Plasmin cleaves rabbit serum apohemopexin (M = 60,000) at a single site producing a heme-binding domain (I, M = 35,000) and a second domain (II, M = 25,000) (W. T. Morgan and A. Smith (1984) J. Biol. Chem. 259, 12001–12005). The absorbance spectra of heme-domain I are indicative of a bis-histidyl coordination complex with the central heme iron atom. Chemical modification of the 5 histidine residues of apo-domain I with diethylpyrocarbonate abolished heme binding, supporting this assignment. Upon binding heme, domain I migrates more rapidly in sucrose gradients, and, in sedimentation velocity experiments, the s value of domain I increases from 3.17 ± 0.04 to 3.71 ± 0.09, a notably large increase which indicates that the domain becomes much more compact. This conformational change which plays a pivotal role in heme transport requires the bis-histidyl coordination with the central heme iron atom and leads to a tighter association between domain I and domain II shown by the co-migration of heme-domain I and domain II in sucrose gradients. In turn, the association of heme-domain I with domain II increases the thermal stability of the heme-domain I chromophore. Results of binding studies using mouse hepatoma cells and isolated domains indicate that domain I not only binds heme but also plays a vital part in the heme-binding interaction. The change in conformation of domain I upon heme binding and the association between domains I and II induced by heme are both notable determinants of the strength of the heme-binding interaction, but an intact “hinge region” between the domains is not necessary for receptor binding. The importance of both domains in bringing about the transport function of hemopexin is confirmed by the ability of three (two specific for domain I and one for domain II) of seven monoclonal antibodies raised against hemopexin to inhibit the heme-binding interaction.

The transport of heme to the liver by hemopexin is mediated by a specific receptor in the plasma membrane of the liver parenchymal cells (1–3). Upon binding heme, hemopexin undergoes a change in conformation which was proposed to be important for increasing the affinity of the heme-hemopexin complex for the hemopexin receptor (4). A heme-linked conformational change is suggested both by an increase in ellipticity at 231 nm (5) and a sharpening of absorbance at 290 nm (6) as well as in an enhanced resistance to proteolysis (7). The interaction of heme-hemopexin with its receptor results in the removal of the heme from hemopexin and its transfer to an intrinsic heme-binding membrane protein which appears to act in intracellular heme transport (8). Two non-disulfide-linked functional domains of rabbit hemopexin have been isolated after limited proteolysis by plasmin (9). The larger, amino-terminal glycopeptide (domain I) is the heme-binding domain, and the smaller carboxyl-terminal glycopeptide (domain II) was proposed to play a part in augmenting the heme-receptor interaction (9). The two domains are linked by a “hinge” region which, like the human hemopexin sequence (10), is rich in histidine and glycine.2 Proteolytic cleavage of hemopexin in this region abolishes the heme-induced increase in ellipticity at 231 nm (9), and this hinge region is made resistant to proteolysis in heme-hemopexin due to a change in conformation not simply to steric hindrance (7, 9). The present study was undertaken to gain further information on heme-linked conformational changes in domain I and on the roles of both domains of hemopexin in its function.

**Materials and Methods**

Hemopexin was isolated from rabbit serum, and its purity (greater than 95%) was checked as previously described (9, 11). The two domains of hemopexin were obtained by ion exchange chromatography after digestion of apohemopexin with plasmin using published procedures (9). Proteins were labeled with 125I (ICN, Imine, CA) using IODO-BEADS (Pierce Chemical Co.) according to the manufacturer’s instructions, and unincorporated 125I was removed by passage over Sephadex G-25 (Sigma) or exhaustive dialysis.

Mesoheme (iron-mesoporphyrin IX) from Porphyrin Products (Logan, UT) was used in place of heme (iron-protoporphyrin IX) in this work since mesoheme is more stable than heme and mesoheme-hemopexin complexes are chemically and biologically equivalent to heme-hemopexin (1–3). Mesoporphyrin (Porphyrin Products) was labeled with 55Fe (Du Pont-New England Nuclear) by refluxing in dimethyfornamide and freed of unincorporated iron and metal-free porphyrin by washing with acid (12).

