# Preferred Heme Binding Sites of Histidine-Rich Glycoprotein†

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ABSTRACT: The heme binding sites of rabbit histidine-rich glycoprotein (HRG), 94 kDa, were studied with rose bengal (RB), a fluorescein derivative that associates with histidine residues. Difference absorbance spectra indicate that HRG binds RB at two thermodynamically preferred sites ($K_d \sim 2$ $\mu$M) that are spectroscopically equivalent. Up to 18–22 equiv of RB can also be bound by a set of lower affinity sites. Mesoheme is capable of displacing RB from the two preferred sites ($K_d = 0.6$ $\mu$M) and provides evidence that the two sites are not identical. Two peptides isolated from plasmin-digested HRG, one 35-kDa peptide rich in histidine (~30 mol %) and one 15-kDa peptide relatively poor in histidine (~4 mol %), also bind RB and mesoheme. The two preferred RB binding sites of HRG are located on the 15-kDa histidine-poor peptide and the lower affinity "class" of sites on the 35-kDa histidine-rich peptide. Mesoheme or RB quenches the tryptophan fluorescence of HRG and the histidine-poor peptide with an apparent binding stoichiometry near 2. Fluorescence quenching also indicates that 1–2 equiv of Cu(II) binds to the 15-kDa peptide, and absorbance spectroscopy provides evidence that Cu(II) is capable of displacing heme from the peptide. The fluorescence lifetimes of RB, determined by phase-modulation fluorometry, indicate that the two preferred sites in the histidine-poor domain are more apolar than the more numerous sites located in the histidine-rich region of the protein.

Rabbit histidine-rich glycoprotein (HRG) is a 94-kDa protein containing 11.2 mol % histidine, present in serum at concentrations near 1 mg/mL (Morgan, 1981). A human congener also exists in serum (78 kDa, 9 mol % histidine), which has considerable N-terminal sequence homology with rabbit HRG as well as similar physical properties; however, it is present in concentrations 8-fold less than its rabbit counterpart (Heimburger et al., 1972; Morgan, 1978).

One of the consequences of the abundance of histidine in rabbit HRG is the protein's ability to bind 15–20 equiv of such divalent metals as Cu, Zn, Ni, Cd, and Co with $K_d$'s of 0.2–1 $\mu$M.[1] In addition, HRG is capable of binding 25 equiv of heme ($K_d = 1$ $\mu$M) at many of the same sites that bind divalent metal (Morgan, 1981). Chemical modification of the histidine residues by diethyl pyrocarbonate results in a greatly diminished metal and heme-binding affinity. In addition, metal binding by HRG is pH dependent, indicating a loss of binding ability as histidine residues protonate (Morgan, 1981).

Another notable feature of HRG is the diversity of its associations in vitro in addition to binding heme and metals; for example, HRG interacts with a subset of T lymphocytes (Lijnen et al., 1983b) and binds the polysaccharide heparin with high affinity ($K_d = 10$ nM) (Lijnen et al., 1983a) as well as plasminogen ($K_d = 1$ $\mu$M) (Lijnen et al., 1980) and thrombospondin (Leung et al., 1984). Perhaps due to this plethora of interactions, no unique physiological function has been directly attributed to HRG; moreover, the protein's metal- and heme-binding capabilities are likely to influence the above interactions and may as well influence metal homeostasis (Guthans & Morgan, 1982). Thus, it is important to characterize more fully the several heme and metal binding sites in HRG and to determine whether "classes" of binding sites occur within the protein, i.e., thermodynamically preferred sites.

A convenient spectroscopic probe for heme binding sites is rose bengal (RB) (Coulson & Yonetani, 1972; Morgan & Muller-Eberhard, 1975), a halogenated fluorescein derivative, which associates with histidine residues via electron transfer (Sidorowicz, 1978). In addition, the fluorescence lifetime of RB is dependent upon the polarity of its environment (Fleming et al., 1977) and provides a means to evaluate the relative hydrophobicity of heme binding sites.

Results obtained by study of peptides isolated from plasmin-digested HRG indicate that HRG contains one domain rich in histidine–proline–glycine contained in a 35-kDa peptide of 30 mol % histidine and another domain with more normal histidine, proline, and glycine contents represented by 15- and 45-kDa peptides of 4 mol % histidine (Morgan, 1985). These peptides provide a means to determine the locale of specific binding sites within the protein.

Reported here are studies of the binding of RB, several other halogenated fluorescein derivatives, and mesoheme to rabbit HRG and isolated peptides. Absorption and fluorescence techniques were used to obtain information on the specificity, hydrophobicity, and locale of the dye and heme binding sites.

