A Protein with Multiple Heme-binding Sites from Rabbit Serum*  

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A 93,000 molecular weight protein (HBP.93) which binds hemin and protoporphyrin IX with high affinity has been isolated from rabbit serum using affinity chromatography on hemin-conjugated agarose. The amino acid composition of this protein is unique in that the proline and histidine contents are remarkably high (16.6 and 9.9 mol %, respectively). A large increase in the absorbance of the Soret region arises from the heme-protein interaction. The spectrophotometric titration showed that the protein can bind 25–35 mol of hemin/mol of protein. The apparent dissociation constant was estimated to be 1–4 \times 10^{-7} \text{M} for hemin at pH 7.4 and \sim 10^{-8} \text{M} for protoporphyrin IX at pH 9.2. The similarity of the difference spectrum of heme-HBP.93 complex to that of heme-hemopexin complex suggests that a bisimidazol-type coordination of heme iron is involved in the binding. The extremely high capacity of HBP.93 to bind heme is also demonstrated by a large increase in the sedimentation velocity of the protein upon heme binding. The native heme-protein complex migrates faster than the heme-free protein in a polyacrylamide gel at pH 8.8; the increased mobility appears to be due to the charge on the carboxyl groups of the bound heme. Although the use of a hemin-agarose column has failed to reveal a protein of similar size and heme affinity in the sera of a number of other species, including man, the heme-binding properties and high histidine level of the human \(\alpha\)-histidine-rich glycoprotein raise the possibility that the two proteins are related.

Until recently only two proteins in mammalian serum, albumin, and hemopexin had been shown to have a high affinity for heme and porphyrins. Serum albumin was first shown to be capable of binding heme by Rosenfeld and Surgenor (1). It was further identified that the heme-binding site of albumin is different from the sites which bind bilirubin (2). Hemopexin, a \(\beta\)-glycoprotein which is a more specific binder of heme and porphyrins, has only one binding site with a dissociation constant for heme that is much lower than \(10^{-8} \text{M}\) (3–5). Studies of the interaction of hemopexin with heme and other porphyrin derivatives, using various spectrometric techniques such as optical absorption (6), fluorescence quenching of tryptophan residues (3), circular dichroism (6), Mossbauer spectra (7), magnetic circular dichroism (8), and ESR (7) have revealed that heme is bound by coordination to histidine residues. Physiologically, hemopexin appears to function as a carrier protein for the disposal of heme that has been released from hemoglobin. The heme moiety of the heme-hemopexin complex has been shown to be taken up by hepatic parenchymal cells and degraded to bilirubin (9).

Recently Morgan (10, 11), in studies of the metal-binding properties of the \(\alpha\)-histidine-rich glycoproteins of human and rabbit sera, has shown that these proteins also bind heme competitively with Cu\(^{2+}\), Hg\(^{2+}\), and Zn\(^{2+}\). In this case, 10 molecules of heme are bound/mol of protein with an affinity that is significantly lower than the heme-albumin interaction (10).

The present paper describes the isolation and properties of a unique serum protein which binds up to about 30 mol of heme/mol of protein with a relatively high affinity. The protein, which is referred to as HBP.93 in this report, was purified from rabbit serum by affinity chromatography on hemin-conjugated agarose. Binding of hemin to the protein was clearly demonstrated by heme-induced changes in the absorption spectra, sedimentation velocity, and electrophoretic mobility of the protein. Amino acid analysis revealed that this protein, like the previously reported \(\alpha\)-histidine-rich glycoproteins of human serum (10), has an unusually high content of histidine and proline; however, both the molecular weight and affinity for heme of this rabbit serum protein appear to be higher than the human \(\alpha\)-histidine-rich glycoproteins. The similarity in properties of these proteins is discussed.

**EXPERIMENTAL PROCEDURES**

**Preparation of Hemin-Agarose**—The preparation of hemin-conjugated agarose was achieved using 1,1'-carbonyldiimidazol as a coupling agent. About 2.2 \mu mol of heme, linked through its carboxyl group, were immobilized/1 ml of aminoethyl agarose. A detailed description of this synthesis will appear elsewhere.