Complexes of hemopexin or of heme-binding domain with mesoheme were prepared by mixing 1 eq of tetrapyrrole with 1 eq of protein. Unbound tetrapyrrole was removed by passage over DEAE-

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1. The abbreviations used are: heme, iron-protoporphyrin IX; mesoheme, iron-mesoporphyrin IX; mAb, monoclonal antibody; PBS, phosphate-buffered saline, pH 7.4; DEP, diethylpyrocarbonate; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid.

2. F. Tatum, A. Smith, and W. T. Morgan, unpublished results.
cellulose (DE52, Whatman, Clifton, NJ) or by exhaustive dialysis. Concentrations were determined spectrophotometrically using extinction coefficients (mM"1 cm"1) of 1.1 × 104 at 280 nm for apohemopexin (13), of 1.3 × 104 at 405 nm for mesoheme-hemopexin and mesoheme-heme binding domain (9), and of 1.7 × 104 at 394 nm for mesoheme in diethylamine sulfobisulfite (14). Absorbance measurements were obtained in a Gilford 2600 spectrophotometer. Mesoheme-hemopexin is in the ferric state under aerobic conditions (15), and reduced spectra were obtained by adding a few crystals of sodium dithionite.

Modifications reactions using DEP were carried out essentially as described previously for hemopexin (16) by adding the indicated amount of DEP to apo- or mesoheme-domain I at a concentration of 4.6 μM in 0.1 M sodium phosphate, pH 6.3, for 1 h on ice. The number of DEP-modified histidine residues was calculated using a millimolar difference extinction coefficient of 3.2 cm"1 at 240 nm (17). Solutions of DEP were prepared in absolute ethanol. Effects of modification on mesoheme binding were assessed by the loss of absorbance in the characteristic Soret band of the mesoheme-domain complex at 405 nm (9).

The temperature stability of mesoheme-hemopexin and mesoheme-domain I in PBS was assessed by measuring the absorbance spectrum in the Soret region of the mesoheme complexes in a thermostatted four-cell holder in a Gilford 2600 spectrophotometer. Samples were equilibrated at the indicated temperatures for 10 min before the spectra were recorded or held at 45°C for the indicated times before measurement. Temperature was measured with a Yellow Springs electronic cuvette thermometer.

Sucrose gradients (5-20% w/v, 5.8 ml) were run in a Beckman VT80 rotor at 4°C and 465,000 × g (80,000 rpm) to a preset cumulative centrifugal effect (ω2) of 3.04 × 107 rad2 s"2. After mixing domain I with ligand (2.5 μM final concentration of each), the mixtures were incubated on ice for 15 min before 200 μl was layered onto the gradient and centrifuged. After the run, 10 drop fractions were collected from the bottom of the centrifuge tube, and their radioactivity was measured. In a parallel run, 125I-labeled chymotrypsinogen (2.5 S), ovalbumin (3.5 S), aldolase (7.9 S), and β-amylose (9.4 S) were used as standards to determine the sedimentation coefficient of the protein.

Velocity sedimentation was performed in a Beckman Spinco Model E ultracentrifuge equipped with absorption optics and a scanner. Double sector cells with charcoal-filled epon center pieces and quartz windows were used. Samples were dissolved in PBS, pH 7.4, and protein concentrations and heme saturation were determined from absorbance spectra (see above). Runs were carried out at 20°C and 60,000 rpm. The absorbance profile of the cell at 280 nm or 405 nm was recorded at 4 min intervals after the protein cleared the meniscus. s values were calculated from the slopes of plots of In R versus t where R is distance from the meniscus and t is time.

