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Studies on the subunits of human myeloperoxidase

Ragnar L. OLSEN and Clive LITTLE
Institute of Medical Biology, University of Tromsø, 9001 Tromsø, Norway

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The subunit composition of human myeloperoxidase was studied with the use of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and gel filtration. The subunit pattern observed depended on the manner in which the enzyme was treated before analysis. Reduction before heat treatment in detergent led to two main protein species (M_r 57000 and 10500), whereas reduction during or after heat treatment yielded an additional species of M_r 39000. Heating without any reductive pretreatment yielded the 39000-M_r form as the major electrophoretic species. Carbohydrate staining showed large amounts of sugar on the 57000-M_r species and little on the 10500-M_r form. Significant amounts of haem were associated with this latter subunit. Haem also seemed to be associated with the 57000-M_r form but not with the 39000-M_r one. These three subunit forms were isolated and their amino acid composition analysed. The 57000-M_r and 39000-M_r forms had very similar amino acid composition and yielded an apparently identical collection of fragments on incubation with CNBr. Once separated, the subunits could not be interconverted. Generally, minor amounts of other molecular-mass forms were observed. The nature of the various molecular-mass forms originating from myeloperoxidase is discussed.

Myeloperoxidase (MPO) is a lysosomal haem-containing glycoprotein present in large amounts in neutrophil granulocytes (Schultz, 1980; Olsen & Little, 1983). As much as 2–5% (w/w) of the total protein in these cells is MPO (Schultz & Kaminker, 1962; Nauseef *et al.*, 1983). The physiological role of this enzyme is not clear, but it has been proposed to have antimicrobial functions (Klebanoff, 1975; Root & Cohen, 1981). Recent studies suggest a role for the enzyme in chemiluminescence in polymorphonuclear leucocytes (Dahlgren & Stendahl, 1983). This enzyme has been isolated from several sources such as canine pus (Agner, 1958; Harrison *et al.*, 1977), pig blood (Odajima & Yamazaki, 1970; Kokryakov *et al.*, 1982), guinea-pig bone marrow (Himmelhoch *et al.*, 1969), human myeloid-leukaemia cells HL-60 (Yamada *et al.*, 1981) and human blood (Schultz & Shmukler, 1964; Olsson *et al.*, 1972; Bakkenist *et al.*, 1978; Andersen *et al.*, 1982). Despite the fact that this enzyme is readily purified from such a wide variety of sources and is available in relatively large quantities, the literature abounds with discrepancies concerning the subunit composition. For the human and the dog enzyme some workers report subunits with M_r of 10000–15000 and 57000–60000 giving a holoenzyme of 136000–146000 consisting of two light and two heavy subunits (Harrison *et al.*, 1977; Andrews & Krinsky, 1981). Others report that the human enzyme, in addition to these two subunits, consists of various amounts of subunits with M_r values of 81000, 39000 and 25000 (Olsson *et al.*, 1972; Andersen *et al.*, 1982; Nauseef *et al.*, 1983). Still others report in the human enzyme two subunits with M_r values of 81000 and 63000 (Bakkenist *et al.*, 1978). In addition, Matheson *et al.* (1981) report that human MPO contains only one kind of subunit (M_r 59000).

We have recently purified the enzyme from human leucocytes and shown, under reducing and denaturing conditions, the presence of three major subunits with M_r values of 57000, 39000 and 15500 (Olsen & Little, 1983). It has been published that two haem groups are present in the native holoenzyme of MPO (Agner, 1958). Suggestions have been made that the two haem groups, or their mode of binding to the protein, are different

Abbreviations used: MPO, myeloperoxidase; SDS, sodium dodecyl sulphate.

(Agner, 1958; Harrison & Schultz, 1978; Odajima, 1980).

In the present paper we report on the subunit pattern of highly purified human MPO and present explanations for many of the previously published discrepancies. The amino acid compositions of the subunits are reported and also show the relationship of one of the haem prosthetic groups to the subunits.

Materials and methods

Materials

Buffy coats from fresh human blood were obtained by centrifugation at 1000*g* for 12min, and MPO was isolated as described previously (Olsen & Little, 1983).

Sources of chemicals and biochemicals are indicated in the text below.