## Materials and Methods

Histidine-rich glycoprotein was isolated from rabbit serum by procedures previously described (Morgan, 1981). Protein solutions were freshly prepared in buffer (vide infra) from lyophilized protein, and the concentration of protein was assessed from the absorbance at 280 nm with an extinction of 55 500 $M^{-1}$ $cm^{-1}$.

Buffers used were 0.1 M or 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.4, $\mu = 0.1$ M (NaCl), or 10 mM potassium phosphate, pH 7.4, $\mu = 0.1$ M (KCl). These were prepared from deionized water, which was then treated with a Barnstead Nano-pure system (final resistivity >15 MΩ). Identical results were obtained with either buffer.

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[1] These numbers differ from those published previously by a factor of 94 000/58 000, which corrects for the most recent molecular weight determination for rabbit HRG (W. T. Morgan, unpublished results).

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Buffered solutions of RB (Eastman Organic Chemicals, 87% pure) were prepared daily, and the dye concentration was determined by the absorbance at 545 nm ($\epsilon_{545}$ = 95 000 $M^{-1}$ $cm^{-1}$) (Coulson & Yonetani, 1972). Solutions of eosin Y and erythrosin B (Aldrich, 85% and 91% pure, respectively) and rhodamine B and fluorescein (both from Sigma, 95% pure) were prepared by weighing appropriate amounts on a Cahn electrobalance and dissolving it in a known amount of buffer. Concentrations were determined from absorbance (fluorescein diacetate, $\epsilon_{493}$ = 75 000 $M^{-1}$ $cm^{-1}$) (Brusman, 1971) or by calculation from known purity.

Iron(III) mesoporphyrin IX chloride (mesoheme, Porphyrin Products) was dissolved in dimethyl sulfoxide and its concentration was assessed from the absorbance at 394 nm ($\epsilon_{394}$ = 170 000 $M^{-1}$ $cm^{-1}$) in dimethyl sulfoxide. $CuCl_2$ (Aldrich) was dissolved in deionized water, and the solution was adjusted to pH 3 to prevent hydrolysis. Standardization of the $CuCl_2$ stock solution was performed by direct titration with primary standard disodium ethylenediaminetetraacetate ($Na_2EDTA$) and with 0.1% pyrocatechol violet indicator (Welcher, 1958).

Absorbance spectra were recorded on a Cary 210 or 219 spectrophotometer in auto-gain mode at ambient temperature with a 0.2 nm/s scan rate and 0.1–1.0-mm slit widths. Baselines were routinely subtracted from spectra.

Fluorescence emission spectra were obtained on a Perkin-Elmer 650-40 spectrofluorometer in the ratio mode with the auto zero at ambient temperature. For study of tryptophan quenching of HRG, the excitation wavelength was 290 nm and the emission was monitored between 310 and 390 nm. Polarization measurements of the emission maximum of the excited dye were performed with Polaroid polarizers, which resolved the vertical and horizontal components of the excitation and emission beams. Fluorescence polarization, $P$, was calculated according to

$$P = (I_{vv} - GI_{vh})/(I_{vv} + GI_{vh})$$

where $I$ is the fluorescence intensity and $G = I_{hv}/I_{hh}$, with the subscripts h and v referring to the vertical or horizontal orientation of polarization of excitation and emission beams, respectively. The $G$ factor corrects for the inequivalent transmittance of polarized light.

HRG was digested with plasmin, and peptides were isolated by reverse-phase high-performance liquid chromatography (HPLC) after reduction and carboxymethylation as described (Morgan, 1985). Plasmin-digested HRG yields two types of peptides: a histidine-rich domain (35 kDa, ~30 mol % histidine) and two peptides (45 and 15 kDa) with more normal histidine, proline, and glycine contents (~4 mol % histidine). Concentration of peptides was assessed from the absorbance at 220 nm, which is approximately 11.5 for 1 mg/mL solutions of these peptides (Morgan, 1985).

Binding titrations were performed with a Hamilton 100-$\mu$L syringe fitted to a Hamilton repeating dispenser, which allows 2 $\mu$L of solution to be dispensed per addition. Concentrations were typically 1–8 $\mu$M for protein and peptide and 0.3 mM for stock solutions of the dyes and mesoheme. Volume changes did not exceed 5% of the initial volume.

The total amount of RB bound to HRG in the difference absorbance experiments was calculated from $\Delta\epsilon_{567}$ = 62.2 $mM^{-1}$ $cm^{-1}$, determined at 50:1 HRG:RB ratios. The amount of free RB was calculated for the Scatchard plots by subtracting the experimentally found differential absorbance at 567 nm from that calculated with the extinction coefficient.

