Isolation of the haemopexin-haem receptor from pig liver cells

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Isolated pig liver plasma membranes interact specifically with the haemopexin-haem complex (K_d 4.4×10^{-7} M). Affinity chromatography was used to isolate a membrane component which binds this complex with high affinity. Pig serum haemopexin was first isolated by affinity chromatography on haemin-Sepharose followed by HPLC gel filtration. Liver membranes solubilized with Triton X-100 were incubated with haemin-Sepharose saturated with haemopexin, and as a control, with affinity gel lacking haemopexin. SDS-polyacrylamide gel electrophoresis of the eluted protein indicated that from the haemin-Sepharose emerglow-molecular-mass haemin-binding proteins whereas the eluate from haemopexin-haemin-Sepharose contained an additional 71 kDa protein, which did not bind free haemin. This protein appears to represent the haemopexin-haem receptor or a part of it. Haem from the haemopexin complex, as also free haemin, was accepted by a binder in the plasma membrane, which in gel filtration behaved like an 80 kDa molecule. This component probably represents a second functional subunit of the haemopexin-haem receptor.

Hemopexin receptor *Hemopexin isolation* *Heme* *Affinity chromatography* *(Pig liver membrane)* *Heme iron*

1. INTRODUCTION

In vivo and in vitro studies have shown that a receptor on hepatocytes adsorbs the haemopexin-haem complex [1,2]. Only haem enters the parenchymal cells and the liberated haemopexin returns to the circulation intact. A specific binding site for haemoglobin-haptoglobin is also located on parenchymal cells, and offers a second possibility for haem to enter the cells [3]. A third mechanism for haem uptake is perhaps provided by the asialoglycoprotein receptor, which binds the asialohaemopexin-haemin complex [4].

Apo-haemopexin does not compete with the holo-protein for the binding to the receptor; thus the receptor can distinguish between the apo- and holo-proteins. A conformational change in the haemopexin molecule [1] after haem binding is probably necessary to form its receptor-binding site. However, the asialoglycoprotein receptor binds both asialohaemopexin and its haem complex in addition to other asialoglycoproteins.

According to Smith and Morgan [5], isolated rabbit liver plasma membranes interact with haemopexin-haem and remove haem from the complex which accumulates in a haem-binding membrane component (HBC). It has a molecular mass of 115 kDa, a Stokes radius of 7.5 nm according to gel filtration, and an acidic isoelectric point. Similar haem-binding proteins (or haem receptors) have also been found in pig duodenal brush border membranes [6], milk fat globules [7] and murine erythroleukemia cells [8].

Here, we used affinity chromatography to isolate the haemopexin-haem receptor from pig liver plasma membranes. In the affinity medium haemin was covalently bound to the matrix by a hydrophilic spacer preventing the release of haem by a releasing factor or haem acceptor in the plasma membrane.

Abbreviations: HSA, human serum albumin; TMBZ, 3,3′,5,5′-tetramethylbenzidine; haem, iron protoporphyrin IX

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2. MATERIALS AND METHODS

The haemin-Sepharose affinity gel was synthesized as described [9]. Ethylenediamine was first coupled to epoxi-activated Sepharose 6B (Pharmacia) and then haemin to the amino group by a carbodiimide reaction. The haem content of the wet gel was of the order of 10 mg/ml and its binding capacity for pig haemopexin 40 mg/ml. Haemopexin was isolated from defibrinated blood by affinity chromatography. 100 ml pig serum was diluted twice with 5 mM Tris-HCl buffer (pH 7.4), containing 0.05% Triton X-100 (buffer A) and then incubated as a batch with 10 ml haemin-Sepharose for 6–12 h. The gel was packed into a small column and then washed with several litres of buffer A until the background absorbance became zero. The column was then washed with 0.1 M phosphate buffer (pH 7.0, 100 ml), and the adsorbed proteins eluted with 0.1 M citrate buffer, pH 2.0. The eluted protein, mainly haemopexin, was immediately neutralised with phosphate buffer and stored at −20°C.

