Interactions of Porphyrins with Rabbit Hemopexin*

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SUMMARY

The formation of complexes of rabbit hemopexin with a representative group of porphyrins was investigated to determine the stoichiometry and estimated dissociation constants ($K_d$) as well as the effect of pH on these interactions. In a 1:1 molar ratio, rabbit apohemopexin (RHx) binds iron-protoporphyrin IX (heme), iron-deuteroporphyrin IX (deuteroheme), iron-mesoporphyrin IX (mesoheme), and cobalt-deuteroporphyrin IX. The estimated $K_d$ values are heme $\simeq$ mesoheme $\simeq$ deuteroheme $\leq 10^{-8}$ M; and cobalt-deuteroporphyrin $\leq 10^{-7}$ M. The relative affinity of RHx was shown to be deuteroheme $>$ nickel-deuteroporphyrin IX $>$ deuteroporphyrin IX, and cobalt-deuteroporphyrin $>$ nickel-deuteroporphyrin, by displacement of ligands with lower affinity employing difference spectrophotometry or gel electrophoresis. The binding strength was influenced more by the presence and nature of the metal chelated to the porphyrin nitrogen than by the substitutions in positions 2,4 of the porphyrin ring. The pH dependence of the association and dissociation of the deuteroheme-RHx complex showed 50% saturation at pH 4.9 $\pm$ 0.1 and 10.7 $\pm$ 0.1. At pH values from 6 to 9.5, binding was equimolar, whereas none occurred below pH 4.0 or above pH 11.2. The circular dichroism spectrum of RHx exhibits a prominent positive maximum at 231 nm, which is tentatively ascribed to tryptophan. The spectrum of RHx is pH-dependent, and the clinical applications of hemopexin have been reviewed (7).

Our objective is to obtain detailed information on the binding of porphyrins by hemopexin to provide the basis for investigation of its role in heme and porphyrin catabolism. The use of heme analogues has provided valuable insight into the structure and function of other hemeproteins (13-19). Here we report on interactions of rabbit apohemopexin with various porphyrins examined by absorption spectrophotometry, and we describe circular dichroism studies which possibly correlate the conformation and function of this protein.

EXPERIMENTAL PROCEDURE

Materials RHx* was purified from rabbit serum, and the purity and homogeneity of the preparations were assessed as previously described (2). Monomeric RHx was utilized in all experiments. This was often verified by polyacrylamide gel electrophoresis (20) by examining protein samples immediately after their use. Protein concentrations were measured employing the millimolar extinction coefficients at 280 nm of 113 and 125 for the apo- and hemoprotein, respectively (3).

Deuteroporphyrin IX, iron-deuteroporphyrin IX, iron-mesoporphyrin IX, cobalt-deuteroporphyrin IX, and nickel-deuteroporphyrin IX were gifts of Dr. B. F. Burnham. Hemin was obtained from Eastman (Lot 691, recrystallized). Metalloporphyrin and porphyrin solutions were prepared by dissolving the porphyrin in a minimal amount of 0.1 M NaOH, diluting it with appropriate solvent to approximately $5 \times 10^{-4}$ M at pH 8.5 to 9.5, and then filtering it through a Millipore 01300 filter. All porphyrin solutions were protected from direct light, and lume was used within 3 hours of preparation. Stock solutions of the other porphyrins were stable for several days (21) as judged by their absorption spectra (22) and by paper chromatography (23).

Concentrations of iron-porphyrin solutions were determined

Hemopexin is a serum $\beta$-glycoglobulin which binds iron-protoporphyrin IX in a 1:1 ratio (1, 2) to form a low spin complex. Human and rabbit hemopexins have been studied in some detail and show no significant differences in their physicochemical properties (2). Both proteins have a molecular weight of 57,000 (3) and contain approximately 20% carbohydrate (2). In common with other heme-proteins (4, 5), hemopexin shows changes in optical rotatory dispersion in the far ultraviolet region upon combining with heme (6).