**Purification of HBP.93**—Rabbit serum (14 ml) was diluted 20-fold with a buffer containing 0.5 M NaCl and 0.02 M sodium phosphate (pH 7.5) (buffer A) and applied to a column (1.0 \times 6.5 cm) of hemin-agarose which had been conditioned with buffer A at 4 °C. After the column was washed with 200 ml of buffer A, the adsorbed protein was eluted with a decreasing pH gradient which was made by introducing a mixture of 5 mM citric acid and 5 mM succinic acid to 50 ml of buffer A placed in a logarithmic (convex) gradient maker. One-ml fractions were collected at a flow rate of 10 ml/h. Protein in the fractions was determined from the absorbance at 280 nm. Aliquots of 30 \mu l were then taken from each fraction and electrophoresed in 7.5% polyacrylamide gels containing 0.1% SDS (12). Fractions containing hemopexin and HBP.93 were pooled separately and dialyzed against buffer A adjusted to pH 4.6 with acetic acid, then against a buffer

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2 The abbreviations used are: SDS, sodium dodecyl sulfate; BSA, bovine serum albumin.
Nuclear, specific activity 2500 Ci/mmol) at 37 °C were fractionated from the bottom into 40 fractions. Each fraction heme to HBP.93 was titrated by adding increasing amounts of heme sulfoxide and then diluted with 1.5 volumes of 0.025 M NaCl acetate buffer. Neither the heme binding or concentration at the end point of titration were measured. Half-cystine and methionine were determined spectrophotometrically (15). The final concentration of the solutions was usually 4-8 × 10^{-4} M. Solutions were used within 5 h of preparation.

Spectrophotometric measurements were performed on a Beckman double beam spectrophotometer (model Acta III). The binding of heme to HBP.93 was titrated by adding increasing amounts of heme in two cuvettes with a 1:cm light path, one containing buffer and the other the protein solution, and recording the difference in absorption at 414 nm. Difference spectra were recorded at the saturating heme concentration point of the heme-HBP.93 complex.

Sedimentation Analysis of Hemin-HBP.93 Complex—HBP.93 was labeled with 32P by incubating it with [α-32P]dCTP (New England Nuclear, specific activity 2500 Ci/mmole) at 37 °C for 50 min and the free 32P was removed by dialysis.2 Neither the heme binding or sedimentation properties of the HBP.93 protein were affected by the labeling protocol. HBP.93 labeled with 32P was then complexed with heme by incubating it with a saturating dose of heme in buffer B at 37 °C for 30 min. 32P-labeled and heme-saturated HBP.93 (0.1 ml) was loaded onto a 5-20% linear sucrose gradient in buffer B (4 ml) and centrifuged in a SW 50 rotor at 38,000 rpm for 22 h at 5 °C. Free HBP.93 and BSA were used as markers and were sedimented separately in identical gradients. After the centrifugation, the gradients were fractionated from the bottom into 40 fractions. Each fraction was analyzed for heme and protein content by absorbance at 410 nm and 32P scintillation counting, respectively.

Electrophoresis of Heme-HBP.93 Complex—HBP.93 was incubated at 37 °C with various concentrations of heme in buffer B. After 30 min, 1/4 volume of 0.25 M Tris-HCl (pH 6.8), 1.0 M sucrose, 0.05% bromphenol blue was added, and the mixture was immediately subjected to electrophoresis in a 7.5% polyacrylamide gel. The gel was prepared according to the procedure of Laemmli (12) without including SDS. Two identical series of samples were run in the same gel (15 × 9 × 0.15 cm) at 20 mA. One series was stained with Coomassie brilliant blue (R-250) and the other with 3,3′-dimethoxybenzidine/H2O2 in 1 M acetic acid (16).