Fluorescence lifetimes of RB were measured by phase modulation (Lakowicz, 1983) with a SLM 4800 spectrofluorometer equipped with an acoustooptic Debye-Sears LM48 light modulator filled with 19% v/v ethanol in water to provide a 30-MHz modulation frequency at 20 °C (Spencer & Weber, 1969). The extinction wavelength used was 550 nm for all samples. Although this wavelength is not the absorbance maximum of all samples, it is well within the major absorbance of rose bengal. Moreover, lifetime values did not significantly differ if the excitation wavelength was changed to other values within that absorbance band. Several different references were used, including an oyster glycogen scattering reference and reference fluorophores of known lifetimes such as 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) and dimethyl-POPOP (Lakowicz et al., 1981). All references gave essentially the same lifetimes with the phase method; however, values for the modulation of the RB sample were always larger than that of the reference (scattering solution or fluorophore reference), prohibiting the use of the phase and modulation values to determine whether the fluorescence observed with RB was a simple exponential. The reason for this modulation problem in determinations of short lifetime is due to photomultiplier artifacts also observed in other laboratories (Lakowicz et al., 1981). Measurement of samples with known lifetimes such as POPOP ($\tau$ = 1.35 ns) vs. a scattering solution gave good agreement with the literature values from both phase and modulation results (Lakowicz et al., 1981). In addition, the lifetime values of RB in a variety of solvents obtained here through phase lag were within 5–20% of literature values obtained by pulse methods (Fleming et al., 1977), indicating that the instrument was correctly tuned.

## Results and Discussion

A titration of the binding of RB by HRG with difference absorbance spectroscopy is shown in Figure 1. During the addition of the first 2 equiv of RB a shift in $\lambda_{max}$ from 566.5 to 569 nm with isosbestic behavior is observed, indicating an equilibrium between two absorbing species. The extinction at 567 nm observed at low RB:HRG ratios is 62.2 $mM^{-1}$ $cm^{-1}$ in good agreement with the value of 65.0 $mM^{-1}$ $cm^{-1}$ reported for RB binding to the heme site of cytochrome $c$ peroxidase (Coulson & Yonetani, 1972). Approximately 20 additional equiv of RB also bind to HRG with a $\lambda_{max}$ at 569–571 nm and a shift in isosbestic point from that observed from 0–2 equiv, suggesting that a different "class" of sites exists. The total binding of RB to HRG is more clearly observed by monitoring the difference absorbance minimum at 505 nm (Figure 1, inset), since absorbance changes observed here are not as large as those at 570 nm. The binding of 20 equiv of RB to HRG is not unexpected since the dye interacts with histidine residues; in addition, this stoichiometry correlates with the reported stoichiometry of heme binding to HRG (Morgan, 1981). A modified Scatchard plot of data gathered at RB:HRG ratios below 2 equiv indicates a binding stoichiometry of 2 and a $K_d$ of 2 $\mu$M (Figure 2A). Data beyond 1.3 equiv are influenced by the next class of sites (see inset of Figure 2A), since the $K_d$ value for the second class of sites is ~10 $\mu$M. However, the inset clearly illustrates the existence of two distinct linear regions representing two classes of RB binding sites in HRG. The existence of two preferred, indistinguishable binding sites for RB in HRG is unexpected since HRG contains no subunits.

If the preferred RB binding sites are heme binding sites of HRG and if RB is bound with lower affinity, heme should displace RB from those sites. Figure 3 illustrates that this does occur. The protein was equilibrated with 1 equiv of RB, then heme was added, and the absorbance of RB was monitored. Throughout the addition of up to 2 equiv of heme an isosbestic point was maintained, and the $\lambda_{max}$ gradually shifted from 559 (no heme added) to 563 nm (1.8 equiv of heme added). After