Monoclonal antibodies were obtained by using mouse FOY myeloma cells with spleen cells from DBA mice (18) immunized with rabbit mesoheme-hemopexin. Seven stable hybridoma lines, JEN-1, JEN-3, JEN-5, JEN-8, JEN-11, JEN-13, and JEN-14, were isolated. All monoclonal anti-hemopexin antibodies were κ IgG, immunoglobulin, and ascites fluid for each was produced in pristane-treated mice (19). The mAbs were purified using a monoclonal antibody purification system from Bio-Rad according to the manufacturer's directions. IgG purity (>90%) was monitored using SDS-polyacrylamide slab gel electrophoresis (20) on 4-20% acrylamide gradient gels, and IgG concentration was estimated using an absorbance at 280 nm of 1.4 for 1 mg/ml solution. After purification by the monoclonal antibody purification system procedure, immunoreactivity was again assessed by immunoprecipitation, by Western blots after either nondenaturing or denaturing electrophoresis, and by immuno-dot blots. Reactivity to the following antigens was evaluated: heme-hemopexin, apohemopexin, hemopexin-domain I, domain II, and domain II. The mAbs did not react with rat or human hemopexin, and no difference in immunoreactivity, as judged by inhibition of immunoprecipitation, between the heme and apo form of rabbit hemopexin was detected. For immunoprecipitation (21), 50 μl of antibody (ascites fluid) were diluted with 50 μl of ascitic fluid from normal mouse and 1 ml 1640 medium as carrier. One hundred μl of [125I]-antigen, e.g. heme-[125I]-hemopexin (specific activity, 10,500 cpm/ng; concentration, 10 ng/ml) is then added followed by incubation at 4°C for 16 h. After addition of 200 μl of 30% polyethylene glycol 6000 (Sigma) and incubation at 4°C for 1 h, the sample is centrifuged at 10,000 × g for 5 min. The supernatant is aspirated off, and the precipitated protein was measured using a Beckman 8000 γ counter.

Western blotting (22) and immunoblotting were carried out essentially as described by Towbin et al. except that affinity-purified alkaline phosphatase-conjugated rabbit anti-mouse IgG from Cappel was used as the second antibody and 5% w/v Carnation non-fat skim milk powder in PBS was used as the blocking solution. After separating the samples by SDS-polyacrylamide gel electrophoresis under nondenaturing conditions or by electrophoresis under denaturing conditions by replacing SDS by Bio-Rad at 4°C. As a positive control for the monoclonal antibodies, polyclonal goat anti-immunoreactivity was assessed by the loss of absorbance in the characteristic Soret band of the mesoheme-domain complex.
A:

with bovine serum albumin as a standard.

the heme-domain extract from each well using the Pierce BCA protein assay system.

Panel structure and properties of the domains was required. First, pexin could be undertaken, additional characterization of the respective proteins to mesoheme-125I-hemopexin and to [66Fe]mesoheme-hemopexin. Under these conditions, specific binding was approximately 80% of the total, i.e. the amount of mesoheme-125I-hemopexin bound to the cells was decreased to approximately 20% by incubation with nonradioactive mesoheme-hemopexin. The mean specific binding and heme uptake were 0.48 and 1.2 pmol/mg of protein, respectively, in control experiments. The values given are the means of three experiments. The standard error of the mean was less than ±15% in each case.

Binding of mesoheme-125I-hemopexin and uptake of [66Fe]mesoheme were measured after preincubation of the cells with the various compounds shown for 30 min at 37 °C. The ratios of the blocker proteins to mesoheme-125I-hemopexin and to [66Fe]mesohemepexin were 20:1 in both cases. The cells were incubated with 50 nM mesoheme-125I-hemopexin or with 250 nM [66Fe]mesoheme-hemopexin for an additional 30 min and then harvested. Additional details are given under “Materials and Methods.” The results are expressed as % inhibition of specific binding and uptake caused by each compound compared to the control, mesoheme-hemopexin. Under these conditions, specific binding was approximately 80% of the total, i.e. the amount of mesoheme-125I-hemopexin bound to the cells was decreased to approximately 20% by incubation with nonradioactive mesoheme-hemopexin. The mean specific binding and heme uptake were 0.48 and 1.2 pmol/mg of protein, respectively, in control experiments. The values given are the means of three experiments. The standard error of the mean was less than ±15% in each case.