RESULTS

Purification of HBP.93 and Hemopexin by Hemin-Agarose Chromatography—Affinity chromatography in a hemin-bound polysaccharide matrix has been shown to be a useful tool for isolating hemopexin from human serum (17) and separating heme-depleted apohemoprotein from its heme-containing counterpart.1 We initially intended to examine the specificity of a hemin-agarose column using rabbit serum as a source of heme-binding proteins, expecting the selective retrieval of hemopexin and albumin from the complex mixture of proteins present in serum. Our preliminary experiments confirmed a highly specific binding of proteins to the column (i.e., only two proteins were eluted from the column by lowering the pH of the medium with acetic acid). Unexpectedly, however, albumin was not adsorbed to the column under the conditions used; instead a new protein of Mr = 93,000 was retained and eluted with a pH close to that expected for the expected hemopexin. These proteins were demonstrated to be held in the column through a specific interaction with the immobilized heme, since their retention was completely precluded by the presence of 0.5 mM hemin in the binding buffer. The relative abundance of the Mr = 93,000 component (HBP.93) in rabbit serum prompted us to isolate and characterize the protein. In order to separate these proteins after adsorption on the heme-agarose, a pH gradient elution was performed (Fig. 1A). Although the proteins were eluted as a large peak with a shoulder, SDS-polyacrylamide gel electrophoresis of the column fractions (Fig. 1B) revealed that the two proteins were fairly well separated by the column, HBP.93 being eluted in the lower pH region of the gradient. The Mr = 65,000 protein (pool I) which is not distinguishable from albumin on the SDS-gel was identified as hemopexin based on the following observations. 1) The apparent molecular weight of the protein estimated by SDS-polyacrylamide gel electrophoresis was close to that of rabbit hemopexin (18). 2) The mobility of the Mr = 65,000 protein in polyacrylamide gel electrophoresis at pH 8.8 in a native condition (the same system as the one used in Figs. 6 and 7) was different from that of albumin which migrates about 2 times faster than the Mr = 65,000 protein. This also precludes the presence of albumin in pool I. 3) The characteristic difference spectrum of the heme-protein complex (Fig. 3C and Ref. 4). 4) The amino acid composition of the Mr = 65,000 protein was very similar to that of rabbit hemopexin (18). The correlation coefficient between the corresponding amino acid residue contents was calculated to be 0.94.

The purity of HBP.93 in pool II was satisfactory for further analysis. In order to improve the recovery of these proteins, however, the proteins bound to the hemin-agarose column were eluted at once with the low pH buffer and subjected to chromatography on SE-Sephadex (Fig. 2). HBP.93 was completely separated from hemopexin which eluted as a double peak at a lower NaCl concentration (see “Discussion”). The yield of HBP.93 was approximately 0.3 mg from 1 ml of rabbit serum which corresponds to a 50-60% recovery. HBP.93 possessed a unique amino acid composition (Table I) in that contents of proline and histidine were unusually high (16.6 and 9.9 mol %, respectively) as compared to the averages from a variety of proteins; methionine was not detected. There

1 It has been observed that both native or SDS-denatured proteins can be labeled nonspecifically with 32P by incubation with [α-32P]dCTP at 37 °C. The labeling, which is due to an impurity in the commercial preparations, does not alter the electrophoretic or physical properties of human proteins. Since the protein labeling can be prevented by semicarbazide, the involvement of some carbonyl-containing sugar derivatives, a breakdown product of the [α-32P]dCTP preparation, is suspected to account for the labeling reaction (29).
were no discernible similarities in amino acid composition between HBP.93 and hemopexin. HBP.93, a rather abundant protein in rabbit serum, is clearly identifiable as a distinct band when whole rabbit serum is resolved by SDS-polyacrylamide gel electrophoresis (Fig. 1B). The protein band of purified HBP.93 in an SDS gel was also stainable with the periodate-Schiff reaction (19), indicating that HBP.93 is a glycoprotein (result not shown).