Isolation of subunits

Subunits of M_r 10500, 39000 and 57000 were isolated by gel filtration on a column (1.6cm × 80cm) of Ultrogel AcA-44 (LKB Produkter, Bromma, Sweden) equilibrated and eluted with 0.1M-sodium acetate buffer, pH5.6, containing 0.5% SDS. For the 57000-M_r form, MPO (20mg/ml) in 0.1M-sodium acetate buffer, pH5.6, containing 5% SDS, was reduced by incubation at room temperature for 15min with 1% (v/v) 2-mercaptoethanol before incubation for 5min on a boiling-water bath. For the 39000-M_r and 10500-M_r subunits, the enzyme in the above buffer was incubated for 5min on a boiling-water bath. The same procedure was used for the 10500-M_r subunit except that, after isolation from the Ultrogel AcA-44 column, the subunit was concentrated and rechromatographed on a column (1.6cm × 70cm) of Bio-Gel P-30 (100–200 mesh) (Bio-Rad Laboratories, Richmond, CA, U.S.A.) to remove a minor contaminant of M_r approx. 22000.

Electrophoresis

Analytical SDS/polyacrylamide-gel electrophoresis was performed in gel slabs with dimensions of 0.25cm × 8.0cm × 8.0cm, by using a modification of the Laemmli (1970) system described in the Pharmacia booklet *Polyacrylamide Gel Electrophoresis* (Pharmacia Fine Chemicals, Uppsala, Sweden). The electrophoresis was performed at a constant current of 50mA per slab with 12.5% polyacrylamide in the separating gel and pH8.3 in the electrophoresis buffer. M_r values were determined by using the 'low-molecular-weight' calibration kit from Pharmacia. To analyse the low-M_r subunit (M_r 10500) SDS/polyacrylamide-slab-gel electrophoresis with 8M-urea present in the sample and the gel was performed (Swank & Munkres, 1971). The BDH Chemicals (Poole, Dorset, U.K.) standard (M_r range 2512–16949) was used, and the electrophoresis was performed at a constant current of 50mA per gel with 12.5% polyacrylamide in the gel.

After electrophoresis the detergent was removed from the gel with 25% (v/v) propan-2-ol/10% (v/v) acetic acid in gel destainer GD-4II (Pharmacia). In the same apparatus the gel was stained with Coomassie Brilliant Blue in 25% (v/v) methanol/10% (v/v) acetic acid and subsequently destained with the same solution without the dye.

Glycoproteins were detected in the SDS/polyacrylamide gel by using periodate/Schiff staining as described by Segrest & Jackson (1972).

Deglycosylation was performed by using trifluoromethanesulphonic acid/anisole at 0°C as described by Edge *et al.* (1981). A reaction time of 3h was used, and then the protein was separated from reagents and low-M_r sugars by extraction and dialysis (method A of Edge *et al.* 1981). *N*-Terminal amino acid analysis was carried out as described by Hartley (1970) and Gray (1972).

For cleavage by CNBr, MPO subunits were freeze-dried from 0.5% SDS and dissolved (1 mg/ml) in 70% (w/v) formic acid. The enzyme was then treated with a 200-fold molar excess of CNBr in a tightly stoppered tube for 24h in the dark at room temperature. The reaction mixture was then frozen and freeze-dried.

Amino acid analysis

The isolated subunits were dialysed extensively against 50mM-NH_4HCO_3 and dried in a vacuum oven. They were then hydrolysed in 6M-HCl in sealed tubes at 110°C for 24, 48 and 72h. The values for threonine, serine and tyrosine were obtained by extrapolation to zero hydrolysis time. Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1956) before hydrolysis. Quantitative amino acid analyses were performed with a JEOL JLC-6AH amino acid analyser.

Tryptophan was determined by using *N*-bromosuccinimide (J. T. Baker, Deventer, The Netherlands) essentially as described by Spande & Witkop (1967) on MPO subunits dissolved in 6M-guanidinium chloride/0.1M-sodium acetate buffer, pH4.6. Lysozyme (Sigma Chemical Co.) was used as an internal standard in the tryptophan determinations. The concentrations of the subunits used for the tryptophan determination were determined by the method of Bradford (1976), with lysozyme as standard.

Solutions of enzyme or subunits were concentrated by ultrafiltration in Diaflo equipment (Amicon Corp., Lexington, KY, U.S.A.) with a PM-10 membrane.

