Lactoperoxidase haem, an iron-porphyrin thiol

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The haem prosthetic group of lactoperoxidase can be prepared from the enzyme in high yield by reductive cleavage with mercaptoethanol in 8 M-urea under mild conditions. The product yields porphyrins, after removal of iron, which show visible spectroscopic properties similar to protoporphyrin but are considerably more polar. In the presence of iodoacetamide, a different product is obtained by reductive cleavage. The proton n.m.r. and mass spectra of this compound indicate that the prosthetic group of the enzyme is the iron complex of 18-mercaptomethyl-2,7,12-trimethyl-3,8-divinylporphyrin-13,17-dipropionic acid. It is proposed that the unusual strength of binding of the prosthetic group to the apoprotein is due to formation of a disulphide bond from a cysteine residue to the porphyrin thiol.

INTRODUCTION

Lactoperoxidase (LPO, EC 1.11.1.7) is a haemoprotein enzyme found in milk, saliva and tears. The enzyme catalyses the oxidation of thiocyanate to a reactive form with antimicrobial properties. Early studies by Morrison *et al.* (1957) indicated that the prosthetic group of LPO is unusually firmly bound to the protein. Whereas low yields of haem can be obtained from the enzyme by alkaline hydrolysis, acid hydrolysis with HI yields an iron-free porphyrin similar in properties to mesoporphyrin. Morell & Clezy (1957) confirmed these studies and obtained higher yields of haem from LPO by treatment of the enzyme with HBr in acetic acid. Removal of iron from this product gave a porphyrin with spectroscopic properties that suggested the presence of an electrophilic substituent on the porphyrin-ring system. More recently Sievers (1979), using Pronase digestion, obtained a porphyrin from LPO. The properties of this porphyrin led him to suggest an identity with protoporphyrin. Resonance-Raman studies on the enzyme, which confirmed the presence of two vinyl groups in the prosthetic group (Manthey *et al.*, 1986), provide further support for this proposal. In our hands, Pronase digestion of LPO gave low yields of a porphyrin with spectroscopic properties similar to those of protoporphyrin. T.l.c. of this porphyrin showed that it was much more polar than protoporphyrin. This fact prompted a reinvestigation of the nature of the LPO prosthetic group.

MATERIALS AND METHODS

Enzyme preparation

LPO was prepared by a modification of the method of Morrison *et al.* (1957). Cheddar-cheese whey (5000 litres) was adjusted to pH 7.0 by addition of solid NaOH. A suspension of Amberlite CG-50 ion-exchange resin (11 kg) in water (40 litres), which had previously been adjusted to pH 7.0 in a similar manner, was added to the whey, the suspension was stirred for 1 h and the whey removed by siphoning. From this point all operations were carried out at a temperature of 4 °C. After washing

the resin four times with water by suspension, settling and decantation, the suspended resin was transferred to a large column (12 cm × 100 cm) and washed with water until the washings were clear. The column was then eluted with 0.5 M-sodium acetate (5 litres) and the dark brown–green band of LPO collected. After adjustment to 80% saturation with $(\text{NH}_4)_2\text{SO}_4$, the precipitate was collected by centrifugation, redissolved in water and dialysed against three changes of water. After centrifugation, the dialysate was chromatographed on a column of Amberlite CG-50 resin (7 cm × 70 cm) by using stepwise elution with 2 litres each of 0.1 M-, 0.25 M- and 0.6 M-sodium acetate. When sodium acetate was used as eluent, no preceding band of red–brown lactoferrin, seen when phosphate buffers were used, was observed. The LPO-containing eluate was dialysed against water, concentrated by poly(ethylene glycol) absorption and freeze-dried. The resulting protein had an A_{412}/A_{280} ratio of 0.62. Spring whey gave about 5 g of freeze-dried enzyme.

Disc electrophoresis using a 7.5%-(w/v)-polyacrylamide gel and a pH 4.3 β -alanine buffer (Reisfeld *et al.*, 1962) gave one major band and an additional minor band of higher mobility after staining with Coomassie Blue. Only the major band was stained by Leucomalachite Green (Burdett, 1977). The preparation was therefore judged to be free of other haemoproteins.

Pronase digestion of LPO

The procedure used was that of Sievers (1979). LPO (0.5 g) was dissolved in 50 mM- $(\text{NH}_4)_2\text{CO}_3$ (50 ml) containing 1 mM- CaCl_2 . After the addition of 50 mg of Pronase (Calbiochem) dissolved in the same buffer (5 ml), proteolysis was allowed to proceed at 37 °C for 15 h under N_2 . After adjusting the pH of the digest to 2.5 with 5 M-HCl, the haemin was extracted with butan-2-one. Removal of iron was achieved by using acetic acid redistilled over FeSO_4 by the method of Morell *et al.* (1961). The iron-free porphyrin was esterified with diazomethane that had been co-distilled with diethyl ether.