Spectrophotometric Characterization of Heme-HBP.93 Complex—It is well known that the absorption spectrum of hemin changes significantly through its interaction with proteins. Changes in the extinction coefficient and wavelength of absorption bands have been observed when heme or porphyrins interact with serum albumin (1) and hemopexin (6). The pattern of the absorption change (i.e., the difference spectrum between protein-bound heme and free heme) also provides useful information on the mode of heme-protein interaction. As shown in Fig. 3A, a characteristic difference spectrum was observed with HBP.93. The peak at 415 nm is due to the Soret band of heme. The negative absorption noticed at 351 nm is attributable to the differential absorption of free hemin whose concentration is higher in the reference cuvette than in the sample side. Protein absorption around 280 nm was can-

**TABLE I**

<table>
<thead>
<tr>
<th>Amino acid composition of HBP.93</th>
<th>HBP.93</th>
<th>HRG*</th>
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<tr>
<td>mol %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartic acid</td>
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<td>Threonine</td>
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<td>3.2</td>
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<tr>
<td>Serine</td>
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<td>Glutamic acid</td>
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<td>11.2</td>
</tr>
<tr>
<td>Glycine</td>
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<tr>
<td>Alanine</td>
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</tr>
<tr>
<td>Half-cysteine</td>
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<tr>
<td>Valine</td>
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<tr>
<td>Methionine</td>
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<td>0.5</td>
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<tr>
<td>Isoleucine</td>
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<td>Leucine</td>
<td>6.2</td>
<td>5.9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.4</td>
<td>2.5</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.6</td>
<td>5.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>9.9</td>
<td>10.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.4</td>
<td>5.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.4</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* a2-Histidine-rich glycoproteins (HRG) calculated from the data of Morgan (10).
Heme-binding Serum Protein

FIG. 3. Difference spectra of heme-HBP.93 complex (A), protoporphyrin IX-HBP.93 complex (B), and heme-hemopexin complex (C). Freshly prepared solutions of hemin and protoporphyrin IX were mixed with 1 to 0.025 M sodium borate (pH 9.2) containing 0.4 nmol of HBP.93 or 6.2 nmol of hemopexin. After 30 min, the difference spectra were recorded at room temperature against the solutions containing the ligands alone. Reduced spectra were determined after adding a small amount of sodium dithionite. Amounts of ligands added were, hemin, 30.7 nmol (A); protoporphyrin IX, 33.4 nmol (B); hemin, 18.8 nmol (C); oxidized spectra (---), reduced spectra (---).

Fig. 3 shows that the apparent dissociation constant (Kd) of the heme-protein complex was calculated using the curve portion of the titration curves (22). The maximum number of heme molecules bound to 1 molecule of the protein (n) can be estimated from the equivalence point on the titration curves. These binding parameters measured in different conditions are summarized in Table II. The protein showed a stronger affinity for hemin at pH 7.4 (Kd = 1.4 × 10^{-7} M) than at pH 9.2 (Kd = 10^{-8} M). The values of n observed in these conditions were remarkably high (n = 25-35) when compared to that of hemopexin (n = 1). The value of n could be overestimated by an extensive aggregation of hemin in the solution (23). However, this seems unlikely since the Kd/nmol of fully saturated protein was also high in HBP.93 (~1300 mmol cm^{-1}) compared to that of hemopexin (~72 mmol cm^{-1}). Thus, we might anticipate that HBP.93 binds heme up to at least 20 mol/1 mol of protein (see “Discussion”). Binding parameters obtained for protoporphyrin IX were comparable to those of hemin at pH 9.2.