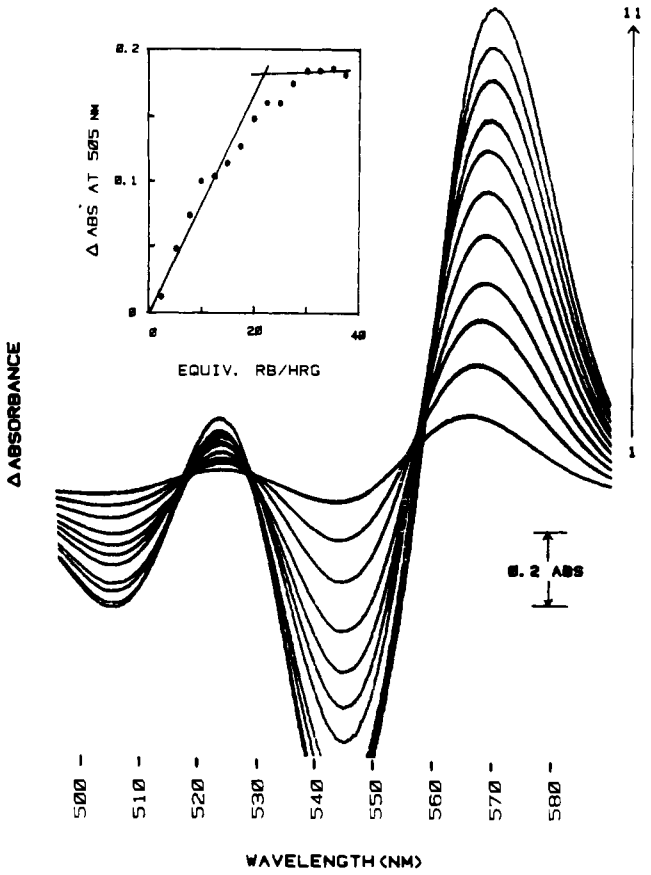


FIGURE 1: Difference absorption spectra of HRG titrated with RB. The sample cuvette contained protein and RB; the reference cuvette, RB only. The RB:HRG ratios and wavelength maxima for curves 1–11 were as follows: 0.5, 566.5 nm; 1.0, 567.5 nm; 1.5, 568.5 nm; 1.9, 569.0 nm; 2.4, 569.0 nm; 2.9, 569.5 nm; 3.4, 569.5 nm; 3.8, 570.0 nm; 4.3, 570.0 nm; 4.8, 570.0 nm; 5.3, 570.5 nm. The concentration of HRG was 4.6 $\mu$M in 0.1 M Hepes, pH 7.4. The inset shows the change in absorbance at 505 nm as a function of added RB. The concentration of HRG was 1.0 $\mu$M in 10 mM phosphate buffer, pH 7.4.

the addition of 2 equiv of heme, there was very little change in the absorbance of RB, indicating that RB was displaced from the first class of sites and then bound to the next class of sites:

$$\text{RB–class 1} + \text{2heme} \rightarrow \text{RB–class 2} + \text{heme–class 1}$$

If the absorbance spectrum of RB bound to the two class 1 sites (preferred) is significantly different from the absorbance spectrum of RB bound to the numerous class 2 sites, then isosbestic behavior of the absorbance spectra throughout the equilibrium will occur. Heme binding to HRG is observed to be significantly tighter than RB binding to HRG since only 2 equiv of heme is required to complete the displacement. More heme would be required if the affinities were similar and RB was effectively competing for the sites. RB bound to the second class of sites can also be seen by the $\lambda_{max}$ = 570 nm of the difference absorbance spectrum observed when RB was added to the 2heme–HRG complex (data not shown). Comparison with the values listed in Figure 1 shows that only after the addition of 2 equiv of RB to HRG does the $\lambda_{max}$ exceed 570 nm.

A peptide ($M_r$ 15 kDa) isolated after plasmin digestion of HRG also binds 2 equiv of RB preferentially (Figure 2B). The difference absorption $\lambda_{max}$ for these titrations never exceeds 569 nm (data not shown), similar to the binding of the first 2 equiv of RB to intact HRG. Therefore, the two preferred RB binding sites appear to be located in this 15-kDa peptide.

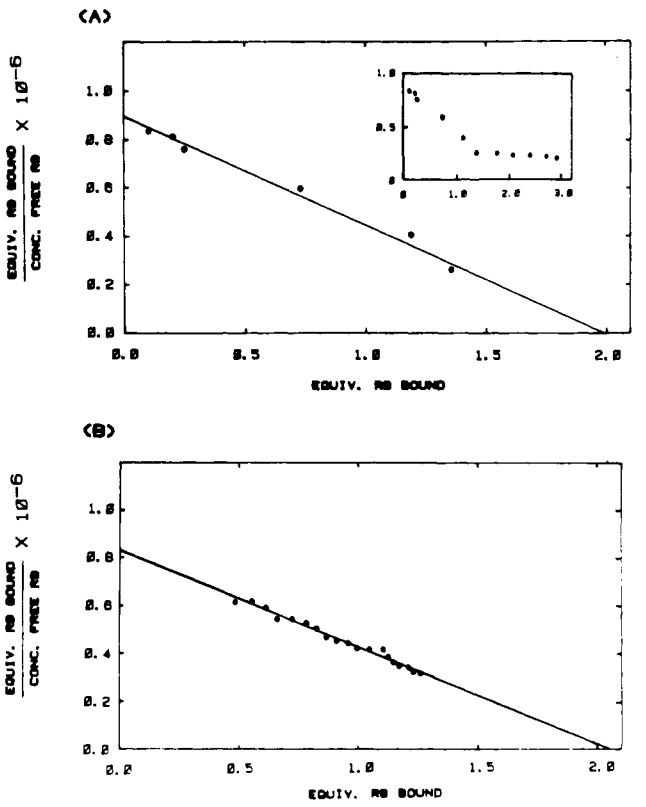


FIGURE 2: Modified Scatchard plots of RB binding to HRG and the 15-kDa peptide. Data were obtained from difference absorption spectral titrations. RB binding to 6.8 $\mu$M HRG (panel A) and RB binding to 3.0 $\mu$M 15-kDa peptide (panel B) are shown. The inverse of the slope gives the $K_d$ value shown in Table I. The inset of panel A shows data at higher equivalents of RB bound (same data as panel A included).