Removal of heme from the circulation is an essential function in the poorly understood early stages of heme catabolism (7, 8). Plasma heme is conveyed to the liver parenchymal cell, the site of its degradation to bilirubin, solely or predominantly by hemopexin (9-11). This protein binds other porphyrins (12), including those prevalent in certain porphyrias, and is likely to be instrumental in their catabolism as well. The biological properties of this protein have been reviewed (7).

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The abbreviations used are: RHx, rabbit apohemopexin; heme, iron-protoporphyrin IX; mesoheme, iron-mesoporphyrin IX; deuteroheme, iron-deuteroporphyrin IX; CD, circular dichroism.
immediately before use in 0.05 m NaOH-pyridine (1:1 v/v) to which solid sodium dithionite was added. The millimolar extinction coefficients used were cobalt-heme, 130 at 406 nm; mesoheme, 140 at 407 nm; and heme, 190 at 419 nm. Concentrations of cobalt-deuteroporphyrin and nickel-deuteroporphyrin solutions were measured in 0.025 m sodium borate buffer, pH 9.1; and deuteroporphyrin, in 0.1 m HCl. The millimolar extinction coefficients used were cobalt-deuteroporphyrin, 90 at 409 nm; nickel-deuteroporphyrin, 52 at 418 nm; and deuteroporphyrin, in 0.1 M sodium borate, pH 9.1, and 0.1 M KOH from a micrometer syringe, and the absorption spectrum taken. The pH was then gradually changed by adding 6 m HCl or 6 m KOH from a micrometer syringe, and the absorption measurements were repeated. The pH was measured with a Radiometer model 25 pH meter following absorbance measurements. Volume increases remained below 5% in all instances. Metalloporphyrin association was examined in the same manner except that 1 eq of deuteroheme was added to the sample after the change in pH.

In binding experiments employing polyacrylamide gel electrophoresis (20), the protein (1.5 nmols) was incubated with a 5-fold molar excess of porphyrin for 10 min at 37°C prior to electrophoresis at 5 ma per gel. Displacement of one porphyrin by another was tested by interrupting the electrophoretic run after the free porphyrin had been separated from the bound, and by then applying 2 eq of the second porphyrin. Samples in triplicate were analyzed: one was stained with benzidine (24); one with Amido black; and the third was photographed under visible and ultraviolet light.

Circular dichroism measurements were carried out on a Cary Model 61 CD spectropolarimeter at 25°C. The slit width of the instrument was programmed to yield constant light intensity throughout the spectral region. Protein samples were examined in 1-cm fused-quartz cells at concentrations (10^-5 to 10^-4 m) chosen to maintain the dynode voltage below 600 volts. The ellipticities, expressed as mean residue ellipticity, [Θ], were based on a mean residue molecular weight of 112 (6). Results in other spectral regions are reported as molecular ellipticity, θ, using a molecular weight of 57,000 for RHx (3). The effect of pH on CD spectra was determined 15 min after adding the protein to phosphate buffer adjusted in pH with 6 m HCl or 10 m NaOH. The pH of the sample was obtained following the CD measurement. The effect of binding on the CD spectra was evaluated after addition of the ligand to the apoprotein and equilibration for 15 min in the instrument. No corrections were made for dilution, which was less than 2%.

RESULTS

Porphydin Binding—The metalloporphyrins, mesoheme, deuteroheme, and cobalt-deuteroporphyrin, readily combine with RHx. These interactions produce absorption spectra which are similar to those of heme-RHx and characteristic of a low spin heme protein (2). These spectral properties are the result of coordination of the metal in the porphyrin with residues on the protein molecule. Fig. 1 illustrates three typical titration curves obtained by measuring the change in absorbance upon addition of ligand to RHx. Both cobalt-deuteroporphyrin and deuteroheme show binding stoichiometries of 1:1, whether the ligand is mixed with protein or vice versa. The addition of cobalt-deuteroporphyrin to RHx causes small increments in absorption even at very high ratios of ligand to protein. This effect could originate in weak secondary interactions of this porphyrin with the protein. The formation of a complex between RHx and nickel-deuteroporphyrin or deuteroporphyrin produces only slight changes in absorption; however, the binding of these porphyrins was demonstrated clearly by gel electrophoresis experiments.