Sedimentation Analysis of Heme-HBP.93 Complex—Saturation of HBP.93 with heme should result in an increase in the molecular weight by approximately 20% which should be large enough to be detected from the increased sedimentation rate of the protein. This was indeed the case as a significant increase in the sedimentation velocity was observed upon heme binding (Fig. 5). This result was not caused by protein aggregation (see “Discussion”). The free protein co-

![Fig. 3 Difference spectra of heme-HBP.93 complex (A), protoporphyrin IX-HBP.93 complex (B), and heme-hemopexin complex (C).](http://www.jbc.org)

![Fig. 4 Spectrophotometric titration of HBP.93 with hemin.](http://www.jbc.org)

**Table II**

<table>
<thead>
<tr>
<th>Ligand/ pH</th>
<th>0.6 μM</th>
<th>0.4 μM</th>
<th>0.2 μM</th>
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<tr>
<td>Hemin</td>
<td>7.4</td>
<td>32</td>
<td>35</td>
</tr>
<tr>
<td>Hemin</td>
<td>9.2</td>
<td>25</td>
<td>35</td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td>9.2</td>
<td>ND</td>
<td>27</td>
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<table>
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<tr>
<th>HBP.93</th>
<th>n</th>
<th>Kd</th>
<th>n</th>
<th>Kd</th>
<th>n</th>
<th>Kd</th>
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</thead>
<tbody>
<tr>
<td>Protobeme</td>
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<td>32</td>
<td>35</td>
<td>0.11</td>
<td>35</td>
<td>0.39</td>
</tr>
<tr>
<td>Protobeme</td>
<td>9.2</td>
<td>25</td>
<td>23</td>
<td>1.2</td>
<td>35</td>
<td>0.96</td>
</tr>
<tr>
<td>Protobeme</td>
<td>9.2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Concentrated solutions were prepared with 0.025 M sodium borate (pH 9.2).
* Buffer B and 0.025 M sodium borate were used for measurements at pH 7.4 and pH 9.2, respectively.
* Not determined.
sedimented with the BSA marker. A good correlation between the peaks of protein (\(^{32}\)P) and hemin (A\(_{410}\)) confirmed the presence of protein-bound heme.

**Electrophoretic Analysis of Heme-HBP.93 Complex**—The electrophoretic mobility of HBP.93 may well be affected by the association of the protein with hemin. Binding of hemin to HBP.93 was tight enough to localize the protein-bound hemin by dimethoxybenzidine staining after electrophoresis (Fig. 6). The protein was incubated with different concentrations of hemin at pH 7.4 prior to electrophoresis. The electrophoretic mobility of the heme-protein complex visualized by both kinds of staining increased with the increasing concentration of hemin, up to the heme/protein molar ratio of 128. The heme/protein ratio at which the maximal mobility increase is attained was much higher than the ratio at the end point of titration (Fig. 4). This discrepancy may be due to the shift of binding equilibrium toward dissociation which is caused by the decrease of free heme concentration in the vicinity of the protein zone. The increased mobility of the heme-protein complex mainly arises from the increased negative net charge on the protein molecule contributed by the carboxyl groups of hemin side chains. The decrease in pl values of hemopexin pl variants upon heme binding has been reported (24). These observations suggest that at least a part of the charged groups of hemin side chains in the heme-protein complex are not neutralized by positive amino acid residues of the protein, and are relatively exposed on the surface of the protein molecule. Comparison of the mobilities of the bands in corresponding lanes revealed the dimethoxybenzidine-stained bands move slightly faster than Coomassie brilliant blue-stained ones except in lane 9. This displacement suggests that during the electrophoresis the electric field continuously propels the negatively charged free heme molecule dissociated from the protein toward the anode and then concentrates the protein-bound hemin towards the front part of the protein band.

The slow moving bands in Fig. 6 are dimeric forms on the heme-protein complex. The appearance of the polymer bands were dependent on the age of the hemin solution used (Fig. 7). With the most aged hemin solution, polymer bands up to pentamers were discernible (Fig. 7a), whereas only dimer bands were seen with a freshly prepared solution (Fig. 7c). The polymeric nature of these slower moving bands was confirmed by a linear relationship between logarithm of the mobility and number of monomers in the polymers. The occurrence of the polymer bands was also dependent on heme/protein ratio. With a higher heme/protein ratio, intensity of the polymer bands decreased. The mechanism of the molecular association is not clear. It appears likely, however, that some forms of heme polymer are involved in this reaction since heme polymers are formed as a solution is aged (23).