RESULTS AND DISCUSSION

Before study of heme-linked conformational changes in domain I and the functional roles of the domains of hemopexin could be undertaken, additional characterization of the structure and properties of the domains was required. First, the heme-domain I complex was studied in more detail. The absorbance spectra of the mesoheme-domain I complex (Fig. 1) closely resemble those of mesoheme-hemopexin (9) and suggest that 2 histidine residues of the domain, like hemopexin, are involved in coordination of the heme iron, forming a low spin complex (15). The lack of an absorbance band at 620 nm (Fig. 1, panel A) characteristic of high spin ferric hemeproteins (24), and the results of modification of the histidine residues of domain I with DEP support this assignment.

As shown in Table I, the number of modified histidine residues in apo- and mesoheme-domain I increases as the DEP concentration used for modification increases. Ethoxy-formylation of the 5 histidine residues of apo-domain I abolishes its heme binding activity, and mesoheme protects 2 residues from modification in the heme-domain I complex. Only 3 histidines can be modified in the complex, and the spectrum of the complex is not influenced by the modification.

FIG. 4. Sucrose gradient centrifugation of domain I. 125I-

Hemopexin and its radiolabeled domains were centrifuged in 5-20% sucrose gradients as described under “Materials and Methods.” The buffer was 0.1 M sodium phosphate, pH 6.3. Values given are from a typical experiment.

FIG. 3. Sedimentation velocity centrifugation of domains I and II. Hemopexin, its isolated domains, and their respective heme complexes were centrifuged at 60,000 rpm, and the absorbance profiles were monitored at 4-min intervals as described under “Materials and Methods.” The buffer was 0.1 M sodium phosphate, pH 7.4. Panel A: o, apo-domain I; □, mesoheme-domain I; and ▲, small domain. Panel B: o, apohemopexin; □, mesoheme-hemopexin.

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As shown in Table I, the number of modified histidine residues in apo- and mesoheme-domain I increases as the DEP concentration used for modification increases. Ethoxy-formylation of the 5 histidine residues of apo-domain I abolishes its heme binding activity, and mesoheme protects 2 residues from modification in the heme-domain I complex. Only 3 histidines can be modified in the complex, and the spectrum of the complex is not influenced by the modification.
The coordination between the histidine residues of domain I and the central iron of heme is weaker than that between those of heme and hemopexin (9). As shown in Fig. 2, heme-hemopexin in PBS, but not 15 mM phosphate buffer, is remarkably stable to heat, with little change in absorbance noted even at 60 °C or after prolonged incubation at 45 °C. In contrast, the heme-domain I complex melts more easily than the intact protein so that one-half of the histidine-heme coordination is abolished at 60 °C. Interestingly, adding domain II to heme-domain I enhances the stability of the heme-domain I complex. The temperature at which half the absorbance is lost is higher for heme-domain I alone (Fig. 2A), and the stability of the mixed domains to incubation at 45 °C is markedly increased (Fig. 2B). Taken together with the increase in mutual affinity of the two domains induced by heme (9), these observations suggest that domain I undergoes a change in conformation upon binding heme which leads to an increased interdomain interaction in turn stabilizing the coordination between domain I and heme. Since a heme-linked change in conformation might serve as a trigger to allow hemopexin to interact with its receptor, a means to document and characterize this change was sought.

No conformational alteration of domain I induced by heme was detected using circular dichroism (9), but a change in conformation of domain I when heme is bound is clearly demonstrated by the exceptional increase in the sedimentation velocity of domain I when it binds heme. The s value increases from 3.17 ± 0.04 to 3.71 ± 0.09 (Fig. 3A), implying that the domain becomes significantly more compact when it binds heme. Intact hemopexin, whose sedimentation shifts from 4.8 S to 5.1 S (Fig. 3B), is also made more compact by heme. The more compact shape of heme-domain I relative to apo-domain I is also apparent in the higher migration rate of the former in sucrose gradients (Fig. 4), with s values of 2.9 for the apo-domain I and 3.8 for the hemoprotein, in satisfactory agreement with the sedimentation velocity results.