In Table I we summarize spectral and binding characteristics of the porphyrin-RHx complexes. The absorption maxima of the iron-porphyrin complexes shift to progressively lower wave lengths from vinyl 2,4-substituents (heme) to ethyl (mesoheme) to hydrogen (deuteroheme). All three iron-porphyrins are rapidly bound by RHx whether dithionite is present or not. However, prolonged exposure to dithionite does affect the iron-porphyrin-RHx complexes.

Effect of Sodium Dithionite on Iron-Porphyrin-RHx Complexes—Marked spectral changes are caused by storage of equimolar complexes of RHx with deuteroheme, mesoheme, or heme in 0.006% dithionite at 4°C for 48 hours. Only minimal spectral changes occurred in control samples not exposed to dithionite. Absorption spectra of a representative complex (deuteroheme-RHx) are shown in Fig. 2. The reduced samples, which had autoxidized during storage, displayed lesser Soret band intensities and modified ultraviolet spectra (Fig. 2, Curve c). The Soret λmax of heme-RHx was shifted from 413 to 410 nm. A comparison of the reduced spectra of the stored complexes (Fig. 2, Curve d) with the original (Fig. 2, Curve b) also shows decreased
TABLE I

Spectral and binding characteristics of metalloporphyrin-RHx complexes

<table>
<thead>
<tr>
<th>Complex</th>
<th>Absorption spectra&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ΔOptical density&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Molar ratio&lt;sup&gt;c&lt;/sup&gt;</th>
<th>$K_d \times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oxidized</td>
<td>Reduced, $\lambda_{max}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$\lambda_{max}$</td>
<td>$\epsilon$</td>
<td>$\lambda_{max}$</td>
<td>$\epsilon$</td>
</tr>
<tr>
<td>Heme-RHx</td>
<td>413</td>
<td>113</td>
<td>427</td>
<td>526</td>
</tr>
<tr>
<td>Mesoheme-RHx</td>
<td>405</td>
<td>115 ± 8</td>
<td>415</td>
<td>520</td>
</tr>
<tr>
<td>Deuteroheme-RHx</td>
<td>403</td>
<td>122 ± 5</td>
<td>413</td>
<td>518</td>
</tr>
<tr>
<td>Cobalt-deuteroporphyrin-RHx</td>
<td>418</td>
<td>120 ± 10</td>
<td>419</td>
<td>1.18</td>
</tr>
<tr>
<td>Nickel-deuteroporphyrin-RHx&lt;sup&gt;z&lt;/sup&gt;</td>
<td>377</td>
<td>120 ± 10</td>
<td>419</td>
<td>1.18</td>
</tr>
<tr>
<td>Deuteroporphyrin-RHx&lt;sup&gt;z&lt;/sup&gt;</td>
<td>389</td>
<td>120 ± 10</td>
<td>419</td>
<td>1.18</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined in borate buffer, 0.025 M, pH 9.1.
<sup>b</sup> Determined immediately after addition of sodium dithionite.
<sup>c</sup> Determined from difference spectra in borate buffer.

These values are expressed as moles of ligand per mole of RHx at the equivalence point.

* Estimated by the method of Cuatrecasas et al. (25). The uncertainty in this method is strongly dependent upon the ratio of total ligand to $K_d$ and upon the protein concentration. Under these experimental conditions, $K_d$ is determined only as regards a factor of about 5 for cobalt-deuteroporphyrin. The RHx concentration was approximately $6 \times 10^{-4}$ M in each case.

† Absorption spectra data of Hrkal and Muller-Eberhard (2) and extinction coefficients of Seery et al. (3).

‡ Wave length maxima were determined by filtering equimolar mixtures of porphyrin and RHx (0.8 ml, $8 \times 10^{-4}$ M) through a column (0.8 X 18 cm) of Sephadex G-25 (Pharmacia, medium grade). Extinction coefficients were not determined because of moderate dissociation of the complexes under these conditions.

RHx exhibited the largest shift in $\lambda_{max}$, which is consistent with the known instability of heme compared with the other iron-porphyrins (21). The distinct shoulder at 290 nm of RHx was notably diminished after similar treatment; however, the protein combined readily with 1 eq of heme and yielded a hemochrome spectrum comparable with the complex prepared with fresh RHx.