Preparation of 'LPO haem' by reductive cleavage

Freeze-dried LPO (1 g) was dissolved in 0.1 M- K_2HPO_4 (100 ml). N_2 was bubbled through the solution for 5 min, after which mercaptoethanol was added to a final concentration of 60 mM. The solution was stirred under N_2 at 4 °C for 15 min, after which time solid urea was added slowly to a final concentration of 8 M. After the mixture had been left for 20 h at 4 °C in the dark, the pH of the brown-red solution was adjusted to 2.5 with 5 M-HCl. LPO haem was extracted with ethyl acetate (twice), which was in turn washed with 0.1 M-HCl (five times) and finally with water, before it was dried over anhydrous Na_2SO_4 . After evaporation to dryness *in vacuo* at 60 °C, the oily residue was dissolved in a few drops of 0.2 M-NaOH and diluted to 1 ml with water. After acidification with 0.1 M-HCl, the precipitated haem was collected by centrifugation, washed with water (four times) and dried over NaOH *in vacuo*. The corresponding porphyrin was prepared by dissolving the residue obtained by evaporation of the ethyl acetate solution of the LPO haem (see above) in acetic acid and removal of iron with $FeSO_4$. After extraction of the porphyrin into diethyl ether, it was further extracted into 3 M-HCl and then with diethyl ether after treatment of the acid extract with saturated sodium acetate. The dried diethyl ether extract was evaporated to dryness at 40 °C and the residue dissolved in aq. 1 M- NH_3 . The solution of the porphyrin anion was washed with diethyl ether (twice), and the porphyrin precipitated by addition of acetic acid. The precipitated porphyrin was collected by centrifugation, washed with water and dried *in vacuo*.

Preparation of the acetamide derivative of LPO haem (Ib, Fig. 1)

The preparation of this derivative was carried out exactly as for LPO haem itself, except that, after addition of urea, the solution of denatured enzyme was left for 3 h. After this period iodoacetamide (2 g) was added and the solution stirred for 30 min before being left under nitrogen in the dark at 4 °C for a further 16 h. The prosthetic group was extracted in exactly the same way as described above. The mass of dried haem obtained in this way was approx. 3 mg.

Fig. 1. Structure of LPO haem and its derivatives

Compound Ia, X = SH; compound Ib, X = $S-CH_2CONH_2$

Yield determinations

Yields were determined by comparing the A_{559} of the pyridine haemochrome prepared from a portion of the enzyme solution before treatment with that of the absorbance at the same wavelength of the pyridine haemochrome of the protein-free haem. Formation of the haemochrome was allowed to proceed for 2 h for the enzyme and 5 min for the isolated haem.

Amino acid analyses were carried out on 6 M-HCl hydrolysates of haem, pre-column derivatization with phenyl isothiocyanate being used. N.m.r. spectra were recorded on a Bruker LXP 300 spectrometer. M.s. was performed on a double-focusing instrument (Darcy *et al.*, 1978; Cullis *et al.*, 1980) operating in the field-desorption mode using an emitter that was a thin wire (10 μ m diameter) covered with carbon microneedles (Neumann *et al.*, 1980).

Electrophoresis of porphyrin-free acids was carried out by the method of Lockwood & Davies (1962).

Attempted derivatization of porphyrins with hydroxylamine and acetic anhydride used methods detailed by Falk (1964). T.l.c. of porphyrin methyl esters was performed as described by Smith (1975). Porphyrin and haem-free acids were separated on Whatman KC18F reverse-phase plates by using as solvent methanol/water (9:1, v/v), which was 1 mM with respect to tetrabutylammonium bromide and 10 mM with respect to acetic acid, and had been adjusted to an apparent pH of 6.5 with aq. 5 M- NH_3 . A similar system has been used for separation of haemins by h.p.l.c. (Tangeras, 1984). The latter system was also used for preparative separations of porphyrin-free acids.

RESULTS

The yields of haem from the three different preparations are shown in Table 1. The yield of haem from Pronase digestion of LPO was extremely variable.

T.l.c. of the haem extracts showed that, although a single compound was produced in each case, no two products were identical (Table 2). All were considerably more polar than protohaem. None of the haem products showed a change in R_F value after reaction with acetic anhydride. $NaBH_4$ caused non-specific reduction of the porphyrin-ring system, as evidenced by loss of Soret absorption. Amino acids were not detected after 6 M-HCl hydrolysis of the haems obtained from mercaptoethanol cleavage of LPO in either the presence or the absence of iodoacetamide.