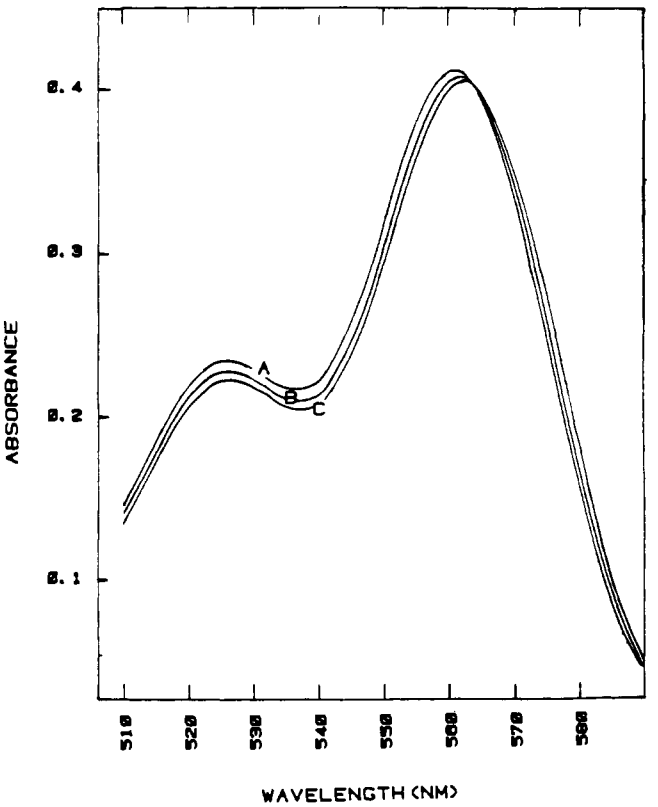


FIGURE 3: Absorption spectra of RB–HRG in the presence of mesoheme. Spectra shown are with (A) 0.6, (B) 1.2, and (C) 1.8 equiv of mesoheme added. The RB concentration was 5.5 $\mu$M, and that of HRG was 5.4 $\mu$M, in 0.1 M Hepes, pH 7.4.

Table I: Summary of Binding of RB and Heme to Preferred Sites in HRG and the 15-kDa Peptide

| | fluorescence | | absorbance | |
|---|---|---|---|---|
| | no. of preferred sites | $K_d$ ($\mu$M) | no. of preferred sites | $K_d$ ($\mu$M) |
| | Rose Bengal Binding[a] | | | |
| HRG | 2 | 0.4 | 2 | 2.2 |
| 15-kDa peptide | 2 | 0.2 | 2 | 2.5 |
| 35-kDa peptide[b] | na[c] | na | na | 10–20 |
| | Mesoheme Binding[a] | | | |
| HRG | 2 | 0.1 | 2 | 0.6 |
| 15-kDa peptide | 2 | 0.2 | 1 | 1 |

[a] From modified Scatchard analysis, i.e., plots of equivalents bound/free conc. vs. equivalents bound. The $x$-axis intercept indicates the number of preferred sites, and $-1$/slope is the $K_d$ value. [b] A histidine-rich peptide that contains no aromatic amino acid residues necessary to perform fluorescence quench experiments (Morgan, 1985). [c] na, not applicable.

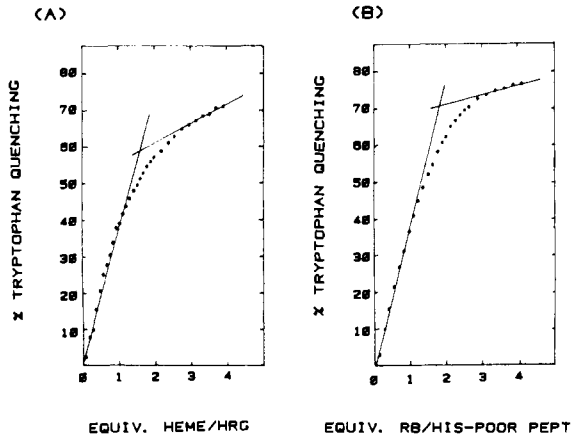


FIGURE 4: Quenching of HRG tryptophan fluorescence by RB and mesoheme. Panel A shows the titration of 2.0 $\mu$M HRG by mesoheme and panel B the titration of the 15-kDa peptide (2.0 $\mu$M) by RB. The buffer was 0.1 M Hepes, pH 7.4.