Results

Polyacrylamide-gel-electrophoretic studies

Purified MPO was subjected to SDS/polyacrylamide-gel electrophoresis with the use of 12.5% separating gel. MPO in 10mM-Tris/HCl buffer, pH8.0, containing 5% SDS, was pretreated in different ways before the electrophoresis. Fig. 1 shows the results. Standard proteins were run in lane 1. MPO samples (20μg) were applied to each well from lane 2 to lane 7. These six samples were pretreated in different ways before being loaded in the wells. MPO in lane 2 was reduced with 10% (v/v) 2-mercaptoethanol for 10min at room temperature and then heated at 100°C for 10min before electrophoresis. This gives rise to two major bands with M_r values 57000 and 15000 in addition to a weak band with M_r 39000. MPO in lane 3 was heated at 100°C for 10min with 10% 2-mercaptoethanol added 1–2min after the sample had been placed in the boiling-water bath. This results in three major protein bands of M_r 57000, 39000 and 15000. In addition, a broad diffuse band with M_r 22000–25000 is also discernible. MPO in lane 4 was heated at 100°C for 10min and after rapid cooling to room temperature was then incubated with 10% 2-mercaptoethanol for about 10min. The results are approximately similar to those obtained in lane 3. MPO in lane 5 was incubated for 10min at room temperature with 10% 2-mercaptoethanol. This gives one strong band with M_r 57000 and a band of M_r 15000. The amount of the latter subunit is less than that of the corresponding band seen in lanes 2–4. In addition, a weak band of M_r 70000 is also seen. MPO in lane 6 was merely heated at 100°C for 10min before electrophoresis (i.e. no reductant). This gives rise to a strong protein band of M_r 39000 and a strong band of the light subunit (M_r about 15000). A weak band with M_r 57000 is seen in addition to a stronger band of M_r 80000. A fifth protein band with an M_r estimated to be 120000 is also seen after boiling as the only pre-electrophoretic treatment. In lane 7 MPO was neither heated nor reduced before electrophoresis. The result is one very strong broad protein band with an estimated M_r of 120000–150000. The other four protein bands seen in lane 6 are also faintly discernible. The light subunit (M_r around 15000) is present in somewhat larger quantities than the other three minor components. Replacing 2-mercaptoethanol by 0.1mM-$NaBH_4$ as reductant resulted in no change in the electrophoresis patterns. Similarly the pretreatment of enzyme samples with 6M-guanidinium chloride (removed by dialysis before electrophoresis) and 5% SDS before reduction and/or heating in 0.5% SDS failed to change to subsequent electrophoretic patterns. Using MPO samples from the same or several different batches of enzyme always gave the same results.

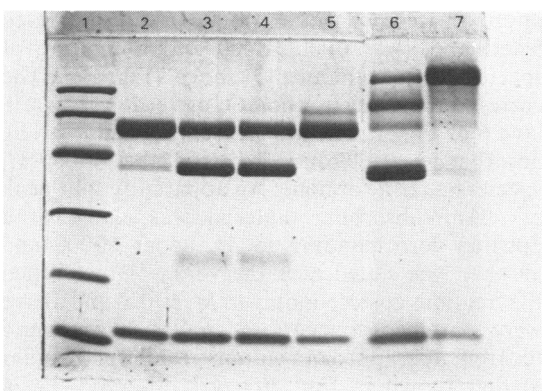

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of MPO after different pretreatments*

MPO (20μg) as a 0.8mg/ml solution in 10mM-Tris/HCl buffer, pH8.3, containing 0.5% SDS, was applied to each well from lanes 2 to 7. Lane 2, MPO reduced for 10min before boiling for 10min. Lane 3, MPO boiled for 10min and reductant added just after boiling had started. Lane 4, MPO boiled for 10min, cooled and then reduced for 10min. Lane 5, MPO reduced for 10min at room temperature. Lane 6, MPO boiled for 10min. Lane 7, MPO with no pretreatment. Lane 1, standard proteins: phosphorylase *b* (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 20000) and lactalbumin (M_r 14400). Other experimental details are given in the Materials and methods section and the Results section.

Prolonged boiling in SDS can cause cleavage of polypeptide chains (Kowit & Maloney, 1982), and consequently the influence of time of exposure at 100°C of samples of MPO on their subsequent electrophoretic behaviour was investigated. Electrophoretic patterns as shown in Fig. 1 were obtained when enzyme samples were placed in a boiling-water bath for 1min. Evidence for polypeptide cleavage in the form of the appearance of new protein bands was obtained only after some 30min exposure of the samples (either MPO or the isolated 57000-M_r or 39000-M_r subunits therefrom) to 100°C in SDS (results not shown).

A sample of MPO was placed on a boiling-water bath for 10min and reductant (10% 2-mercaptoethanol) was added just before the heating had started. This MPO was run on SDS/polyacrylamide-slab-gel electrophoresis in two lanes, one of which was stained for protein, the other for carbohydrate by using periodate/Schiff staining. The results (see Fig. 2*a*) show the presence of three main protein bands of M_r 57000, 39000 and 15000.

