1.7 μ kat/mg (4-aminoantipyridine oxidation) and 63 and 70 nkat/mg (cysteamine oxidation).

DISCUSSION

In order to ensure the neutrophil origin of MPO, we prepared purified neutrophil suspensions (96-97%), and, since the enzyme is localized in the azurophilic granules

(Spitznagel *et al.*, 1974), we used granules for the extraction. Direct detergent extraction of the leucocytes has been reported, but the subsequent $(\text{NH}_4)_2\text{SO}_4$ fractionation then gives a poor recovery (Bakkenist *et al.*, 1978; Andersen *et al.*, 1982).

CM-Sepharose CL-6B chromatography revealed four MPO forms. This cation-exchange chromatography procedure was adopted from Yamada *et al.* (1981a), who obtained three forms of MPO extracted from HL-60 cells. We observed that the steepness of the potassium phosphate gradient was crucial for the fourth form to appear. Therefore the present results do not necessarily indicate a difference in heterogeneity between MPO isolated from HL-60 cells and normal neutrophils. Also, we found the same heterogeneity in chronic-myeloid-leukaemia MPO. Since the isolation procedure for the latter MPO is different (Olsson *et al.*, 1972), comparison of the relative proportions of the forms is not possible. An analogous heterogeneity of normal MPO has been observed by Pember *et al.* (1982), who found three forms by using CM-cellulose and a gradient of NaCl. The differences in elution behaviour from cation-exchangers indicate charge heterogeneity of the MPO forms. Some differences in charged amino acid composition have been observed (Pember *et al.*, 1983). Also, the MPO forms have been suggested to have different carbohydrate contents, since they were eluted at different concentrations of methyl α -D-mannoside from concanavalin A-Sepharose (Yamada *et al.*, 1981a).

The four MPO forms from normal neutrophils were eluted at the same position upon gel filtration, which indicates similar M_r values. Contaminating eosinophil peroxidase was separated from MPO during the differential $(\text{NH}_4)_2\text{SO}_4$ precipitation and the CM-Sepharose CL-6B chromatography. This lower solubility and higher affinity for the cation-exchanger of eosinophil peroxidase have previously been observed by Wever *et al.* (1981). The present purification procedure for MPO gives highly purified MPO forms in high yield and is also suitable for large-scale isolation of the forms.

In contrast with normal MPO, the chronic-myeloid-leukaemia MPO yielded two small additional gel-filtration peaks with estimated M_r values of 90000-95000 and 230000-240000. The elution characteristics of the MPO of lower M_r seem to be identical with

those of the small MPO isolated from HL-60 cells (Yamada *et al.*, 1981a,b). Atkin *et al.* (1982) also found an abnormal form of MPO from a patient with chronic myeloid leukaemia. The higher- M_r form may be an aggregate, but rechromatography indicated that it is stable and has a defined M_r . Cockle & Harkness (1983) have previously suggested that the changes observed in the heterogeneity of salivary peroxidase during the menstrual cycle are due to the formation of oligomeric aggregates.

The MPO forms isolated from HL-60 cells showed about the same specific enzyme activity as measured by guaiacol oxidation (Yamada *et al.*, 1981a). In contrast, Pember *et al.* (1983), when examining normal MPO, found a lower peroxidase activity towards guaiacol and iodide by the form that was eluted with the lowest salt concentration from the cation-exchanger, whereas towards *NN*-dimethyl-*p*-phenylenediamine, pyrogallol and chloride the specific activities were about the same. They also found a difference in inhibition-sensitivity to 3-amino-1,2,4-triazole. However, their MPO forms had A_{430}/A_{280} absorption ratios of 0.60–0.72, which indicates impure enzymes (Agner, 1958).

The purified MPO forms were virtually pure as measured by the A_{430}/A_{280} absorption ratios of 0.80–0.87 (Agner, 1958; Andersen *et al.*, 1982), and the specific enzyme activities with 4-aminoantipyrine and guaiacol as substrates were compatible with pure enzymes (Matheson *et al.*, 1981; Yamada *et al.*, 1981a). The forms had the same specific peroxidase and peroxidase-oxidase activities with the various substrates used and showed the same dependence on H_2O_2 concentration for the three peroxidase substrates tested. Also, we found no difference in inhibition-sensitivity to 3-amino-1,2,4-triazole by the MPO forms. Thus we conclude that both normal and chronic-myeloid-leukaemia leucocytes contain four MPO enzyme forms differing in their elution behaviour on cation-exchangers but with the same enzymic properties. In contrast, horseradish peroxidase isoenzymes have been found to have different enzyme activities (Nakajima & Yamazaki, 1979, and references cited therein). Despite the difference in size, the abnormal forms of the enzyme found in chronic-myeloid-leukaemia MPO had the same spectral characteristics as and similar enzyme activity characteristics to normal MPO.

The MPO-dependent oxidation of cysteamine in the presence of chelator but in the absence of H_2O_2 shown in Table 2 is remarkable. The peroxidase-oxidase reactions of MPO in Table 2 have not previously been described; however, with horseradish peroxidase some other peroxidase-oxidase substrates have been reported (reviewed by Yamazaki & Yokota, 1973).

It has been argued that multiple forms of normal MPO are artifacts arising during isolation, since neutrophils contain a lot of degradative enzymes (Andersen *et al.*, 1982). However, heterogeneity on cation-exchange chromatography has also been observed in the presence of proteinase inhibitors (Yamada *et al.*, 1981a; Pember *et al.*, 1983) and with different isolation procedures (Pember *et al.*, 1982). MPO heterogeneity in neutrophils has also been suggested on the basis of cytochemical experiments (Lippi & Cappelletti, 1982). In any event, MPO is probably exposed to various degradative conditions *in vivo*, for example in the killing of micro-organisms and in inflamed tissues. This stresses

the importance of studying the characteristics of the enzyme forms in order to understand the function(s) of MPO. The present knowledge of the different forms of MPO is as yet insufficient to assign different functions to the various forms. However, the finding that the enzyme forms have the same enzymic activities suggests that different functions may be related to, for example, different affinities for various types of cell structures *in vivo*, rather than by different enzymic activities. MPO is a very basic protein with an isoelectric point greater than 10 (Agner, 1941), which implies that the enzyme may attach to negatively labelled structures. Selvaraj *et al.* (1978) have shown that smaller amounts of MPO and H_2O_2 were needed to obtain equivalent bactericidal activity if the enzyme was absorbed on the bacterial surface. The previously reported differences in distribution in the light and heavy azurophilic granules (Kinkade *et al.*, 1983), extractability by hyperosmotic salt solution (Pember *et al.*, 1983; Olsen & Little, 1984) as well as degranulation (Pember & Kinkade, 1983) suggest that the enzyme forms occupy different compartments in the neutrophil.

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