In vitro studies on some parameters of the binding of the rat hemopexin–heme complex with the hepatic membrane reeptor

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The binding of [^{125}I]Hpx–heme with the rat hepatic plasma membrane receptor was studied at 37°C as well as different parameters such as plasma membrane concentration, calcium dependence, optimal pH and specific binding. A Scatchard plot revealed the existence of one binding for [^{125}I]Hpx–heme on the isolated liver plasma membrane with a $K_d = 3.2 \times 10^{-8}$ M.

Hemopexin–heme complex *Binding* *Hepatic membrane receptor*

1. INTRODUCTION

Hemopexin (Hpx) is a plasma β-glycoprotein which binds heme with high affinity and is considered to be a transport protein carrying heme to the liver [1] in the same way as haptoglobin (Hp) is a transport protein for hemoglobin [2]. In the case of severe hemolysis, when the hemoglobin level is higher than the binding capacity of haptoglobin, it dissociates into globin and heme and the latter is immediately bound by hemopexin [3]. In vivo, as reported by Smith and Morgan, the hemopexin–heme complex (Hpx–Heme) interacts with the liver by a receptor-mediated process and hemopexin returns intact to the circulation after delivering its ligand to the hepatocyte [4]. Heme is then catabolized and the iron rapidly incorporated into hepatic ferritin [5]. Additional evidence for the existence of a receptor has been obtained using isolated hepatocytes [6].

This work, using isolated rat liver plasma membrane and [^{125}I]Hpx–Heme, extends our studies on rat hemopexin [7,8] and on the uptake of the asialohemopexin–heme complex by isolated rat hepatocytes [9]. To characterize the specific receptor of the Hpx–Heme, it was important to define the parameters necessary for an optimal binding in vitro of the [^{125}I]Hpx–Heme first.

2. MATERIALS AND METHODS

Rat Hpx and rat Hp were prepared from the plasma of male rats (Wistar, 400–450 g) injected with turpentine as in [10,11].

Labeled [^{125}I]Hpx was prepared as in [12] with minor modifications. The reaction mixture, containing 1 mg Hpx, 1 mCi ^{125}I (CEA, Saclay) and 10 μg fresh chloramine-T (Sigma) in 0.2 ml 50 mM sodium phosphate buffer (pH 7.4) was stirred for 1 min. The reaction was stopped by the addition of 0.1 ml sodium metabisulfite (Sigma) 2.5 mg/ml (w/v) in 50 mM sodium phosphate buffer (pH 7.4) and 0.2 ml KI (Prolabo) 0.2 mg/ml (w/v). Separation of labeled [^{125}I]Hpx was carried out by gel filtration using a 6 g Sephadex G-25 column (Pharmacia), 50 mM Tris–HCl buffer (pH 7) was utilised for the equilibration of the gel and for elution. Before using the Sephadex column, 1 ml human plasma was passed through it and the column was washed with 500 ml Tris–HCl buffer. The specific activity of labeled [^{125}I]Hpx was 400 ± 40 μCi/mg.

The hemopexin–heme complex (Hpx–Heme) was prepared as follows: hemin (Sigma) was dissolved in a small amount of 0.1 N NaOH and then adjusted to pH 7 with 0.1 M phosphate buffer. Its concentration was measured in the hydroxyl form at 385 nm using a molar absorption coeffi-

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cient of $\epsilon = 5 \times 10^4\ M^{-1}.cm^{-1}$. The Hpx–Heme was obtained by adding an equimolar amount of heme to Hpx.

The haptoglobin–hemoglobin complex (Hp–Hb) was obtained by adding horse hemoglobin to rat haptoglobin. Separation of Hp–Hb from the reaction mixture was carried out by gel filtration using an AcA 4 (LKB) column equilibrated with 50 mM Tris–HCl buffer (pH 7) (2.5 × 100 cm). Elution of the Hp–Hb complex with the same buffer was followed at 420 nm.

Removal of sialic acid from fetuin (Sigma) was performed using formic acid 1 N at 80°C for 60 min. The reaction mixture was dialysed 4 times against 0.1 M Tris–HCl buffer (pH 7).

