Further characterization of human eosinophil peroxidase

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1. The large and the small subunits (M_r 50000 and 10500 respectively) of human eosinophil peroxidase were isolated by gel filtration under reducing conditions. The subunits were very strongly associated but not apparently cross-linked by disulphide bridges. 2. During storage, the large subunit tended to form aggregates, which required reduction to dissociate them. Amino acid analysis of the performic acid-treated large subunit showed the presence of 19 cysteic acid residues. 3. The small subunit of eosinophil peroxidase had the same M_r value as the small subunit of myeloperoxidase. However, although these subunits have very similar amino acid compositions, they showed different patterns of peptide fragmentation after CNBr treatment. 4. The carbohydrate of eosinophil peroxidase seemed associated exclusively with the large subunit and comprised mannose (4.5%, w/w) and *N*-acetylglucosamine (0.8%, w/w). 5. The far-u.v. c.d. spectrum of the enzyme indicated the presence of relatively little ordered secondary structure.

Eosinophil peroxidase (EPO) is a haem-containing glycoprotein present in large amounts in the specific granules of eosinophils (Enomoto & Kitani, 1966; Bainton & Farquhar, 1970). The function of the enzyme is not known, but it has been suggested as a possible modulator in hypersensitivity reactions (Henderson *et al.*, 1982; Goetzl, 1982). Another possible function is in antiparasitic action (McLaren *et al.*, 1977; Buys *et al.*, 1981; Nogueira *et al.*, 1982). The enzyme was first obtained from rat eosinophils (Archer *et al.*, 1965) and guinea-pig bone-marrow leucocytes (Desser *et al.*, 1972). Later the enzyme was isolated in highly purified form from both human (Wever *et al.*, 1981) and horse eosinophils (Jörg *et al.*, 1982). The isolation and characterization of the enzyme have been hampered by its tendency to aggregate and to bind to different materials (Wever *et al.*, 1981; Jörg *et al.*, 1982; Bolscher *et al.*, 1984).

We have previously reported an isolation procedure that gives highly purified EPO in an unaggregated form that does not bind to gel-chromatography media, dialysis tubing or other materials commonly used in isolation schemes (Olsen & Little, 1983). EPO is composed of two

subunits, with approximate M_r values of 50000 and 15000 (Olsen & Little, 1983; Bolscher *et al.*, 1984). In addition it has been suggested that the prosthetic group of EPO is protohaem IX, the same as found in lactoperoxidase (Wever *et al.*, 1980).

In the present paper we report on the isolation of the subunits of EPO and present the amino acid compositions, carbohydrate contents and other data on the purified subunits. The results are compared with those for myeloperoxidase (MPO), the peroxidase found in neutrophil granulocytes, and other haem-containing peroxidases.

Materials and methods

Materials

Buffy coats from fresh human blood were obtained by centrifugation at 1000*g* for 12 min, and EPO was purified essentially as described previously (Olsen & Little, 1983). After the gel-filtration step the EPO peak was concentrated and rechromatographed on the same gel-filtration column, and finally EPO was re-run on a small concanavalin A-Sepharose column (2 ml of gel/10 mg of EPO) under the same conditions as in the initial stage of the purification. The A_{415}/A_{280} ratio of the preparations used in this work was 1.05–1.10:1.

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

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The subunits of EPO were isolated by gel filtration on a column (1.6 cm $\times$ 80 cm) of Ultrogel AcA-44 (LKB Produkter, Bromma, Sweden). The elution buffer was 0.1 M-sodium acetate, pH 5.6, containing 0.5% SDS. EPO in the same buffer, but with 5% SDS, was reduced for 5 min with 1% (v/v) 2-mercaptoethanol at room temperature and subsequently incubated for 3 min on a boiling-water bath, before the gel chromatography. The small subunit of MPO (MPO III) was isolated as described in Olsen & Little (1984).

Methods

SDS/polyacrylamide-slab-gel electrophoresis with 8 M-urea present in sample and gel (dimensions 0.25 cm $\times$ 8.0 cm $\times$ 8.0 cm) was performed essentially as described by Swank & Munkres (1971). As standards, a kit for determination of the M_r values of polypeptides (M_r range 2560–17200) was used (Pharmacia Fine Chemicals, Uppsala, Sweden). Before electrophoresis, the samples were reduced for 5 min with 1% (v/v) 2-mercaptoethanol at room temperature and heated on a boiling-water bath for 3 min. The electrophoresis was performed at a constant current of 50 mA/gel with 12.5% (w/v) polyacrylamide in the gel.