Pig liver was obtained from a slaughterhouse. Immediately after excision 100 g liver was cut into small pieces with scissors in ice-cold Hank's solution (Orion, Helsinki). The medium was changed twice to eliminate blood. The pieces were homogenized with an Omni mixer (Sorvall) in 1 mM $NaHCO_3$, 0.5 mM $CaCl_2$ buffer (pH 7.5), containing 0.01% phenylmethylsulphonyl fluoride (PMSF) (Sigma) as protease inhibitor. The homogenate was diluted with the buffer to 8 l and filtered through straining silk. Plasma membranes were collected by centrifugation at 1800 × *g* and 4°C for 30 min, and washed twice by resuspending the pellet in bicarbonate-$CaCl_2$ buffer followed by centrifugation [11].

Haemopexin-haemin-Sepharose gel was prepared by binding pure haemopexin to haemin-Sepharose. The frozen haemopexin solution was thawed, sterilised by filtration and then fractionated using an HPLC column (TSK G3000 SW). The fractions corresponding to the haemopexin monomer peak were pooled and incubated with haemin-Sepharose until the gel was saturated. Unbound protein was removed by washing the gel with buffer A containing 0.02% NaN_3 and 0.01% PMSF. During storage of haemopexin at 4°C a smaller molecule (about 30 kDa) began to appear. According to the literature it is produced by the action of plasmin [10]. It was purified by HPLC gel filtration and used in some experiments.

For uptake studies the membrane pellet was suspended in RPMI 1640 medium (Difco), containing 0.01% PMSF. The membranes were incubated at 37°C with different concentrations of haem-saturated haemopexin and then collected by centrifugation at 3000 × *g* for 30 min. The haem content of the supernatants was assayed with TMBZ (see below) using as standards haemin or haemin-haemopexin. The membrane-bound fraction of haemin was calculated and used for Scatchard analysis.

For isolation of the haemin-haemopexin receptor plasma membranes were solubilized with Triton X-100. The membrane pellet was suspended in water containing PMSF (1% suspension), an equal volume of 1% Triton X-100 in buffer A added and the mixture sonicated twice for 5 s with a 150 W ultrasonic disintegrator (MSE) and then incubated for 2 h in an ice bath. The unsolubilized fragments were removed by centrifugation at 100000 × *g* for 1 h, and the extract then diluted 1:10 with buffer A containing PMSF and NaN_3 and incubated with haemopexin-haemin-Sepharose overnight at 4°C in a slowly rotating mixer. The gel was packed into a small column and washed by running buffer A through the column until the absorbance at 280 nm became zero. As a control, a part of the extract was incubated with haemin-Sepharose gel to which no haemopexin had been bound. Both columns were eluted at 50°C with 1% SDS in Laemmli sample buffer [12].

SDS electrophoresis [12] was performed in 12 or 7.5% polyacrylamide gels using as standards with known molecular masses ovalbumin, human serum albumin, transferrin and haemoglobin. The gels were stained for protein with a silver reagent [13] and with TMBZ-H_2O_2 [14]. Before electrophoresis, the samples were incubated with 1 μM haemin. Usually the same gel was first stained with TMBZ and then, after washing with 30% isopropanol and 50% methanol, with silver.

Gel filtration through a Sephacryl S-300 column was used to demonstrate the transfer of haem from haemopexin to the haem binder of the liver plasma membrane extract. The running buffer was 50 mM Tris-HCl, 0.15 M NaCl (pH 7.4) containing 0.05%

Triton X-100, 0.01% NaN_3 and 0.01% PMSF.