Removal of sialic acid from haptoglobin was performed using immobilized neuraminidase (Sigma) at 37°C for 24 h in 0.1 M citrate buffer (pH 5.3).

Rat liver plasma membrane was isolated from male Wistar rats (250–300 g body wt) by the modification [13] of the method in [14]. Before plasma membrane isolation, liver was perfused with Ca^{2+}-free Hank's solution containing 5 mM EGTA. Isolated plasma membranes were suspended in 0.1 M Tris–HCl buffer (pH 7) and the concentration determined as in [15]. The purity of the plasma membrane preparation was examined by the enzyme markers: 5′-mononucleotidase, Mg^{2+}-stimulated ATPase and $(Na^+ + K^+)$-activated ATPase by the method in [13] and the P_i liberated was assayed as in [16]. Glucose 6-phosphatase was assayed as in [17] using 0.1 M sodium acetate buffer pH 6.1 at 30°C for 15 min and glucose 6-phosphate as substrate. Succinate dehydrogenase was assayed by the method in [18].

The assay for the binding of [^{125}I]Hpx–Heme to plasma membrane was done in triplicate in test tubes (13 × 75 mm). [^{125}I]Hpx–Heme and plasma membrane were incubated in 0.1 M Tris–HCl buffer (pH 7), BSA 1%, $CaCl_2$ 20 mM at 37°C for 60 min. The control measurements were made at 4°C. At the end of the incubation, 2 ml 0.1 M Tris–HCl buffer containing BSA 0.1%, KCl 0.15 M and polyethylene glycol (PEG) 4% (w/v) were added. The reaction mixtures were centrifuged at 1500 × g for 20 min. The supernatants were discarded and the pellets were suspended and washed 3 times with 3 ml of the same washing buffer. The final pellets were counted in a γ-counter (Packard, Autogamma 3002). Amounts of [^{125}I]Hpx–Heme bound to plasma membrane were obtained after subtracting the control values.

3. RESULTS AND DISCUSSION

3.1. *Purity of rat liver plasma membranes*

Purified plasma membranes were prepared by a two-phase aqueous (Dextran 500; polyethylene glycol 6000) polymer system as in section 2. The purity of the plasma membrane preparations may be evaluated by the measurement of marker enzymes shown to be characteristic of subcellular components; 5′-mononucleotidase, $(Na^+ + K^+)$-activated ATPase and Mg^{2+}-stimulated ATPase activities were chosen as markers for plasma mem-

Table 1

Purity of rat liver plasma membrane

Marker enzyme activity	Method of plasma membrane preparation		
	Sucrose density method [13]	Two phase system [13]	Two phase system [Here]
Mg^{2+}-Stimulated ATPase	20.49 ± 3.32	20.56 ± 2.62	18.83 ± 3.16
$(Na^+ + K^+)$-Activated ATPase	2.62 ± 0.23	2.53 ± 0.24	2.48 ± 0.26
5′-Mononucleotidase	29.81 ± 3.90	28.38 ± 3.90	30.02 ± 4.10
Glucose 6-phosphatase	0.62	0.64 ± 0.05	0.50 ± 0.05
Succinate dehydrogenase	0.011 ± 0.001	0.013 ± 0.003	0.008 ± 0.003

Enzyme activities were determined as in section 2 and expressed as μmol formazan produced.mg protein^{-1}.min^{-1} for succinate dehydrogenase and as μmol phosphate released.mg protein^{-1}.30 min^{-1} for the other enzymes. The results are given as means ± SE

brane. The degree of contamination by endoplasmic reticulum was determined by the measurement of glucose 6-phosphatase and mitochondrial contamination was determined by the measurement of succinate dehydrogenase (section 2). Results from these experiments are shown in table 1. Present values agree with those in [13].

3.2. *Effect of plasma membrane concentration on the binding of Hpx–Heme*

Examination of the stoichiometric and kinetic properties of the system revealed that, in the presence of a constant and excess amount of [^{125}I]Hpx–Heme (3 μg), binding was a linear function of plasma membrane concentration over 0–120 μg plasma membrane (fig.1).