Notably, this peptide is relatively poor in histidine (4 mol %) compared to intact HRG (11.2 mol %).

RB also binds to the histidine-rich 35-kDa peptide ($\sim$30 mol % histidine) obtained after plasmin digestion of HRG producing difference absorbance spectra with $\lambda_{max}$ = 570 nm (data not shown). This implies that the second class of RB binding sites, not unexpectedly, resides primarily on the histidine-rich domain of the protein. These binding sites are not as tight as those residing on the 15-kDa peptide, as the $K_d$ is 10–20 $\mu$M for RB binding to the histidine-rich peptide (see Table I). This peptide has also been shown to bind 20–30 equiv of heme (Morgan, 1985).

In addition to absorbance measurements, quenching of the intrinsic tryptophan fluorescence of HRG as a function of added ligand was also used to monitor binding of RB or mesoheme. Previous work on heme binding to HRG showed a total of about 25 hemes bound per 94-kDa molecule by absorbance spectra of the heme chromophore.[1] However, only 1–2 equiv of heme was necessary to fully quench the intrinsic tryptophan fluorescence of the protein, consistent with the existence of preferred heme binding sites (Morgan, 1981). Figure 4 illustrates the fluorescence quenching of HRG by mesoheme (panel A) and of 15-kDa peptide by RB (panel B). Likewise, the 15-kDa peptide was titrated by mesoheme and HRG by RB, with all titrations indicating a stoichiometry near 2 equiv of either heme or RB per HRG or peptide. Further decreases in fluorescence beyond 2 equiv can be attributed to

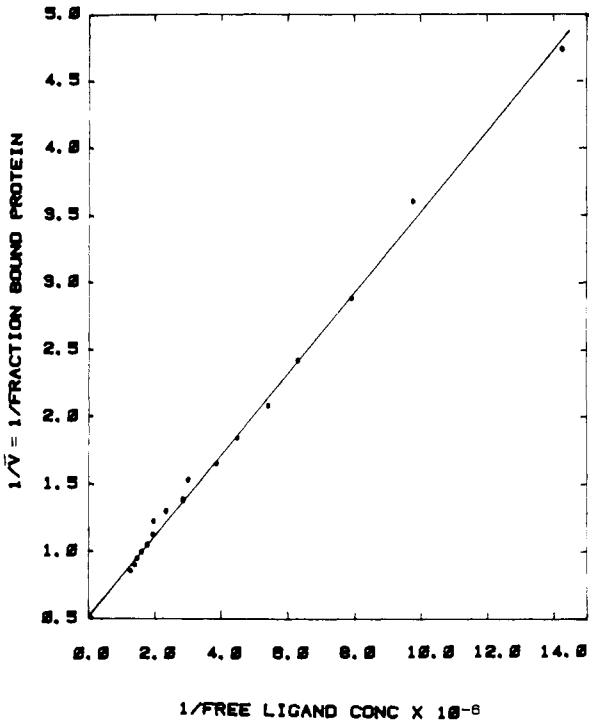


FIGURE 5: Double-reciprocal plot of mesoheme–HRB binding. The titration of 2.5 $\mu$M HRG with mesoheme in 10 mM sodium phosphate buffer, pH 7.4, was monitored by changes in the Soret absorbance band. In this plot, 1/intercept is the number of preferred sites, and the $K_d$ is the slope/intercept.

screening effects of added chromophores, which have not been subtracted out of Figure 4. With excitation at 290 nm, the emission maximum is 342 nm for HRG and 350 nm for the 15-kDa peptide. The red shift in emission maximum implies a greater exposure to solvent of the tryptophan residues of the peptide than in the intact protein. It should be noted that the histidine-rich peptide does not contain aromatic residues (Morgan, 1985) and, hence, does not fluoresce. The intrinsic fluorescence of the 45-kDa peptide is quenched by the addition of 2 equiv of RB, identical with quenching observed in the 15-kDa peptide, indicating that the 15-kDa peptide may be a fragment of the 45-kDa peptide that contains the two preferred binding sites.

The binding of heme, measured with changes in absorption of the Soret band of heme as it complexes with HRG, can be represented by

$$\frac{1}{\bar{v}} = \frac{1}{n} + \frac{K_d}{n[\text{free ligand}]}$$

where $\bar{v}$ is the fraction of bound protein and $n$ is the number of binding sites (see Figure 5; Thompson & Klotz, 1971). From this analysis it is clear that two sites are equivalent and preferred with a $K_d$ of 0.6 $\mu$M (Table I).