Analytical SDS/polyacrylamide-gel electrophoresis without urea was performed on the high- M_r subunit of EPO (M_r 50000) as described previously (Olsen & Little, 1983). After the electrophoresis the gels were stained for protein and carbohydrate (Olsen & Little, 1984).

The cleavage of subunits by CNBr was performed as described previously (Olsen & Little, 1984). G.l.c. for the determination of carbohydrate was performed as described by Bolton *et al.* (1965), with minor modifications.

C.d. spectra of native EPO were obtained at $23 \pm 1^\circ\text{C}$ in a Jasco J500 spectropolarimeter under conditions similar to those described by Little (1978). The ellipticity is expressed as the mean-residue-weight ellipticity:

$$[\theta] = \frac{M \cdot \phi}{10 \cdot c \cdot l}$$

in degrees $\cdot \text{cm}^2 \cdot \text{dmol}^{-1}$, where M is the mean residue weight (112), ϕ the observed ellipticity in degrees, c the protein concentration in g/ml and l the path length in cm. The solvent used for these studies was 0.1 M-potassium phosphate, pH 7.4.

Protein concentrations were determined as described by Olsen & Little (1983), and for spectrophotometric measurements a Hitachi 557 recording spectrophotometer was used. Solutions of enzyme and isolated subunits were concentrated by ultrafiltration in Diaflo equipment (Amicon

Corp., Lexington, KY, U.S.A.) with a PM-10 membrane.

For amino acid analysis the isolated subunits were dialysed extensively against 0.05% SDS and freeze-dried. They were then hydrolysed at 110°C for 24 h in 6 M-HCl containing 0.05% (v/v) thioglycolic acid (Fluka, Buchs, Switzerland) in tubes sealed under N_2 . Half-cystine was determined as cysteic acid after performic acid oxidation (Hirs, 1956). After the hydrolysis the samples were dried in a vacuum oven, and the quantitative amino acid analyses were performed in a JEOL JLC-6AH amino acid analyser with the samples dissolved directly in 0.2 M-sodium citrate buffer, pH 2.2, containing 0.5% (v/v) thiodiglycol (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) and 0.01% (v/v) octanoic acid (Merck, Darmstadt, Germany).

Results

Subunit isolation

The subunits of EPO were separated by gel filtration after reduction and boiling of the enzyme (Fig. 1). The more-retarded peak is the low- M_r subunit (EPO II), and it appeared homogeneous in SDS/polyacrylamide-gel electrophoresis (Fig. 3, lane 5). The high- M_r subunit (EPO I) was concentrated and rechromatographed on the same column and the main fractions were pooled, and analysis by SDS/polyacrylamide-gel electrophoresis showed homogeneity. Several unsuccessful attempts were made to separate the subunits by gel filtration in 0.1 M-sodium acetate buffer, pH 5.6, with 0.5% SDS without reductant in the sample, heating (100°C for 3 min) of the sample only,

Fig. 1. Gel filtration of denatured EPO. Gel filtration of EPO (10 mg) on an Ultrogel AcA-44 column (1.6 cm $\times$ 80 cm) with 0.5% SDS/0.1 M-sodium acetate, pH 5.6, as elution buffer. The enzyme in this buffer, but including 5% SDS, was reduced with 1% 2-mercaptoethanol and heated on a boiling-water bath for 3 min before the chromatography.

heating (100°C for 3 min) with 8 M-urea included in the sample, or increasing the SDS concentration in the sample to 5% (w/w). The resulting elution patterns showed one peak, with a shoulder on the lower-*M_r* side. On the other hand, EPO in SDS/polyacrylamide-gel electrophoresis could be separated into the large and the small subunit without the use of reductants.

It was also noted that the homogeneous high-*M_r* subunit in 0.5% SDS/0.1 M-sodium acetate buffer, pH 5.6, had a tendency to aggregate. The isolated subunit was usually stored at -20°C, and when a stored sample was thawed and then applied directly to the gel-filtration column two protein peaks were found (Fig. 2), one with an elution volume corresponding to EPO I and the other as a considerably less-retarded peak. When the latter was subjected to SDS/polyacrylamide-gel electrophoresis it was found necessary to add reductant (1% 2-mercaptoethanol) to this aggregate to generate an apparent *M_r* equal to that of EPO I.

Properties of the subunits

Subunits of EPO with *M_r* values of approx. 50 000 (EPO I) and 10 500 (EPO II) were isolated as described in the Materials and methods section. The amino acid compositions of the isolated subunits are presented in Table 1. For comparison with EPO II, the amino acid composition of the small subunit of MPO, MPO III (Olsen & Little,

1984), is also included. The amino acid compositions of EPO II and MPO III are in general similar, although minor differences in, for example, the number of arginine residues can be noted; arginine

Fig. 2. Gel filtration of the large subunit of EPO. After concentration and storage at -20°C for a week, the large subunit of EPO (7 mg) was chromatographed on the Ultrogel AcA-44 column (1.6 cm × 80 cm) with 0.5% SDS/0.1 M-sodium acetate, pH 5.6.