The following assays were carried out with 3 μg [^{125}I]Hpx–Heme and 90 μg plasma membrane.

3.3. *Effect of calcium on the binding of Hpx–Heme to plasma membrane*

Fig.2 shows that even in the absence of Ca^{2+} binding of Hpx–Heme to the plasma membrane is possible. But an increase in [$CaCl_2$] (5–50 mM) considerably raises the degree of binding until an equilibrium is reached.

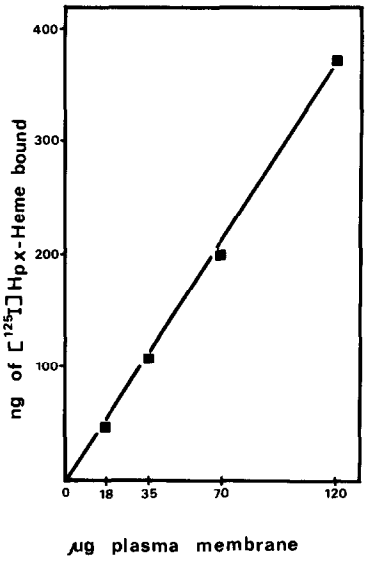

Fig.1. Effect of plasma membrane concentration on the binding of Hpx–Heme. Each tube contained 2.5 μg [^{125}I]Hpx–Heme, BSA 1%, 20 mM $CaCl_2$, 0.1 M Tris–HCl buffer (pH 7) and variable amounts of plasma membrane in 0.3 ml final vol. Incubation at 37°C was stopped after 60 min and tube contents were treated as in section 2. Values are means of 3 expt.

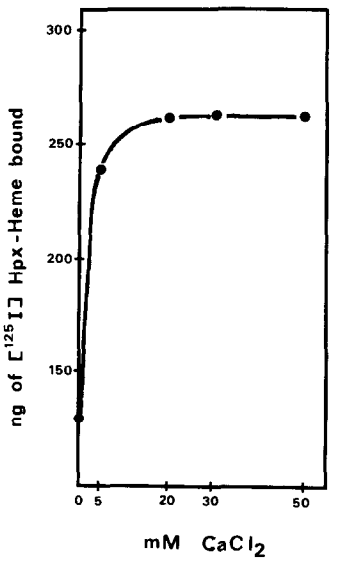

Fig.2. Effect of calcium on the binding of Hpx–Heme to plasma membrane. Each tube contained 80 μg plasma membrane protein, 2.8 μg [^{125}I]Hpx–Heme, 0.1 M Tris–HCl buffer (pH 7), BSA 1% and variable amounts of $CaCl_2$ in 0.3 ml final vol. Incubation at 37°C was stopped after 60 min and tube contents were treated as in section 2. Values are means of 3 expt.

3.4. *Optimum pH for the binding of Hpx–Heme to plasma membrane*

The effect of alterations of pH on the stability of the Hpx–Heme plasma membrane binding was examined. A preliminary investigation showed that binding was optimal between pH 6.5–7.5 with 0.1 M Tris–HCl buffer. After incubation of [^{125}I]Hpx–Heme with plasma membrane at pH 7 for 30 min, the pH was adjusted to the values shown in fig.3 and reincubated for an additional 30 min. The binding was optimal between pH 6.8–7.2. Below and above these values the complex was unstable.

3.5. *Effect of temperature on the kinetics of binding of Hpx–Heme to plasma membrane*

The time course of binding of Hpx–Heme to plasma membrane was examined at different temperatures. Fig.4 shows that binding of Hpx–Heme on plasma membrane does not occur at 4°C, it is low at 20°C and much more important