The fluorescence and absorbance data are summarized in Table I. It is apparent that by fluorescence quenching and absorbance measurements there are two equivalent sites for RB in the peptide and in the protein; however, there is a discrepancy in $K_d$ values obtained by different physical methods. Whereas fluorescence quenching of tryptophan residues measures binding only to sites near the tryptophan residues (the preferred sites), the difference absorbance titrations cannot effectively separate the two classes of sites and there may be some overlap that would artificially raise the $K_d$ value for the preferred sites. This does not appear to be the case from Figure 2, but it is a consideration. Mesoheme binding to HRG gives similar values for $K_d$ and two preferred

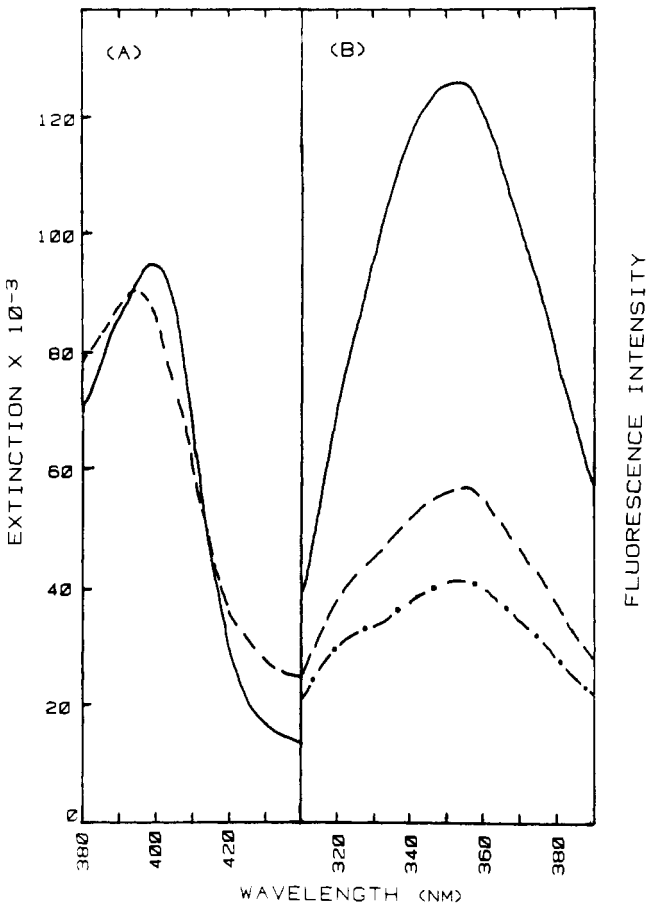


FIGURE 6: Panel A illustrates the change in the Soret band of heme in the presence of excess 15-kDa peptide as a function of added Cu: (—) no Cu; (--) 1.5 equiv of Cu. The concentration of 15-kDa peptide was 5.7 $\mu$M, and the heme concentration was 0.13 $\mu$M, in 10 mM phosphate, pH 7.4. Panel B is the emission fluorescence spectra of tryptophan in the 15-kDa peptide as a function of added copper. The spectra shown are with (—) no Cu, (--) 0.9 equiv of Cu, and (-·-) 2.1 equiv of Cu added. The excitation wavelength was 290 nm. The concentration of 15-kDa peptide was 6.3 $\mu$M in 10 mM phosphate buffer, pH 7.4.

sites of interaction. However, binding of mesoheme to the 15-kDa peptide gives rise to different results with the different techniques. One explanation of this difference is that the absorption band in the Soret region is sensitive to the type of axial coordination to the heme iron. If the histidine residues are not bound to the heme iron, the absorption spectra may be similar to that of free heme. However, if the mesoheme is loosely associated with the binding sites perhaps through hydrophobic interactions of the porphyrin ring, this can just as effectively quench tryptophan fluorescence as can a mesoheme bound to histidine residues. Figure 6A shows the absorbance spectra of mesoheme bound to excess 15-kDa peptide. Addition of heme beyond 0.5 equiv yields a spectrum indicating a considerable amount of free heme is present. It is not surprising that this peptide does not retain the full structural integrity of the intact protein, but it is curious that the preferred RB binding sites are conserved. Perhaps the plasmin cleavage site is contiguous to one preferred binding site, and this site may not bind mesoheme through histidine ligation but through $\pi$ interactions.