Two major porphyrins were obtained upon the removal of iron from the haems derived from LPO by reductive cleavage, in either the presence or absence of iodoacetamide. The two pairs of porphyrins were separated by scraping off the appropriate zones and eluting with methanol. Only the upper band showed a

Table 1. Yield of ethyl acetate- or butan-2-one-extractable haem from LPO

Method of preparation	Yield (%)
Pronase digestion	6
Mercaptoethanol/urea	31
Mercaptoethanol/urea/iodoacetamide	63

Table 2. Reverse-phase t.l.c. behaviour of LPO-haem extracts and porphyrin-free acids

For conditions, see the text.

Haem or product	R_F
Pronase-digest haem	0.71
Mercaptoethanol/urea-treated haem	0.85
Iodoacetamide-treated haem	0.78
Protohaem	0.32
Mercaptoethanol/urea porphyrin products	0.90 (0.78)*, 0.84
Iodoacetamide porphyrin products	0.74 (0.60)*, 0.64

* The value in parentheses represents the R_F value after acetic anhydride treatment.

change in R_F value on treatment with acetic anhydride in each case. None of the products showed a change in spectrum or R_F value after treatment with hydroxylamine. Porphyrin-free acids produced by Pronase digestion appeared to be unstable and could not be detected on the reverse-phase plates. The positions of absorption maxima for selected porphyrin products are shown in Table 3. All spectra were of the aetio type (band IV > III > II > I). The spectrum of LPO haem in diethyl ether was indistinguishable from that of protohaem, except for the position of the major Soret band, which was 383 nm for LPO haem and 380 nm for protohaem.

Mass spectrometry

Porphyrin mass spectra were determined by field desorption. The positions of the major high-mass peaks are shown in Table 4. It is significant that the mass of the major peak for the less-polar components is that of protoporphyrin plus sulphur, or sulphur plus CH_2CONH_2 (iodoacetamide derivative).

N.m.r. spectra

After separation of porphyrin products by preparative reverse-phase t.l.c., yields were too low to give useful n.m.r. spectra. The porphyrins were quite insoluble in chloroform, methanol or dimethyl sulphoxide. Although they could be dissolved in pyridine, the lengthy runs required in this solvent gave altered products with evidence of rhodo-type visible spectra. It was therefore

Table 4. Position of highest-mass peak for field-desorption mass spectra of porphyrins from LPO

Porphyrin	Mass (m/z)
Pronase-treated-LPO porphyrin	666
Mercaptoethanol-treated-LPO porphyrins	614, 594
Iodoacetamide-alkylated-LPO porphyrin	652

decided to examine the spectra of diamagnetic iron(II) complexes derived from the haem.

The spectra of the cyanohaemochromes were obtained by using 0.1 M- NaO^2H in $^2\text{H}_2\text{O}$ as solvent, which was 0.025 M with respect to KCN. Reduction to the iron(II) state was achieved by using a few crystals of sodium dithionite and spectra were recorded under N_2 .

Pyridine haemochrome spectra were obtained by dissolving the haem in (^2H)pyridine and reducing to the iron(II) state under N_2 by adding ascorbic acid to a concentration of 0.015 M. Peaks due to ascorbate and dehydroascorbate were assigned on the basis of the spectra of these compounds in pyridine. The haem concentrations for all spectra were in the range 0.005–0.01 M.

The spectra of the product of simple reductive cleavage of the enzyme showed broad peaks in the region 2–10 p.p.m., which were not suitable for structural assignment. The spectra of the alkylated derivative (iodoacetamide product), on the other hand, gave well-resolved spectra. The positions of peaks and structural assignments for this haem derivative were as follows: δ (p.p.m.) ($^2\text{H}_2\text{O}$ /KCN after dithionite reduction) 2.81 (3H, s, ring CH_3), 2.83 (4H, m, $2 \times \text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 3.17 (3H, s, ring CH_3), 3.23 (3H, s, ring CH_3), 3.62 (2H, t, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 3.88 (4H, m, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$, $\text{SCH}_2\text{CONH}_2$), 5.72 (2H, m), 6.08 (2H, m) ($2 \times \text{CH}=\text{CH}_2$), 8.01 (2H, m, $2 \times \text{CH}=\text{CH}_2$), 9.12 (1H, s), 9.20 (1H), 9.25 (1H, s), 9.35 (1H, s) ($4 \times$ methine protons). The region 4.55–5.0 was obscured by a water peak.