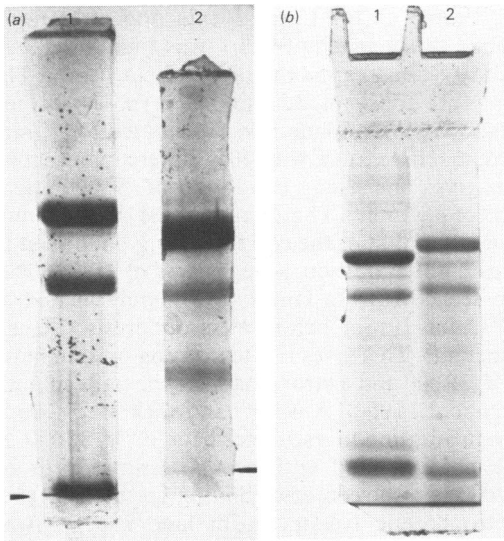

Fig. 2. *Carbohydrate on the subunits of MPO*
SDS/polyacrylamide-gel electrophoresis was performed as described in the text. (*a*) Carbohydrate staining. 2-Mercaptoethanol (10%) was added to the enzyme and the samples, and the mixture was boiled for 10min. Lane 1, MPO (27μg) was stained for protein. Lane 2, MPO (90μg) was stained for carbohydrates as described in the Materials and methods section. (*b*) Deglycosylated MPO. MPO was exposed to a chemical deglycosylation treatment (see the Materials and methods section) and then analysed in SDS/polyacrylamide-gel electrophoresis after reduction and boiling. Lane 1, deglycosylated enzyme. Lane 2, native enzyme.

Carbohydrates are present on the 57000-M_r and the 39000-M_r subunits and also in the 22000–25000-M_r region. No carbohydrate could be detected on the light subunit (M_r 15000). Densitometric scanning of the protein-stained lane and of the carbohydrate-stained lane showed that the ratio between carbohydrate and protein was twice as high for the 57000-M_r species as for the 39000-M_r one (results not shown). When the 39000-M_r subunit was separated without use of reductant (as in lane 6 in Fig. 1), subsequent periodate/Schiff staining indicated the subunit to be very rich in carbohydrate (results not shown). Under these conditions the 22000–25000-M_r carbohydrate-rich species noted above when reductant had been used (lanes 3 and 4 in Fig. 1) was absent.

Since MPO is glycoprotein it is conceivable that the various forms of pretreatment used in connection with gel electrophoresis might cleave some of the glycosidic links on the enzyme and thereby cause electrophoretic heterogeneity. A sample of enzyme was therefore submitted to a deglycosylating treatment involving trifluoromethanesulphonic acid. Subjecting this deglycosylated enzyme to reduction and denaturation followed by SDS/polyacrylamide-gel electrophoresis gave electrophoretic patterns very similar to those obtained with the native enzyme except that the forms of original M_r 57000 and 39000 became about 52000 and 37000 in M_r after deglycosylation (Fig. 2*b*).

Gel-filtration studies

Enzyme that had been reduced but not heated before gel filtration produced an elution profile consisting of three peaks of approximate apparent M_r 70000, 60000 and 10000–15000, with the two larger having absorbance at 406nm in addition to 280nm absorbance (Fig. 3*a*). This elution profile corresponds to the electrophoretic pattern shown in lane 5 of Fig. 1. Reduction treatment followed by boiling of the enzyme produces an elution profile with protein peaks corresponding to approximate apparent M_r values of 60000, 40000 and 10000–15000, with only the first of these showing absorbance at 406nm (Fig. 3*b*). This elution profile corresponds to the electrophoretic pattern shown in lane 2 of Fig. 1. Simply boiling the enzyme before application produces a major fraction of approximate apparent M_r 40000, a well-separated fraction of M_r 10000–15000 and a mixture of species of M_r 60000 and above (Fig. 3*c*). The corresponding electrophoretic pattern is shown in lane 6 of Fig. 1. The low-M_r species had a strong absorbance at 406nm, whereas the 40000-M_r species had little or none. An apparently split peak of 406nm-absorbing material was eluted at a position corresponding to M_r about 60000 and another was eluted near the void volume. When the fractions corresponding to M_r 40000 and above were combined, concentrated, boiled for 5min and rechromatographed, an elution profile very similar to that shown in Fig. 3(*c*) was observed, i.e. a 406nm-absorbing species of M_r 10000–15000 was released (results not shown). When the 10000–15000-M_r-peak material in Fig. 3(*c*) was collected, concentrated and run in urea/SDS/polyacrylamide-gel electrophoresis, the brown colour (Soret peak 406nm) migrated with the protein-staining material to a position corresponding to M_r 10500. Externally added haem in SDS migrated to a position equivalent to M_r <2000 in this system. The 60000-M_r and 40000-M_r material from Fig. 3 was also run in SDS/polyacrylamide-gel electrophoresis, and it migrated in accordance with M_r values 57000 and 39000 respectively (Fig. 5).