For every measurement of haemin in solutions, a fresh solution of 50 mg TMBZ (Merck) in 33 ml methanol was prepared; just before use it was mixed with 77 ml of 0.25 M Na acetate buffer (pH 5.0) to produce the colour reagent. 1 ml of the standards (haemin or haemopexin-haem) and samples, respectively, were mixed with 1 ml colour reagent and incubated for 15 min at room temperature. 5 μl H_2O_2 (Merck) was added to the test tubes and the absorbance at 650 nm read after 30 min.

Haemin (Sigma, III) was dissolved in 0.1 M NaOH and then diluted with 0.1 M phosphate buffer to the concentration required and filtered through a 0.22 μm Durapor filter (Millipore). Protein was measured by the method of Lowry et al. [15], using human serum albumin (Kabi) as a standard.

5'-Nucleotidase, as a marker enzyme for plasma membranes, and glucose-6-phosphatase for microsomal membranes, were assayed according to Aronson and Tauster [16].

3. RESULTS AND DISCUSSION

3.1. *Uptake studies*

The plasma membranes isolated from liver cells interacted with the haemopexin-haem complex either by binding the complex or detaching haem from haemopexin. As shown in fig.1, the results

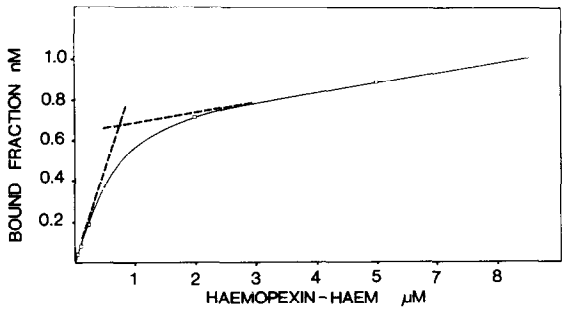

Fig.1. Binding of the haemopexin-haem complex to liver cell membranes was assayed in RPMI 1640 culture medium at 37°C and pH 7.4. After incubation for 30 min the membranes were spun down and the unbound complex assayed from the supernatant by the TMBZ-H_2O_2 method. The results indicate both saturable high-affinity binding (K_d 4.4 × 10^{-7} M according to a Scatchard plot) and unsaturable low-affinity binding.

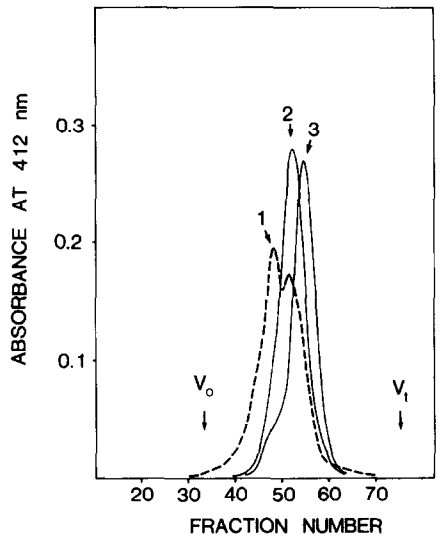

Fig.2. Gel filtration profiles in a Sephacryl S300 superfine column (1.6 × 90 cm). Buffer B, flow rate 12 ml/h, absorbance of 2.4 ml fractions detected at 412 nm. (1) Liver plasma membrane extract incubated with porcine haemopexin-haemin complex, (2) haemopexin saturated with haem, (3) plasmin-degraded haemopexin saturated with haem. V_0, totally excluded volume; V_t, volume of the column.