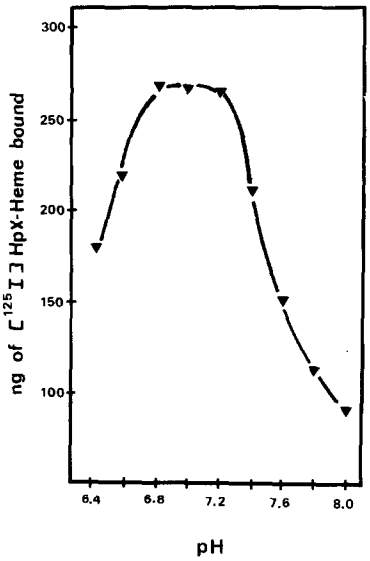

Fig.3. Optimum pH for the binding of Hpx–Heme to plasma membrane. The reaction mixture, containing 90 μg plasma membrane protein, 3 μg [^{125}I]Hpx–Heme, 0.1 M Tris–HCl buffer (pH 7), BSA 1%, 20 mM $CaCl_2$ per 0.3 ml solution, was incubated at 37°C for 30 min. Aliquots were then removed and adjusted to pH 6.4–8.0 by the addition of 0.5 M Tris–HCl buffer solutions. The incubation was continued for an additional 30 min at 37°C prior to being stopped and tube contents were treated as in section 2. Values are means of 3 expt.

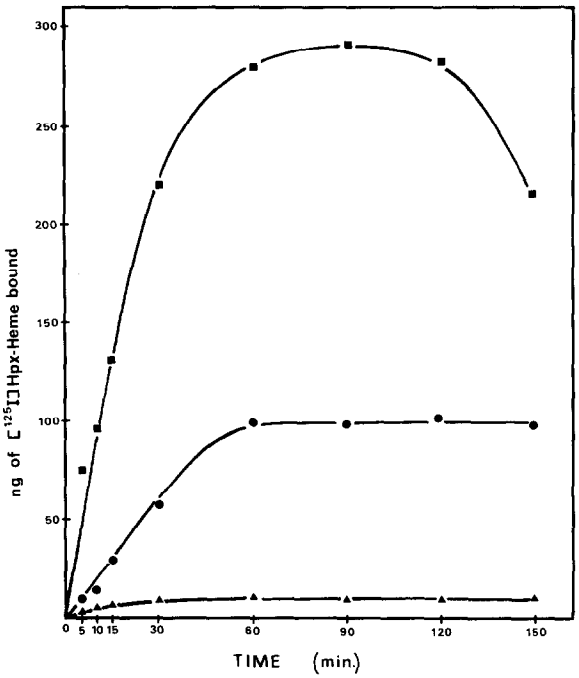

Fig.4. Effect of temperature on the kinetics of binding of Hpx–Heme to plasma membrane. The reaction mixture, containing 90 μg plasma membrane protein, 3 μg [^{125}I]Hpx–Heme, BSA 1%, 20 mM $CaCl_2$, 0.1 M Tris–HCl buffer (pH 7) per 0.3 ml solution, was incubated at different temperatures: 4°C (▲—▲), 20°C (●—●) and 37°C (■—■). At 5, 10, 15, 30, 60, 90, 120 and 150 min, 3 aliquots of each incubation were removed and treated as in section 2. Control values were performed at 4°C. Values are means of 3 expt.

at 37°C. At this temperature there is a time dependent increase: the maximum is reached at 60 min. However the Hpx–Heme–plasma membrane complex degrades after 120 min. Thus assays were carried out at 37°C for 60 min.

3.6. *Specific binding of Hpx–Heme to plasma membrane*

[^{125}I]Hpx–Heme was incubated in competition with following proteins: fetuin, asialofetuin, haptoglobin, asialohaptoglobin, Hp–Hb, Hpx and unlabeled Hpx–Heme for the determination of specific binding to rat liver plasma membrane.

Table 2 shows that haptoglobin, fetuin and Hpx do not compete with the binding of [^{125}I]Hpx–Heme by the plasma membrane.

Asialofetuin and asialohaptoglobin which are bound by the asialoglycoprotein receptor [19,20] and Hp–Hb which is bound by the Hp–Hb receptor [21,22], compete very little (15%).

The data could be explained by a steric hindrance mechanism.

On the contrary, an increased concentration of unlabeled Hpx–Heme (30–100 μg) gives a high competitive inhibition of binding. These data argue for the existence of a specific receptor for the Hpx–Heme complex.