We were unable to extract haem from MPO by the use of organic solvents and acidic conditions (Teale, 1959; Fuhrhop & Smith, 1975). Reductions

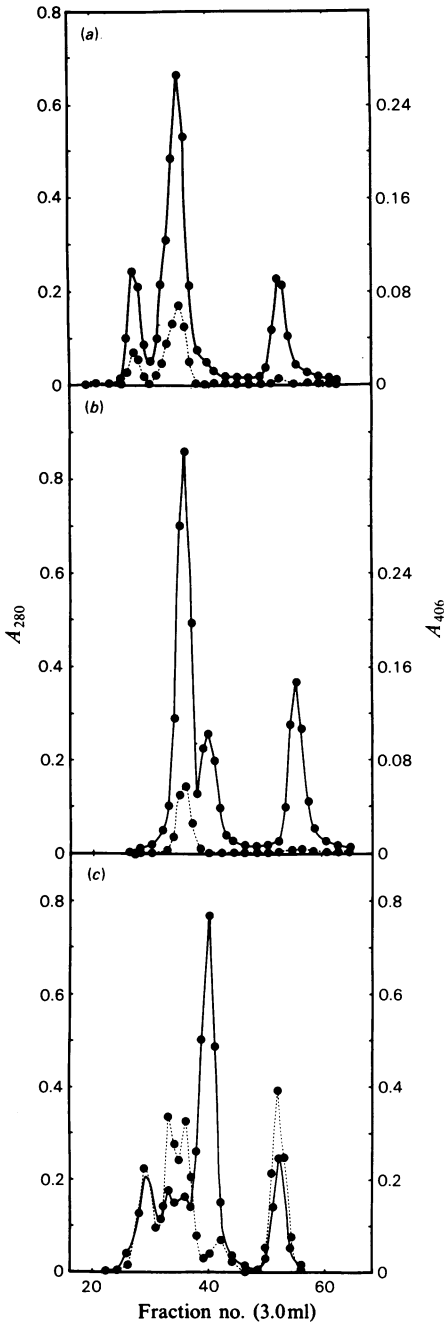

Fig. 3. *Elution profile on Ultrogel AcA-44 of MPO after different pretreatments*

MPO in 0.1M-sodium acetate buffer, pH 5.6, containing 0.5% SDS was pretreated in different ways before gel chromatography on an Ultrogel AcA-44 column (1.6cm × 80cm) in room temperature. (*a*) Reduced with 1% 2-mercaptoethanol at 23°C. (*b*) Reduced with 1% 2-mercaptoethanol and then boiled for 5min. (*c*) Boiled for 10min. Fractions were collected and tested for protein [A_{280} (——)] and haem [A_{406} (-----)].

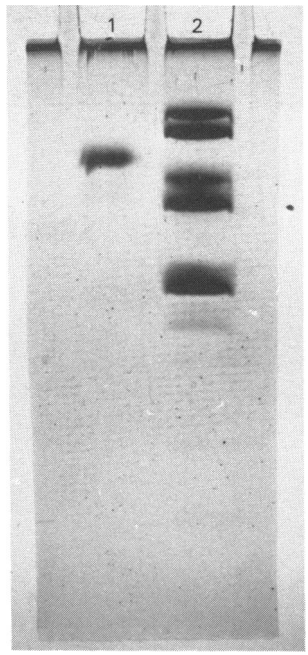

Fig. 4. *Urea/SDS/polyacrylamide-gel electrophoresis of the light subunit of MPO*

The samples in 8M-urea/1% SDS, pH6.8, were reduced and heated for 5min at 60°C before electrophoresis in the presence of 0.1% SDS and 8M-urea. Lane 2, standard peptides, CNBr-cleavage fragments of horse heart myoglobin, approx. M_r 17000, 14400, 8200, 6200 and 2500. Lane 1, light subunit (M_r 10500) of MPO (25μg).

of the enzyme seemed to be a necessary pretreatment for haem release.

The enzyme was also subjected to gel filtration on Sephacryl S-200 (superfine grade) in the presence of up to 5.5M-guanidinium chloride in 0.1M-sodium acetate buffer, pH5.6. No dissociation of the enzyme could be detected.

Properties of the three isolated subunits

Subunits of MPO with M_r values 57000, 39000 and 10500 were isolated as described in the Materials and methods section and appeared to be homogeneous in SDS/polyacrylamide-gel electrophoresis (Figs. 4 and 5). Once isolated, the subunits could not be interconverted to any significant extent by any combination of reduction and heating in SDS. Very small amounts of the 39000-M_r form could, however, be generated on occasions from the 57000-M_r subunit.