**DISCUSSION**

The data presented here clearly demonstrate the presence of a unique heme-binding protein, HBP.93, in rabbit serum. However, we failed to identify analogous proteins in sera from other species tested so far: human, calf, and rat. All the rabbit sera from normal male and female animals and from those immunized with several different antigens contained the protein. Since it is improbable that this protein is strictly specific to rabbits, further search for the presence of this protein in various species will be required. It is possible that we have overlooked this protein simply because the sera from other animals contain very little HBP.93. Hemin-binding proteins which exist in low concentration would not be picked up by the hemin-agarose column since hemopexin might compete...
with these proteins for the binding sites on hemin-agarose when large volumes of serum are used.

Purification of HBP.93 and hemopexin was achieved in one step using hemin-agarose affinity chromatography. The yield of hemopexin varied depending on the extent of hemolysis of the serum used. This seems to be caused by a partial saturation of hemopexin due to the transfer of heme from hemoglobin to hemopexin (5), probably during the preparation. These preparations were apparently homogeneous judging from SDS-polyacrylamide gel electrophoresis and further purification was not performed routinely. When a higher yield of these proteins was desired, however, the two proteins could be separated completely by additional chromatography on SE-Sephadex at pH 5.2. The basis for the separation of hemopexin into two species on this column is unclear (Fig. 2). Since several variants of neuraminidase-treated hemopexin with different isolectric points are known (24), some change modification of amino acid residues is likely as well as a difference in the extent of sialidation. However, we have not pursued this phenomenon any further.

A large heme-binding capacity of HBP.93 is the most remarkable feature of the protein. There seems to be two alternative mechanisms for the binding: binding of hemin aggregate (micelle) to a single binding site on the protein and binding of monomeric hemin to multiple binding sites. Since highly aggregated forms of hemin molecules are known to exist in a neutral solution (23), the former possibility is not unreasonable. However, the following arguments strongly support the latter model. Aggregation of hemin normally accompanies a decrease in the extinction coefficient of the Soret band (25), but the absorbance change observed upon heme binding was a large increase in the Soret absorption, implying that hemin molecules bound to the protein are in a dispersed state. Moreover, the similarity between the difference spectrum of reduced heme-HBP.93 complex and the reduced spectrum of the heme-hemopexin complex (4) suggests that heme molecules bound to HBP.93 are coordinated by two axial imidazol groups of histidine residues as proposed in the heme-hemopexin complex (4). A high content of histidine in HBP.93 is consistent with these data. If we assume that each heme-binding site on HBP.93 is identical with that of hemopexin and that the increase in the absorption intensity of the Soret band \( \Delta E_{414} \) caused by binding of hemin to a single site has an additive property, then we can expect a value as large as 1800-2500 \( \text{M}^{-1} \text{cm}^{-1} \) for \( \Delta E_{414} \) of HBP.93 which is saturated with 25-35 molecules of hemin (Table II). However, the number of binding sites in HBP.93 should be about 18, according to the assumption, since the observed \( \Delta E_{414} \) was 1300 \( \text{M}^{-1} \text{cm}^{-1} \) (see "Results"). This disagreement in the estimated number of binding sites may be settled either by assigning a smaller \( \Delta E_{414} \) for a single binding site in HBP.93 or by assuming an interaction between binding sites which might abolish the additivity of \( \Delta E_{414} \). Although further study is needed to determine more accurately the number of binding sites in HBP.93, we might tentatively conclude that HBP.93 has multiple, probably more than 20, and as high as 30, binding sites for monomeric heme. A large increase in the sedimentation velocity of HBP.93 elicited by heme binding (Fig. 6) indicates an appreciable contribution of bound heme to the molecular weight of the complex. The complex observed in the gradient should not be a dimeric form of HBP.93, because at such a high heme/protein ratio, the heme-HBP.93 complex was predominantly in a monomeric state, even when an aged solution of hemin was used as shown by electrophoretic analysis (Figs. 6 and 7). The sedimentation coefficient of hemopexin does not change when the protein binds heme (26).