To provide additional insight into the role of heme-induced conformational changes in domain I in the binding of hemopexin to its receptor and in hemopexin-mediated heme uptake, the ability of isolated domains to interact with the hemopexin receptor of mouse Hepa cells was examined. This cell line has been shown to synthesize hemopexin and to possess all the cellular components needed for specific, hemopexin-mediated cell uptake of heme. As summarized in Table II, the heme-domain (I + II) complex is as effective as intact heme-hemopexin in competitively inhibiting heme-hemopexin binding although somewhat less effective in inhibiting heme uptake. Why heme uptake is less affected than binding will require additional experiments to answer, but may be related to a role of the intact hinge region in heme release for transport. Each apo-domain, whether alone or in a stoichiometric mixture with each other is without effect. Remarkably, heme-domain I alone possesses significant ability to block binding. These results are striking since they indicate that tight binding to the receptor is coupled to the more compact conformation of domain I produced by heme binding and that domain I possesses in addition to heme-binding activity some of the determinants for interaction with the receptor. Nonetheless, both domains of hemopexin are required for full binding to the receptor, and the increased interaction between domains which is induced by heme would then be important for the production of complementary surfaces between hemopexin and its receptor. In addition, these
findings demonstrate that the heme-induced increase in ellipticity at 231 nm of hemopexin (5) reflects, but is not directly linked to, the conformational changes required for a tight interaction of hemopexin with its receptor since no change in ellipticity at 231 nm occurs upon binding of heme to domain I either alone or when associated with domain II (9).

To further characterize the role of each of the two domains of hemopexin for receptor binding and heme release for cellular transport, a series of experiments employing a battery of monoclonal antibodies raised against heme-hemopexin was conducted. A partial characterization of the seven monoclonal antibodies used, all IgG1, k type, is summarized in Table III. The results of immunoprecipitation assays and immuno-dot blots revealed that JEN-1, JEN-3, JEN-5, JEN-8, JEN-13, and JEN-14 specifically recognize epitopes on domain I and that JEN-11 reacts with an epitope on domain II. Only JEN-14 gave any reaction with isolated domain antigen on Western blots after non-denaturing electrophoresis, indicating that the epitopes recognized by the majority of the mAbs are conformationally sensitive. When tested for their ability to inhibit the interaction of intact heme-hemopexin with cells, JEN-14 effectively inhibited both specific binding of heme-hemopexin to its receptor and specific heme transport. JEN-8 had a similar but slightly smaller effect (Table IV). These data provide additional evidence that domain I not only binds heme but also participates in receptor binding. Nonetheless, the reproducible inhibition of heme uptake (but not binding) produced by JEN-11 (Table IV) supports the involvement of domain II in the heme-hemopexin-receptor interaction. Why JEN-11 does not inhibit binding is unclear. Perhaps JEN-11 binds to a site on hemopexin that must undergo a change in conformation as part of the heme release mechanism, and JEN-11 prevents that release. The fact that the other four antibodies (JEN-1, JEN-3, JEN-5, and JEN-13) did not noticeably affect the heme-receptor interaction demonstrates that the inhibitory effects caused by JEN-8, JEN-11, and JEN-14 arise from binding of these three antibodies to functionally significant portions of hemopexin rather than from a general effect caused by binding of antibody to hemopexin per se.

The results presented here enable us to more fully visualize the changes which occur in hemopexin upon binding heme which are vital for its interaction with the receptor. Our current view of the reaction sequence of hemopexin is shown schematically in Fig. 5. Upon binding heme, domain I dramatically changes conformation and assumes a more compact shape which in turn enhances interactions between the two domains presumably by producing a surface that is complementary with that of domain II. This change renders the hinge region (stippled area in Fig. 5) inaccessible to plasmin. Both domains of hemopexin contain features that are necessary for binding to the hemopexin receptor and heme uptake, as shown by the inhibitory effects of the heme-domain (I + II) complex and of monoclonal antibodies to either domain. An intact hinge region is not required for receptor binding, but the hinge region mediates certain conformational changes which are detected by the change in circular dichroism at 231 nm.

Further study of the heme-associated conformational changes in hemopexin and of other molecular determinants of hemopexin important for its interaction with the hemopexin receptor is in progress based on the results above. Of special interest is the apparently crucial role in receptor recognition played by a portion of the domain I structure which includes the epitopes for JEN-8 and JEN-14.

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Determinants of Hemopexin-mediated Heme Transport

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