The amino acid composition of the isolated subunits was examined, and the results are shown in Table 1. The heavy chain A and the heavy chain B are the subunits with M_r 57000 and 39000

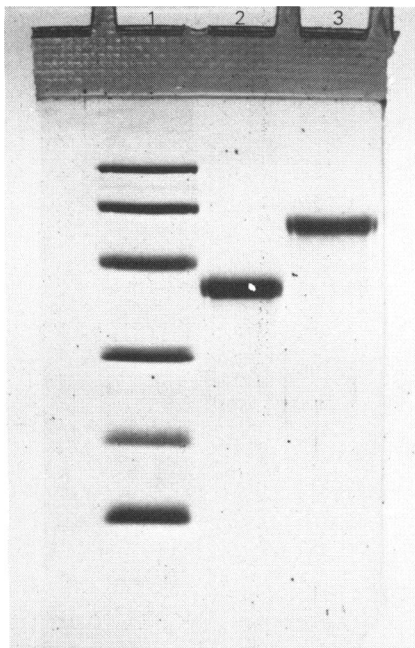

Fig. 5. *SDS/polyacrylamide-gel electrophoresis of the two purified heavy subunits of MPO*
Electrophoresis was performed on the samples in 10mM-Tris/HCl buffer, pH8.3, containing 0.5% SDS as described in the Materials and methods section and the Results section after reduction of samples with 1% 2-mercaptoethanol followed by boiling for 10min. Lane 1, standard proteins, as in Fig. 1. Lane 2, 39000-M_r species (10μg). Lane 3, 57000-M_r subunit (8μg).

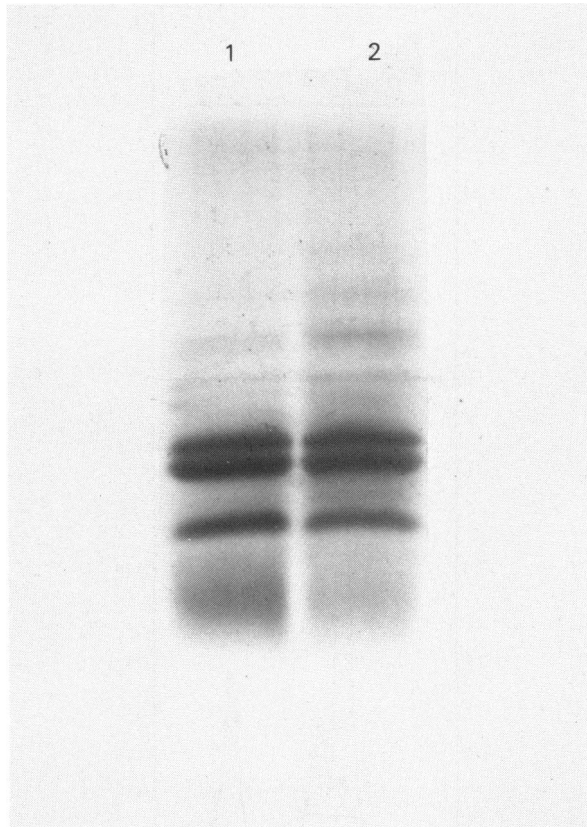

Fig. 6. *SDS/polyacrylamide-gel electrophoresis of CNBr-cleavage fragments of the 57000-M_r and 39000-M_r species*
The isolated subunits were treated with CNBr as described in the Materials and methods section. A 100μg portion of each CNBr-fragmented subunit was run in SDS/gel electrophoresis after reduction and boiling. Lane 1, 39000-M_r species. Lane 2, 57000-M_r species.

respectively, and the light subunit is the 10500-M_r form. The amino acid compositions are expressed as number of residues per molecule subunit.

The result show that 8–10% of the residues in the subunits are proline, and an abundant amount of proline seems to be a characteristic for many peroxidases (Nishikawa *et al.*, 1983). The major basic amino acid is arginine, which accounts for about 10% of the total amino acids in the subunits.

N-Terminal studies on the 57000-M_r subunit with dansyl (5-dimethylaminonaphthalene-1-sulphonyl) chloride showed a single *N*-terminal residue, valine. Equivalent studies on the 39000-M_r species failed to indicate an *N*-terminal residue.

CNBr fragmentation was performed on the 57000-M_r and 39000-M_r subunits, and the electrophoretic patterns of the two fragmented subunits are shown in Fig. 6. In each case three major bands and six minor ones are observed. The M_r values of the major fragments were estimated more accurately by using 12.5%-acrylamide gels containing 6M-urea and 1% SDS and the same M_r standards as were used in Fig. 4 (results not shown). In this system the three major bands each appeared as doublets and the estimated M_r values were 16000 and 14000, 9500 and 7500, and 3700 and 2900. No difference was discernible in either electrophoretic system between the fragments from the 57000-M_r and the 39000-M_r species.