In pyridine, after ascorbate reduction, the positions of peaks were as follows: δ (p.p.m.) [(^2H)pyridine] 3.32 (2H, t), 3.30 (2H, t) ($2 \times \text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 3.35 (3H, s), 3.50 (3H, s), 3.59 (3H, s) (ring methyl groups), 3.73 (2H, t, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 4.18 (2H, $\text{SCH}_2\text{CONH}_2$), 4.20 (2H, t, $\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$), 5.49 (2H, s, $\text{CH}_2\text{SCH}_2\text{CONH}_2$), 5.91

Table 3. Absorption maxima (λ_{max}) of porphyrin products from LPO (all are non-esterified)

Product	λ_{max} (nm)	
	Base in methanol	Cation in 3 M-HCl
Protoporphyrin	402,504,537,575,629	408,554,598
Mercaptoethanol/urea-treated porphyrin		
High R_F	406,508,543,576,630	412,561,605
Low R_F	407,508,546,576,632	414,562,608
Iodoacetamide-treated porphyrin		
High R_F	405,507,545,575,628	412,561,605
Low R_F	406,507,545,575,630	414,562,607
Pronase-treated porphyrin	406,506,541,577,634	409,559,604

(2H, m), 6.03 (2H, m) ($2 \times \text{CH}=\text{CH}_2$), 8.44 (1H, m), 8.51 (1H, m) ($2 \times \text{CH}=\text{CH}_2$), 10.18 (1H, s), 10.27 (1H, s), 10.37 (1H, s), 10.45 (1H, s) ($4 \times$ methine protons).

Assignments are based on the spectra of similar compounds (Janson & Katz, 1979).

DISCUSSION

The procedure used in the present work for the preparation of LPO haem and its derivatives gives higher yields than any other published method. It further suggests that the prosthetic group is bound to the enzyme by a disulphide linkage which, when broken, gives a haem thiol. This is confirmed by the formation of an alkylated derivative when reductive cleavage is carried out in the presence of iodoacetamide. M.s. shows that the porphyrins derived from these two products have M_r values equal to that of protoporphyrin plus sulphur and that alkylation, presumably of a thiol group, occurs in the presence of iodoacetamide. Satisfactory n.m.r. spectra of the haem and porphyrins derived from simple reductive cleavage were not obtained. Taking into account the solubility properties of these products, it is likely that dimerization or polymerization has occurred, either by formation of disulphide bridges, or by bonding of sulphur to iron, or both. In addition, evidence was seen in some spectra of the presence of hydroxyethyl groups. This probably accounts for the formation of two or more products on removal of iron from all of the haem products. Hydration of vinyl functional groups was also seen in control experiments where myoglobin was treated by using exactly the same cleavage conditions as for LPO. The products of these experiments were protoporphyrin, haematoporphyrin and 3(8)-(1'-hydroxyethyl)-8(3)-vinyldeuteroporphyrin. No other products were seen in these experiments, indicating that the porphyrin thiols from LPO are not artefacts.

By using the iodoacetamide-alkylated haem, well-resolved n.m.r. spectra were obtained for both the diamagnetic cyanohaemochrome and the pyridine haemochrome. The spectra show the presence of only three ring methyl groups and that the two propionic acid groups attached to the porphyrin ring are non-equivalent. Owing to the presence of a large water peak, which could not be completely suppressed in the spectrum of the cyanohaemochrome, a peak due to the presence of a ring methylene attached to sulphur could not be seen. This peak was, however, clearly seen in the spectrum of the pyridine haemochrome at δ 5.49 p.p.m. On this basis, the structure **1a** (Fig. 1) is proposed for LPO haem. Although it is not possible to make a definite assignment of the mercaptomethyl group to ring D rather than ring C, the former is suggested on the basis of analogy with haem a. In this compound the methyl group of ring D has been oxidized to a formyl function (Grassl *et al.*, 1963).

The structure provides a basis for the unusually strong binding of the LPO prosthetic group to its apoprotein. The absence of amino acid residues in the products indicates that this is due solely to disulphide-bond

formation to the protein. It is also consistent with resonance-Raman studies on LPO, which indicate the presence of two vinyl substituents (Manthey *et al.*, 1986). As these two substituents are the major influence on the visible spectrum of LPO haem and its derivatives, the spectra of the porphyrins derived from LPO under mild conditions are very similar to that of protoporphyrin, although small red shifts, especially in the position of band III, are seen.

It is now clear that the unusual visible spectrum of LPO is largely due to the nature of the axial ligands binding to iron in LPO haem rather than to the nature of LPO haem itself. The possible presence of positively charged groups in the haem environment which interact with axial ligands may be of relevance (Behere *et al.*, 1985).

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