Table 1. Amino acid compositions of the subunits of human EPO and of the small subunit of human MPO. The subunits were isolated as described in the Materials and methods section and hydrolysed for 24 h at 110°C with 6 M-HCl and 0.1% thioglycolic acid. Cysteine/cystine were determined as cysteic acid after performic acid oxidation. For comparison the light subunit of MPO was also examined.

Amino acid residue	EPO I (residues/50 000 Da)	EPO II (residues/10 500 Da)	MPO III (residues/10 500 Da)
Asx	47	8	9
Thr	19	4	6
Ser	24	5	4
Glx	29	8	9
Pro	27	8	10
Gly	29	7	6
Ala	36	7	7
Cys	19	3	3
Val	18	4	5
Met	13	2	3
Ile	25	3	2
Leu	52	9	8
Tyr	10	3	3
Phe	22	6	5
His	9	1	1
Lys	13	2	2
Arg	51	11	9
Trp	(—)*	(—)*	1
Total	443	91	93

* Tryptophan was shown by its absorption at 292 nm to be present in both EPO subunits.

accounts for about 12% of the total number of amino acids. The content of proline is very high in both subunits. It was also noted that the ratios of threonine to serine, residues that are eluted next to each other from the amino acid analyser, are 0.8:1 and 1.5:1 for EPO II and MPO III respectively. Cysteine and cystine were determined as cysteic acid, and the results show that as many as 19 such residues are found in the large subunit of EPO.

The properties of the light subunit of EPO (EPO II) were further compared with those of the light subunit of MPO (MPO III). These two subunits were analysed by SDS/polyacrylamide-gel electrophoresis in the presence of 8M-urea, and it seems that these two subunits have identical M_r values of 10 500 (Fig. 3, lanes 4 and 5). Both subunits were treated with CNBr as described in the Materials and methods section, and after urea/SDS/polyacrylamide-gel electrophoresis two different fragmentation patterns were found (Fig. 3, lanes 2 and 3). Only one strong band (M_r 6500) was seen after the CNBr cleavage of EPO II, whereas for MPO III one strong band (M_r 7300) and one much weaker

band (M_r 5000) were found. These cleavage patterns were reproducible with subunits isolated from different batches of enzymes. A 10-fold increase in the amount of the CNBr-fragmented subunits loaded on the gel did not reveal any other bands; in particular no bands of very low M_r (<5000) were seen.

The carbohydrate content of EPO was determined by g.l.c. and showed approx. 4.5% (w/w) mannose and 0.8% (w/w) *N*-acetylglucosamine. No other sugars, except for possible traces of glucose, were found in the intact enzyme. The low- M_r subunit, EPO II, was isolated and analysed for carbohydrate, but none was detected. A similar result was obtained for the low- M_r species (MPO III) of MPO. In addition, carbohydrate staining of the EPO subunits after SDS/polyacrylamide-gel electrophoresis showed the presence of sugar on the large subunit only.

C.d. of eosinophil peroxidase

The far-u.v. c.d. spectrum of native EPO is shown in Fig. 4. The secondary structure of the enzyme was estimated by the method of Rosenkranz & Scholtan (1971), and it was calculated that the enzyme contains about 15–22% β -structure and about 72–80% non-periodic structure. No α -helical structure was detected.

Discussion

Human EPO has been shown to consist of two subunits with M_r values of approx. 50000 and 10500 (Olsen & Little, 1983; Bolscher *et al.*, 1984). In the present work separation of the two subunits could be obtained by gel filtration in 0.5% SDS/0.1 M-sodium acetate buffer, pH 5.6, but only

Fig. 3. Urea/SDS/polyacrylamide-gel electrophoresis of the intact and CNBr-fragmented small subunit of EPO and MPO

The intact and CNBr-fragmented subunits in 8.0M-urea/1% SDS were reduced and heated, and electrophoresis was performed as described in the Materials and methods section. Lane 1, standard peptides, of approx. M_r 17200, 14600, 8200, 6400 and 2560. Lane 2, CNBr-fragmented small subunit of EPO (30 μ g). Lane 3, CNBr-fragmented small subunit of MPO (30 μ g). Lane 4, intact small subunit of MPO (30 μ g). Lane 5, intact small subunit of EPO (30 μ g). Further details are given in the Materials and methods section.