Discussion

MPO is a very stable enzyme. Previous studies measuring protein fluorescence showed no marked unfolding of the enzyme in the presence of 10M-urea or 5M-guanidinium chloride (Olsen & Little, 1983). Incubation and subsequent gel filtration or polyacrylamide-gel electrophoresis in the presence

Table 1. *Amino acid compositions of the subunits of human MPO*

The subunits were isolated and hydrolysed as described in the Materials and methods section. Values for serine, threonine and tyrosine were obtained by extrapolation to zero time. Tryptophan was determined by using *N*-bromosuccinimide, and half-cystine as cysteic acid after performic acid oxidation.

Amino acid residue	Heavy chain B (residues/39000 daltons)*	Heavy chain A (residues/57000 daltons)	Light chain (residues/10500 daltons)
Asx	33 (49)	51	10
Thr	19 (28)	28	7
Ser	18 (27)	28	6
Glx	35 (51)	49	10
Pro	24 (36)	40	9
Gly	24 (36)	35	7
Ala	23 (33)	36	8
Cys	4 (7)	9	3
Val	20 (30)	30	5
Met	3 (5)	5	1
Ile	20 (29)	32	2
Leu	41 (60)	60	9
Tyr	13 (18)	16	2
Phe	15 (22)	26	6
His	4 (6)	5	1
Lys	10 (15)	12	2
Arg	34 (51)	47	9
Trp	3 (5)	3	1
Total residues	343 (508)	512	97

* Amino acid composition of the 39000-M_r species expressed as numbers of residues per 57000 daltons is given in parentheses.

of SDS, without heating or reduction, gave virtually no disaggregation. When disaggregation is achieved either by heating and/or reduction in the presence of SDS, the resultant subunit profile is very dependent on the precise nature of the pre-treatment used. The major forms of subunits appear to be of M_r 120000–140000, 57000, 39000 and 10500. The minor forms also seen are of approximate M_r 80000, 68000 and 22000–24000. Of these the 120000–140000-M_r protein appears to be the intact enzyme and possibly also some enzyme where one or both of the small subunits have been lost. Of the major subunits, it is the presence of the 39000-M_r species that is rather perplexing. It would seem that reduction before heat treatment virtually abolishes this band in favour of the 57000-M_r band. Similar findings have been reported by Matheson *et al.* (1981) and Nauseef *et al.* (1983). In addition, we report that adding the reductant during boiling of the sample or afterwards results in a marked presence of the 39000-M_r band, giving an approximately equal amount to the 57000-M_r band. This 39000-M_r band is also seen in great amounts when the sample is boiled but not reduced. Similar results were obtained with or without 5mM-EDTA in the sample or when 2-mercaptoethanol was replaced by $NaBH_4$ as reductant. The 39000-M_r band is only seen when enzyme is boiled before electrophoresis. This might explain the total absence of this subunit in some reports (Harrison *et al.*, 1977; Bakkenist *et al.*, 1978). We find that once formed and separated the 57000-M_r and 39000-M_r species could not be interconverted by any combination of reduction and heating with SDS of concentrations up to 5%, except that traces of 39000-M_r material could be generated by reduction and heating of the 57000-M_r species.

Of the minor subunits the high-M_r forms (M_r 68000 and 80000) might be the 57000-M_r species with one or both of the light subunits attached. The presence of subunits of such molecular weights has been reported by Bakkenist *et al.* (1978), Andersen *et al.* (1982) and Nauseef *et al.* (1983). The minor band of M_r 22000–25000 seen when the enzyme is boiled before reduction is more difficult to explain, but the presence of such a species has also been noted by other groups (Andersen *et al.*, 1982; Nauseef *et al.*, 1983). Harrison *et al.* (1977) observed a significant amount of a species with M_r 22000 after cross-linking purified dog MPO with dimethyl suberimidate treatment, and they attribute it to two cross-linked light subunits.

Andersen *et al.* (1982) observed that multiple electrophoretic forms of MPO could be produced by incubation of the enzyme with trypsin, and suggested that proteolytic degradation of the enzyme accounted for its electrophoretic hetero-

geneity. Our results suggest that this latter view is unlikely. The presence of proteinase inhibitors during the isolation of the enzyme has no effect on the enzyme's subsequent electrophoretic properties (Olsen & Little, 1983). Furthermore, the same electrophoretic behaviour was noted with enzyme isolated from freshly donated blood (R. Olsen & C. Little, unpublished work). If the postulated proteolytic degradation arises from a contaminating activity operating during work up of the samples for electrophoresis, then this activity would have to be inactivated by reducing pretreatment and also be capable of working in 5% SDS and/or 6M-guanidinium chloride.