Relative Affinities—Estimated $K_d$ values for the various metalloporphyrin-RHx complexes were calculated by the method of Cuatrecasas et al. (25) and are listed in Table I. Although there is unavoidable uncertainty in these estimates under the experimental conditions, strong binding is indicated for the iron-porphyrins ($K_d \approx 10^{-4}$ M) and cobalt-deuteroporphyrin ($K_d \approx 10^{-7}$ M). Since the $K_d$ (dissociation constant) of deuteroporphyrin-RHx<sup>z</sup> is about $5 \times 10^{-4}$ M, the relative affinity of RHx for these ligands is expected to be deuteroheme > deuteroporphyrin and cobalt-deuteroporphyrin > deuteroporphyrin. This was supported by the observations that in the presence of 5 eq of deuteroporphyrin, the formation of a 1:1 complex of RHx with deuteroheme or cobalt-deuteroporphyrin is unimpaired and that addition of deuteroporphyrin to equimolar complexes of deuteroheme-RHx or cobalt-deuteroporphyrin-RHx did not produce appreciable changes in the difference spectra. Similar experiments employing nickel-deuteroporphyrin demonstrated that RHx has a greater affinity for either deuteroheme or cobalt-deuteroporphyrin than for nickel-deuteroporphyrin. No displacement from an RHx-iron-porphyrin complex by another iron-porphyrin or by cobalt-deuteroporphyrin could be detected spectrally after 16 hours in phosphate buffer at room temperature.

Displacement experiments employing gel electrophoresis corroborate the spectrophotometric results. Deuteroheme (benzidine positive) displaces cobalt-deuteroporphyrin (color only), nickel-deuteroporphyrin (color only), or deuteroporphyrin (fluorescent). None of the last three displaced deuteroheme. Although cobalt-deuteroporphyrin and nickel-deuteroporphyrin are not distinguished by gel electrophoresis, the intensity of the

Soret band intensity and shift of the $\lambda_{max}$ to shorter wave length. The results obtained for all three iron-porphyrin-RHx complexes are summarized in Table II.

Addition of a small amount of the respective iron-porphyrin to the treated samples restored most of the lost Soret band intensity and demonstrated a residual binding capacity. Heme-

![Optical absorption spectra of deuteroheme-RHx complex.](image-url)
Effects of sodium dithionite on absorption spectra of iron-porphyrin-RHx complexes

<table>
<thead>
<tr>
<th>Complex</th>
<th>Time stored</th>
<th>Oxidized</th>
<th>Reduced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hrs</td>
<td>λ&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Decrease&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Deuteroheme-RHx</td>
<td>0 hrs</td>
<td>403</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48 hrs</td>
<td>403</td>
<td>1</td>
</tr>
<tr>
<td>Deuteroheme-RHx + Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0 hrs</td>
<td>403</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>48 hrs</td>
<td>403</td>
<td>45</td>
</tr>
<tr>
<td>Mesoheme-RHx</td>
<td>0 hrs</td>
<td>405</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48 hrs</td>
<td>405</td>
<td>1</td>
</tr>
<tr>
<td>Mesoheme-RHx + Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0 hrs</td>
<td>404</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>48 hrs</td>
<td>404</td>
<td>35</td>
</tr>
<tr>
<td>Heme-RHx</td>
<td>0 hrs</td>
<td>413</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48 hrs</td>
<td>413</td>
<td>5</td>
</tr>
<tr>
<td>Heme-RHx + Na&lt;sub&gt;2&lt;/sub&gt;SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>0 hrs</td>
<td>410</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>48 hrs</td>
<td>410</td>
<td>27</td>
</tr>
</tbody>
</table>

<sup>a</sup> Complexes of RHx with iron-porphyrins were made by incubating equimolar amounts (3 × 10<sup>-3</sup> moles) of each constituent in 5.0 ml of 0.1 sodium phosphate buffer, pH 7.4, for 10 min at room temperature. One-milliliter portions of each complex were examined in parallel experiments.

<sup>b</sup> The 48-hour samples were stored in the dark at 4°.