Fig. 4. Far-u.v. c.d. spectrum of EPO
The Figure shows the far-u.v. c.d. spectrum of the native enzyme (3.7 mg/ml) in 0.1 M-potassium phosphate buffer, pH 7.4.

after prior reduction of the sample (Fig. 1). However, subunit separation of the non-reduced enzyme could be effected by SDS/polyacrylamide-gel electrophoresis, indicating that the two subunits are bound only by strong non-covalent forces. Previously we have reported strong non-covalent association between the subunits of human MPO (Olsen & Little, 1984). The large subunit isolated as described had a tendency to aggregate on storage. In this case, since it was necessary to use reductant to obtain monomeric EPO I by SDS/polyacrylamide-gel electrophoresis, we suggest that these aggregates are formed by oxidation of cysteine residues to intersubunit disulphide bonds.

The amino acid compositions of the two subunits of EPO show the same general pattern. Arginine, leucine and proline are present in relatively high abundance (Table 1). A similar general agreement in amino acid composition was found between the heavy and the light subunits of MPO (Olsen & Little, 1984). In the large subunit of EPO, cystine/cysteine, determined as cysteic acid, constitutes about 4.8% of the total number of residues. This is about twice as many as were found in the large subunit of MPO (Olsen & Little, 1984), in bovine lactoperoxidase (Carlström, 1969) and in pig intestinal peroxidase (Kimura & Jellinck, 1982). To prevent loss of methionine, thioglycolic acid was included during hydrolysis, and the conditions used might possibly lead to an overestimation of the proline content (Gardner, 1984). When the amino acid compositions of the two EPO subunits are added, the results fit very well with the amino acid composition for the intact enzyme as published by Bolscher *et al.* (1984).

Previously it was shown that the small subunits of EPO and MPO had similar M_r values (Olsen & Little, 1983). The M_r value for the small subunits of these two enzymes is 10 500. To investigate further the relationship between these two subunits, they were fragmented with CNBr, and found to yield different cleavage patterns (Fig. 3). Except for a small fraction of non-fragmented subunit, only one band (M_r 6500) was found in CNBr-fragmented EPO II. A possible explanation is that a susceptible methionine residue is in the middle of the polypeptide chain, producing two peptides of similar size. MPO III is split into one strong band (M_r 7300) and one much weaker-staining band (M_r 5000). EPO II and MPO III appear to contain two and three methionine residues respectively. The apparent discrepancy between the CNBr-fragmentation pattern and the amino acid analysis may indicate that one or two methionine residues are in such positions in the polypeptide chain that some of the fragments produced are so small that they are not detected by the SDS/polyacrylamide-gel electrophoresis, or they exist as methionine sul-

phoxide, which is not cleaved by CNBr but is reduced to methionine by the thioglycolic acid during the hydrolysis for amino acid analysis (Savage & Fontana, 1977; Fliss *et al.*, 1983). The presence of methionine sulphoxide residues in proteins has been reported (Hudson & Spiro, 1972; Kikuchi & Tamiya, 1981; Fliss *et al.*, 1983). This is especially interesting, since activated granulocytes produce large quantities of agents capable of oxidizing methionine residues (Root & Cohen, 1981; Brot & Weissbach, 1982, 1983).

EPO binds to concanavalin A (Olsen & Little, 1983) and is probably therefore a mannose-containing glycoprotein (Sharon & Lis, 1972). Furthermore, staining of EPO in polyacrylamide gels showed the presence of carbohydrate on the large subunit, but not on the small subunit. This latter observation is at variance with the results published by Bolscher *et al.* (1984), who reported the presence of carbohydrate on both subunits. It has been reported previously that the small subunit of MPO is devoid of sugar (Harrison *et al.*, 1977; Olsen & Little, 1984). In the present work g.l.c. analysis of the small subunits of EPO and MPO indicated that both lacked sugar, although the presence of SDS in the samples made these interpretations somewhat difficult. Intact EPO was shown to contain mannose (4.5%), *N*-acetylglucosamine (0.8%) and possibly glucose. Relatively high contents of mannose and also the presence of *N*-acetylglucosamine have been reported for other mammalian peroxidases such as human MPO (Bakkenist *et al.*, 1978) and bovine lactoperoxidase (Carlström, 1969).

From the far-u.v. c.d. spectrum only 15–22% ordered secondary structure was inferred. This is in marked contrast with bovine lactoperoxidase, where 88% ordered secondary structure was calculated from c.d. studies (Sievers, 1980). It should, however, be pointed out that such calculations based on c.d. spectra are only approximately correct, because, particularly for haemoproteins, with relatively small amounts of ordered secondary structure, great deviations from the true values are possible (Myer & Pande, 1978).

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