The apparent multiplicity of subunits shown by this enzyme could, in principle, arise from multiple forms of the enzyme. In this respect it is noteworthy that Pember *et al.* (1983) do indeed present evidence for such multiple forms of human MPO. MPO form I was separated from forms II and III by using the differential extraction procedures described by Pember *et al.* (1983). Form I and the mixture of forms II and III showed identical behaviour in SDS/polyacrylamide-gel electrophoresis regardless of the particular type of pretreatment used (R. Olsen & C. Little, unpublished work). Our results indicate that the complex subunit pattern is entirely the result of the response of the enzyme and its subunits to the various forms of treatment used before analysis of the subunit profile.

The finding that carbohydrate is attached to the 57000-M_r species but not to the light subunit was also reported for the dog enzyme by Harrison *et al.* (1977). In addition, we find quite large amounts of carbohydrate present on the protein band with M_r 22000–24000. This argues against this band being a dimer of the light subunit.

When the enzyme is reduced and then boiled in SDS, the resultant 39000-M_r band contains relatively little carbohydrate. Under these circumstances a very carbohydrate-rich species of M_r 22000–24000 is also observed. However, when the enzyme is boiled without having any contact with reductant, no band around M_r 22000–24000 is seen, but, even so, a large amount of the 39000-M_r species is present. Subjecting the enzyme to a chemical deglycosylating pretreatment had only a small effect on the molecular masses of the electrophoretic forms of the enzyme, but did not alter the basic patterns observed. It is therefore very unlikely that the 39000-M_r species derives from the 57000-M_r form by deglycosylation during normal pretreatment of the samples for electrophoresis.

The amino acid compositions of the heavy subunit (M_r 57000) and the light subunit (M_r 10500) of human MPO are in general agreement with the values published for the dog enzyme (Harrison *et al.*, 1977). The only major deviation is for methionine in the heavy subunit, where 12 residues/molecule were found in the dog enzyme whereas five/molecule are reported in heavy subunit in the present paper. When the composition of the 39000-M_r subunit is expressed as number of residues per 57000 daltons, the numbers of each amino acid found are strikingly similar to the ones found for the 57000-M_r species. The *N*-terminal amino acid on the 57000-M_r subunit was found to be valine, in agreement with the results obtained by Atkin *et al.* (1982). However, using the dansyl chloride method, we failed to detect the *N*-terminal residue on the 39000-M_r form. CNBr fragmentation of these two subunits yielded an identical series of fragments from both forms when analysed in SDS/polyacrylamide-gel electrophoresis. These results suggest that both these two heavy subunits are composed of essentially the same polypeptide chain. Indeed, in view of the extremely high resistance of MPO to unfolding and denaturation, it would seem possible that the 39000-M_r species is a tightly folded and haem-free form of the 57000-M_r subunit, probably containing intact disulphide bridges in a highly inaccessible interior region. Unless the 57000-M_r subunit is reduced before denaturation, heating even in SDS may cause it to fold up tightly rather than open up, and once in this state reduction of the disulphide bridges by borohydride or 2-mercaptoethanol seems impossible.

Earlier workers have indicated that the two haem groups of MPO are not identical (Agner, 1958; Harrison & Schultz, 1978; Odajima, 1980). Under certain non-reductive denaturing conditions we find that MPO is partly dissociated into subunits and the haem groups are separated. Haem is associated with some high-M_r subunits, and also seems to be bound to the light subunit. Under these conditions all the haem from the enzyme is co-eluted with the subunits or aggregates thereof. No evidence for the release of free haem was found. Harrison & Schultz (1978) showed that, when dog MPO was reduced, but not heated, in the presence of 6.0M-guanidinium chloride, only the 57000-M_r subunit had haem associated. Atkin *et al.* (1982) studied MPO in the neutrophil granulocytes from a patient with chronic myelotic leukaemia. These workers made the qualitative observation that reduced boiled MPO had haem associated to the 57000-M_r subunit. We note in the present work that reduction seems to cleave the haem prosthetic group from the enzyme but that some haem still adheres to the 57000-M_r subunit after the enzyme has been reduced and denatured. Haem was never found associated with the 39000-M_r subunit even in the absence of reductive pretreatment (Fig. 3*c*).

In view of the present results and taking into

consideration the previously published work on this enzyme, we consider the most likely structure for human MPO to be a tetramer composed of two identical carbohydrate-rich 57000-M_r subunits and two identical carbohydrate-free small subunits of M_r 10500. The subunits do not seem to be covalently joined together. The haem residues seem to be very tightly bound to the enzyme, and not released without the addition of reductants. At least one of the haem residues seems to be attached to the small subunit. Under certain conditions, especially after reduction, haem was also associated with the 57000-M_r subunit. It is possible that haem released from the small subunit by reduction may subsequently adhere to the large subunit. These results concerning the haem residues of human MPO differ considerably from those of dog MPO, where non-reductive treatment can release haem associated with the small subunit (Harrison & Schultz, 1978). The dog enzyme also seems to be much easier to denature than human MPO.

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