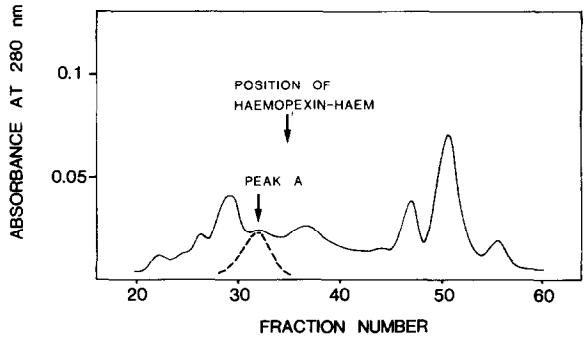

Fig.3. HPLC gel filtration in a TSK G3000 SW column of the Triton X-100-solubilized liver plasma membrane fraction incubated with haemin (1 μM). Detection at: 280 nm (——), 412 nm (– – –). Haem was eluted in the position of protein peak A, which had a smaller elution volume than the haemopexin-haem complex (65 kDa). Free haem was adsorbed by the silica matrix of the precolumn, thus the haem in the position of peak A was protein-bound. Haem could be washed from the precolumn with 20% pyridine.

indicate two different kinds of interaction, one having high affinity (K_d = 4.4 × 10^{-7} M) as expected for a receptor-mediated mechanism. We did not study whether the whole complex was bound to the membranes or only haemin. The incubation medium (RPMI 1640) contained 0.01% PMSF, thus proteolytic liberation of haem from the complex was unlikely. Solubilised membranes were incubated with free or bound haemin and subjected to gel filtration through Sephacryl S-300 and TSK G3000 SW (figs 2,3). A new haem-containing peak appeared, but its position always corresponded to 80 kDa irrespective of whether haemin, haemopexin-haem or a plasmin-degraded haemopexin were used as haem donor. Thus only haem was bound to the membrane component.

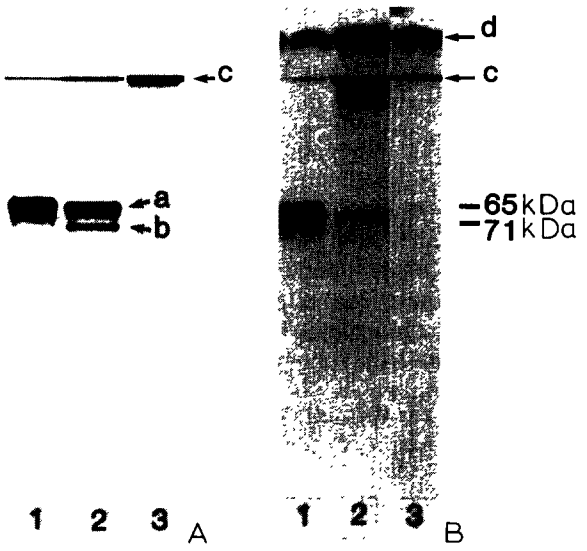

Fig.4. Polyacrylamide (7.5%) SDS gel electrophoresis according to Laemmli [12]. (A) Silver staining for proteins, (B) TMBZ staining for haem. Before the SDS run, samples were incubated with 1 μM haemin for 1 h at room temperature. (1) Porcine haemopexin, (2) eluate from the haemopexin-haemin-Sepharose column (used to absorb the liver plasma membrane extract), (3) corresponding eluate from the haemin-Sepharose column (control). The gel was first stained with TMBZ, and then the bands destained by washing with 50% methanol and finally stained with silver ammonium complex. The TMBZ stain did not interfere with the silver staining. (a) Haemopexin, (b) receptor for haemopexin-haem complex, (c) low-molecular-mass haem-binding protein of liver plasma membranes, (d) free haem. Component b was not seen after haem staining.

3.2. *Isolation of the haemopexin-haem receptor*

Plasma membranes were prepared from 100 g pig liver, the yield being 265 mg, or about 20% based on 5′-nucleotidase activity. Glucose-6-phosphatase activity was below 5% of the starting activity. The membranes were solubilized with Triton X-100 and one part of the extract incubated with haemin-Sepharose and another with haemopexin-saturated haemin-Sepharose. Fig.4 presents the results of the purification. Haemin- Sepharose adsorbed only low-molecular-mass proteins, which in SDS electrophoresis had a mobility similar to that of haemoglobin (16 kDa). In 7.5% polyacrylamide gel they moved as one band (band c) but in 12% polyacrylamide several bands appeared (not shown). If the sample was incubated with haemin before the SDS run these low-molecular-mass proteins (band c) also stained with TMBZ, indicating the existence of a haemin-binding site in them and explaining their affinity for haemin-Sepharose.