<sup>c</sup> The oxidized spectra of the samples stored in the presence of dithionite are those of the autoxidized samples. Reduced iron-porphyrin-RHx samples autoxidize readily in air after elimination of excess dithionite.

<sup>d</sup> Reduced spectra were obtained by adding sodium dithionite as in Footnote <sup>f</sup>.

<sup>e</sup> The per cent decrease was calculated from the ratio of Soret optical density to optical density at 280 nm ratio at zero time to Soret optical density to optical density at 280 nm ratio at 48 hours. The optical density at 280 nm value (71) was that of RHx prior to porphyrin addition.

<sup>f</sup> Dithionite (0.3 mg) was added to the samples from a freshly prepared 1% sodium dithionite solution in 0.1 sodium phosphate buffer, pH 7.4.

benzidine stain in gels on which cobalt-deuteroporphyrin was applied first was notably lighter than those on which nickel-deuteroporphyrin was applied first. Thus, the relative affinity of RHx for these ligands can be inferred to be heme ≈ mesoheme ≈ deuteroheme > cobalt-deuteroporphyrin > nickel-deuteroporphyrin > deuteroporphyrin.

Effect of pH on Deuteroheme-RHx Interactions—The pH dependence for the dissociation of deuteroheme from RHx between pH 3.5 and 11.5 is given in Fig. 3. Results nearly identical (not shown) were obtained for the association reaction. From pH 6.0 to 9.5, the binding of deuteroheme by RHx is equimolar and shows essentially the same hemochrome spectrum. No binding ensues below pH 4.0 or above pH 11.2, and half-maximal binding occurs at pH 4.9 ± 0.1 and 10.7 ± 0.1. Thus, ionization of groups with these apparent pK values and/or conformational changes are indicated. Based on the assumption of one binding and one non-binding form of RHx, treatment of the data using double log plots (26) yields slopes greater than 2. This suggests that at least two ionizable groups participate in the binding process. Approximately 65% of the original hemochrome spectrum of deuteroheme-RHx previously exposed to pH 3.9 or to pH 11.1 was restored by dialysis for 2 hours with phosphate buffer at 4°. It should be noted that abolition of the hemochrome spectrum does not demonstrate loss of all protein-ligand interactions.

Circular Dichroism Spectra—The CD spectrum of deuteroheme-RHx complex. A positive maximum at 231 is observed for RHx, the ellipticity of which increases nearly 50% upon binding of 1 eq of deuteroheme. The inset in Fig. 5 illustrates that this increase in ellipticity is directly proportional to the degree of saturation up to a 1:1 binding ratio. Similar results were found with heme and mesoheme, but not with deuteroporphyrin or
nickel-deuteroporphyrin, even if a 3-fold molar excess of either were added to RHx.

The mean residue ellipticity at 207 nm of apo- and deuteroheme-RHx decreases from $-6.7 \times 10^3$ to $-7.3 \times 10^3$ deg cm$^2$ per dmole and the 231 nm band disappears when the pH is lowered from 6.5 to 2 (Fig. 5). The pH dependence of the 231 nm band is shown in Fig. 6. The midpoints of the transitions are at pH 3.8 $\pm$ 0.2 and 10.0 $\pm$ 0.2 for RHx and at pH 4.5 $\pm$ 0.2 and 10.2 $\pm$ 0.2 for RHx in the presence of deuteroheme. Below pH 4 or above pH 11, the CD spectra of RHx and RHx in the presence of deuteroheme are nearly identical. This would be expected since no binding of deuteroheme seems to occur beyond these pH values (Fig. 3). The presence of deuteroheme shifts the acidic pH dependence, in terms of per cent maximum $[\theta]_{231}$, to less extreme pH values (Fig. 6A). This reflects dissociation of deuteroheme. On the other hand, the alkaline pH dependence is shifted to slightly more extreme pH values (Fig. 6B), possibly because of stabilization of the RHx molecule by deuteroheme.

The 231 nm bands of RHx and deuteroheme-RHx were also abolished by treatment with a 3:1 molar ratio (in terms of tryptophan residues) of the tryptophan oxidizing agent, N-bromosuccinimide, at 25° for 15 min in phosphate buffer.