The fact that the electrophoretic mobility of the heme-HBP.93 complex increases continuously with an increasing heme/protein ratio (Fig. 6) also seems to be consistent with the multibinding site model since heme-protein complexes with intermediate mobilities would not be observed if the protein had a single site for heme aggregation.

The dissociation constants \( K_d \) estimated from the titration data (Table II) are only approximate. Difficulties in determining the \( K_d \) correctly arise from the inherent insufficiency of the titration method to estimate \( K_d \) values smaller than the protein concentration (27), and the presence of multiple binding sites. Because neither cooperativity nor multiaspheric saturation phenomenon were noticed in the titration curve (Fig. 4), we may presume that the majority of the heme-binding sites in HBP.93 have a similar magnitude of affinities for hemin. However, the possibility that a few of the sites have a higher affinity than the others cannot be ruled out.

Despite the higher molecular weight of HBP.93 compared to BSA, HBP.93 co-sedimented with BSA in the sucrose gradient (Fig. 5), implying that the HBP.93 molecule has a high axial ratio. The elongated molecular shape is consistent with the high content of proline in HBP.93 (Table I) since proline residues tend to produce rigid and extended structures as evidenced by the structure of collagen. At present there is no direct evidence for the possible conformational change of the HBP.93 molecule induced by heme binding. The increase in sedimentation velocity of HBP.93 (about 40%, see Fig. 5) might be too great if it is caused solely by an increase in the molecular weight (about 20%) due to heme binding. Part of the increment could be accounted for by a more compact conformation of the heme-protein complex induced by heme binding.

In 1978, an \( \alpha_2 \)-histidine-rich glycoprotein which had been isolated from human serum and described as 3.8 \( \alpha_2 \)-histidine-rich glycoprotein (28) was shown to bind about 10 hemes/molecule of protein (10). Although the estimated affinity of \( \alpha_2 \)-histidine-rich glycoproteins for heme \((K_d = 1.5 \mu M)\) was about 10 times weaker than that of HBP.93, the absorption pattern of the \( \alpha_2 \)-histidine-rich glycoprotein complex was quite similar to the difference spectrum of heme-HBP.93 complex. The amino acid composition of these proteins also showed significant similarity in that both proteins have an extremely high content of histidine and proline residues (Table I). Despite these similarities \( \alpha_2 \)-histidine-rich glycoprotein is reported to have a native molecular weight of 50,000 and is composed of two subunit polypeptides with similar size (10).

More recently, Morgan has described the interaction of heme and divalent metal ions with an \( \alpha_2 \)-histidine-rich glycoprotein purified from rabbit serum (11). The rabbit \( \alpha_2 \)-histidine-rich glycoprotein, however, was reported to have heme-binding properties similar to human \( \alpha_2 \)-histidine-rich glycoprotein and a molecular weight of 58,000. Although these points would tend to exclude an identity between the HBP.93 and the \( \alpha_2 \)-histidine-rich glycoprotein, it remains possible in view of the similarities in amino acid composition that the two are related. Degradation studies and immunological characterization will be required to settle this question.

The physiological role of HBP.93 is yet obscure. Since HBP.93 binds protoporphyrin IX as well as heme, it must be kept in mind that some porphyrins or porphyrin-related compounds...
pounds in serum could equally be the natural ligand for HBP.93. Changes in the serum level of HBP.93 should be looked for in disturbances of porphyrin metabolism like porphyria or in conditions leading to massive hemolysis. Judging from the fact that rabbit serum albumin showed a weaker interaction with hemin-agarose than HBP.93 and that rabbit serum albumin has a relatively small affinity ($K_d \approx 0.5 \mu M$) for deuteroheme (4) compared to human serum albumin, HBP.93 may have a greater role than albumin in the heme metabolism of rabbit serum.

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