Haemopexin-saturated haemin-Sepharose adsorbed from the plasma membrane extract a protein which had a somewhat higher molecular mass than haemopexin (fig.4, band b). Using the standard proteins HSA (67 kDa) and ovalbumin (43 kDa) its molecular mass was estimated to be 71 kDa. In contrast to band c it did not stain with TMBZ. The other protein (band a) was identified

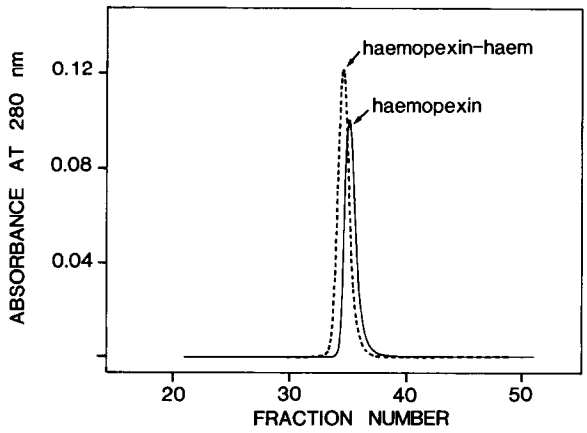

Fig.5. Two gel filtration runs using a TSK G3000 SW HPLC column, 0.1 M phosphate, 0.15 M NaCl buffer (pH 7.2). Pig apo-haemopexin and the haemopexin-haem complex emerged from the column in different positions. The amount of protein was the same in both runs but the specific adsorptivity of the complex was higher at 280 nm.

as haemopexin (dissociated by warm SDS-solution from haemin-Sepharose) considering its molecular mass and haem-binding capacity. In addition to bands a and b, the haemopexin-haemin-Sepharose eluate contained low-molecular-mass haem-binding proteins (band c) but less than the haemin-Sepharose eluate.

Among the known haem-binding proteins haemopexin has the highest affinity for haem, the K_d being about 10^{-13} M [17]. After the binding of haem it has been observed to undergo a conformational change [18], which probably results in the creation of its receptor-binding site. We noticed different elution volumes for the apo- and holo-proteins in HPLC runs through the TSK G3000 SW column (fig.5), apparently reflecting a change from a globular to a more rod-shaped form.

According to Smith and Morgan [5] the binding constant for the rabbit liver HBC is of the order of 10^{-7} M, and our results indicate a similar magnitude for the pig liver HBC. The binding to the receptor probably affects the conformation of haemopexin reducing its affinity for haem. The liberation of haem from the haemopexin-receptor complex changes the conformation of haemopexin back to the apo form, its affinity for the receptor decreases, and apo-haemopexin is liberated into the circulation. This explains why the half-life of the haemopexin molecule in the circulation (7–8 h) is much longer than for most plasma proteins [19].

In contrast to the view of Smith and Morgan [5], we are inclined to believe that the haem-binding component of the receptor or haemin acceptor of the membrane is a relatively small molecule, as is the haemin binder of hog duodenal brush border membranes [6]. Being a hydrophobic protein, in Triton solutions it forms complexes with itself or other membrane components.

We estimated the molecular size of the hog haemopexin isolated by affinity chromatography (and also rabbit haemopexin, unpublished) to be 65 kDa which is considerably higher than the 60 kDa reported by Smith and Morgan [10] but in accordance with the results of Tsutsui and Mueller [20].

We interpret our present findings as indicating that the receptor for the haem-haemopexin complex consists of a non-haem-binding 71 kDa molecule closely associated with a smaller haem-binding molecule which is also able to accept haem from other sources and which perhaps occurs alone in other tissues such as intestine.

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