Conformation parameters were estimated by the method of Greenfield and Fasman (27) to be 8% $\alpha$ helix, 22% $\beta$ structure, and 70% random chain at pH 7.4 and 10% $\alpha$ helix, 10% $\beta$ structure, and 80% random chain at pH 2.

**DISCUSSION**

Some of the essential features of the interactions of porphyrins with RHx were revealed by using porphyrins of varying structures. Our results emphasize the importance of the presence and nature of the metal coordinated with the porphyrin nitrogens. The iron-porphyrins are bound with indistinguishable strength by RHx in a 1:1 molar ratio. Cobalt-deuteroporphyrin IX contains a metal which, like iron, can form octahedral complexes, and also strongly interacts with RHx. Considerably weaker complexes are formed with metal-free deuteroporphyrin IX or with nickel-deuteroporphyrin IX, whose metal forms square-planar complexes only. On the other hand, the substituents in the 2,4 positions of the porphyrin rings are of lesser consequence for the binding process. These substituents do modify the optical absorption spectra, but do not alter the fundamental interaction with the apoprotein.

The estimated $K_d$ values for metalloporphyrin-RHx complexes (Table I) and the $K_d$ value obtained from deuteroporphyrin-RHx also demonstrate that suitable coordination with a metal intensifies the porphyrin-RHx interaction. Although the $K_d$ values are not exact, their order of magnitude indicates tight binding. As discussed by Weber (28), a protein-ligand equilibrium can be accurately assessed only when the protein concentration is comparable with, or smaller than, the $K_d$. The strong binding ($K_d = 10^{-4}$ to $10^{-8}$) observed and the RHx and ligand concentrations ($10^{-4}$ M) required for measurement prevented precise determinations. Nevertheless, the titration curve of RHx with deuteroheme (Fig. 1) presents a sharp break, indicating that the $K_d$ is less than $10^{-8}$ M (29). In addition, the very high affinity of hemopexin for heme was predicted by earlier data on the transfer of heme from albumin (8, 30-32), as well as from cytochrome P-450 (63) to hemopexin.

Our observations on the properties of iron-porphyrin-RHx complexes stored at 4° for 48 hours after reduction by dithionite (Table II, Fig. 2) extend those of Drabkin (8). He noted shifts to shorter wavelength and decreased intensities in the absorp-

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**Figure 5.** Ultraviolet CD spectra of RHx with various equivalents of deuteroheme. Shown are: 0 (--), 0.55 (-----), 1.1 and 2.4 (----) eq of deuteroheme added. The spectrum of RHx in the presence of 1 eq of deuteroheme at pH 2.0 is also shown (----). The insert depicts the percentage of increase in $[\theta]_{231}$ at the indicated ligand to RHx ratios. Measurements were in 0.025 M sodium borate, pH 9.1, at 25°.

**Figure 6.** The pH dependence of the ellipticity at 231 nm of RHx. The symbols used are (O—O), RHx; (•—•), RHx in the presence of 1 eq of deuteroheme. The acid pH dependence is shown in Panel A and the alkaline pH dependence in Panel B. The CD spectra were recorded at 25° from 230 to 220 nm 15 min after mixing the sample with 0.1 M sodium phosphate at the pH indicated. Equimolar amounts of RHx and deuteroheme were used. The term Percent Maximum $[\theta]_{231}$ is defined as $1 \times ([\theta]_{231 \text{ max}} - ([\theta]_{231 \text{ at pH observed}})) / ([\theta]_{231 \text{ max}} - ([\theta]_{231 \text{ min}})) \times 100$. The value used for $[\theta]_{231 \text{ max}}$ was $1.0 \times 10^4$ degree-cm$^2$ per dmole for RHx and $1.5 \times 10^8$ degree-cm$^2$ per dmole for RHx in the presence of deuteroheme. The value used for $[\theta]_{231 \text{ min}}$ was $-1.4 \times 10^4$ degree-cm$^2$ per dmole.
tion spectra of glycoprotein hemochromogens (considered to be hemopexin) and of purified heme-RHx. He proposed that a secondary interaction of the apoprotein with the 2,4-vinyl groups of the heme moiety was responsible for these changes. This view is not supported by our data, since iron-porphyrin-RHx complexes that contained no vinyl groups produced similar spectral alterations. Modification of the porphyrin during storage is probably responsible for most of the spectral changes, for the original hemochromogen pattern is largely restored by addition of freshly prepared iron-porphyrin.