3.7. *Affinity of binding*

To determine the affinity constant of the Hpx–Heme specific receptor binding, the plasma membrane was incubated with increasing amounts of [^{125}I]Hpx–Heme (fig.5A). The binding data were analyzed according to the graphical method of Scatchard [23]. This plot shows one class of binding sites (fig.5B). The K_d obtained using a linear regression analysis (correlation coefficient r =

Table 2

Specific binding of [^{125}I]Hpx–Heme (3 μg) to plasma membrane (90 μg protein)

Inhibitors (μg)		Radio-activity (%) recovered in membrane
None		100
Unlabeled Hpx–Heme	(30)	49
Unlabeled Hpx–Heme	(70)	30
Unlabeled Hpx–Heme	(100)	14
Asialofetuin	(100)	80
Asialofetuin	(200)	81
Asialohaptoglobin	(30)	86
Hp–Hb	(100)	87
Haptoglobin	(100)	98
Hpx	(100)	96
Fetuin	(100)	101
Fetuin	(200)	102

The original incubation, containing 90 μg plasma membrane, 0.1 M Tris–HCl buffer (pH 7), BSA 1%, 20 mM $CaCl_2$ and the above proteins, was incubated at 37°C for 15 min; 3 μg [^{125}I]Hpx–Heme was added and incubations were continued for an additional 60 min prior to being stopped and tube contents were treated as in section 2 (values are means of 3 expt)

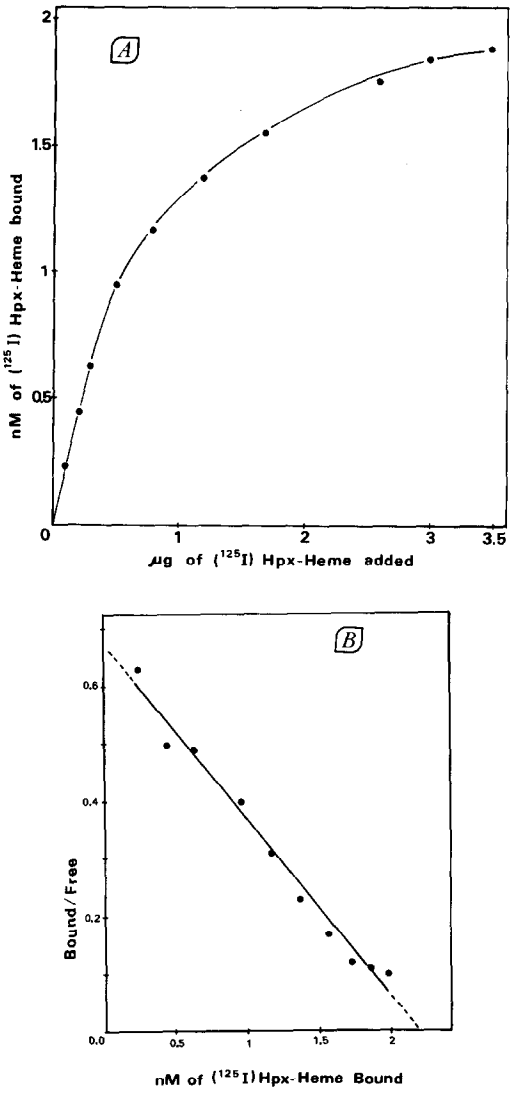

Fig.5. Scatchard plot of binding of [^{125}I]Hpx–Heme to liver plasma membranes. (A) Liver plasma membranes (90 μg) were incubated with various amounts of [^{125}I]Hpx–Heme (100–3500 μg) at 37°C for 60 min and tube contents were treated as in section 2. (B) The binding data from (A) were plotted according to Scatchard [23]. The K_d obtained was 3.2×10^{-8} M.

−0.988) was 3.2×10^{-8} M. This value is of the same order of magnitude as that estimated in [6] by competitive inhibition experiments and as that in [24] working on isolated hepatocytes.

This specific interaction represents a high affinity system comparable with hormonal systems such as insulin–hepatocytes [25].

Here, we have determined the experimental parameters for the uptake of the Hpx–Heme complex by its hepatic membrane receptor. We have shown this receptor to be functional in vitro.

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