Draffin also reported that heme was not bound well in the absence of dithionite, either by purified RHx or by his glycolbulin preparations (8). From this, he inferred that binding is possible only upon reduction of a sulfhydryl group which had been oxidized. On the contrary, neither rabbit nor human hemopexin has any free sulfhydryl groups, and RHx readily binds both the oxidized and reduced forms of the iron-porphyrins (Reference 2 and this work).

The CD spectra of RHx in the ultraviolet region show characteristics uncommon to hemoproteins. Whereas RHx has a shoulder at 280 nm and a peak at 292 nm like many hemoproteins, it does not show as much fine structure in the ultraviolet region as, for example, horse heart cytochrome c (34) or cytochrome b5 (35). The CD maximum at 231 nm of RHx, in addition to its 280 nm shoulder and 290 nm peak, are tentatively ascribed to tryptophan residues, which constitute 15 residues per mole or 5% of the protein, as, for example, horse heart cytochrome c (34) or cytochrome b5 (35). Both the oxidized and reduced forms of the iron-porphyrins (Reference 2 and this work).

The far-ultraviolet CD curves of RHx do not resemble those of hemoproteins consisting mainly of α helix such as myoglobin (37), but they do resemble those of glycoproteins with a high carbohydrate and random chain content (38). Formation of the heme-RHx complex increases the negative optical rotation at 223 nm (6), but we observed no significant difference in ellipticity from 225 to 200 nm between RHx and deuteroheme-RHx. Consequently, the estimated structural parameters of RHx at pH 7.4 (8% α helix, 22% β structure, and 70% random chain) change little upon complexing the deuteroheme. It should be noted that the method of Greenfield and Faeman (27) used for evaluating the conformation of RHx is weakest when applied to proteins that are largely “random chain.” Nevertheless, this suggests that although binding of deuteroheme by RHx effects easily discernible changes in tertiary structure, it produces only minimal changes in secondary structure.

The effects of pH on the interactions of RHx with deuteroheme and on the ellipticity at 231 nm demonstrate the interdependence of conformation and binding. As the pH is decreased from neutrality, the per cent binding of deuteroheme diminishes (Fig. 3), and the per cent maximum [θ]m decreases (Fig. 6A). Between pH 6.5 and 4, the decreases in binding and in ellipticity at 231 nm probably reflect ionization of residues which are directly or indirectly involved in binding. In the presence of deuteroheme, the ellipticity at 231 nm is more sensitive to acid pH. This decline in ellipticity represents loss of the ellipticity induced by the binding of deuteroheme, as well as loss of the intrinsic ellipticity of the apoprotein. Below pH 4, where no hemochrome is formed, the ellipticity at 231 nm is the same in the presence and absence of deuteroheme. The decreases in ellipticity below pH 4 represent loss of the intrinsic ellipticity at 231 nm of RHx. At pH 2 (Fig. 5), this intrinsic ellipticity is completely lost. Thus, the interaction of RHx with deuteroheme seems to require both a suitable state of ionization of key residues and of conformation, and the interaction induces tertiary conformational changes.

These observations may well bear on the mechanism for the intracellular release of heme from the heme-hemopexin complex. Since hemopexin strongly binds both ferri- and ferrethrohe, it is unlikely that an oxidation-reduction is responsible. The dissociation of heme in the liver could be mediated by a membrane-protein interaction, by a specific uptake protein (39), by a microsomal enzyme (40), or by localized conditions. Local pH has been suggested to effect the release of iron from transferrin in the red blood cell (41) and that of membrane-bound glycoproteins into the cytoplasm of parenchymal cells (42). The results discussed above are consistent with the hypothesis that a pH-dependent process operates in vivo in the dissociation of heme from hemopexin.

REFERENCES