Since ~20 equiv of Cu(II) bind to HRG (Morgan, 1981), determining whether classes of sites exist for this and other divalent metal ions and whether sites are capable of accomodating several different ions is of interest. The Soret band of the heme complex of the 15-kDa peptide shifts to that of free heme upon the addition of Cu(II) (Figure 6A). In addition, 1–2 equiv of Cu(II) fully quench the intrinsic fluorescence of the 15-kDa peptide (Figure 6B). These observations imply that the preferred heme binding sites are also sites of Cu(II) binding. However, equilibrating HRG with 15 equiv of Cu(II) prior to titration with RB does not alter the difference absorption spectrum from that of RB and HRG alone. It is plausible that in the presence of a small Cu(II) ion RB can still form charge-transfer complexes with histidine residues. Recent EPR and absorption spectroscopy experiments on HRG demonstrate conclusively that the preferred heme binding sites are affected by the addition of Cu(II), as the heme is observed to change from low to high spin. This spin-state change of heme does not occur until at least 3 equiv of Cu(II) have been added, indicating that the Cu(II) may occupy "preferred" sites of its own, perhaps in the histidine-rich domain of the protein.[2]

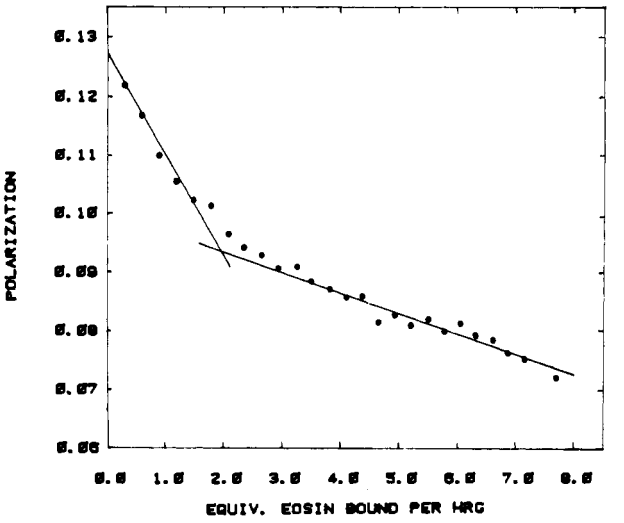


FIGURE 7: Changes in polarization of the eosin Y fluorescence emission upon binding to HRG. Eosin Y was added to 1.7 $\mu$M HRG in 10 mM Hepes, pH 7.4, and the fluorescence polarization measured. Free eosin Y in buffer has a polarization of 0.06.

Other fluorescein derivatives also bind to HRG. Both erythrosin B (the same structure as RB without chlorine) and eosin Y (in which bromine is substituted for iodine in erythrosin B) bind to HRG (12 equiv) and produce quenching of intrinsic fluorescence and changes in absorbance identical with that observed with RB (data not shown). Figure 7 illustrates the binding of eosin Y to HRG as monitored by changes in the polarization of the eosin Y emission. As with RB, 2 equiv of eosin Y are seen to bind preferentially to HRG with a $K_d$ similar to that of RB and HRG. Interestingly, fluorescein and rhodamine B are not bound by HRG, suggesting that only halogenated derivatives of fluorescein are bound

The fluorescence lifetime of RB has been shown to be highly dependent on the solvent (Fleming et al., 1977). In fact, the lifetime of RB in 2-propanol is 10 times longer than that in water. Therefore, the fluorescence lifetime of RB was used to probe the hydrophobicity and polarity of the RB binding sites in HRG. Table II summarizes the results of fluorescence phase lag measurements of RB in buffer, RB bound to HRG at various ratios, and RB bound to isolated peptides. It is apparent that 1 equiv of RB bound to HRG or to the 15-kDa peptide, at one or the other of the two preferred sites, is in a more hydrophobic environment than RB bound at higher ratios of RB to HRG or RB bound to the histidine-rich peptide.

In conclusion, HRG is capable of binding 20–25 equiv of heme or RB. RB ($K_d$ ~ 2 $\mu$M) and heme interact with two

[2] M. K. Burch and W. T. Morgan, unpublished results.

Table II: Fluorescence Lifetimes of Rose Bengal by Phase Modulation

| environment | lifetime (ps) |
|---|---|
| water[a] | 95 ± 15 |
| methanol[a] | 655 ± 85 |
| ethanol[a] | 820 ± 95 |
| 2-propanol[a] | 1015 ± 110 |
| 10 mM phosphate buffer, pH 7.4 | 363 ± 94 |
| HRG + 1 equiv of RB[b] | 593 ± 50 |
| HRG + 5–6 equiv of RB[b] | 510 ± 50 |
| His-Pro-Gly-rich peptide (35 kDa) + 1 equiv of RB[b] | 350 ± 81 |
| His-Pro-Gly-poor peptide (15 kDa) + 1 equiv of RB[b] | 530 ± 140 |

[a] Confirmed values from Fleming et al. (1977). [b] Measured in $\mu$ = 0.1 M, 10 mM phosphate buffer, pH 7.4.

"preferred" apolar sites in a region of HRG that is not rich in histidine. The preferred interaction with RB was demonstrated by visible absorbance spectroscopy and by the quenching of tryptophan fluorescence of HRG upon RB binding. The fluorescence lifetimes of RB bound to HRG or to isolated domains indicate that the two preferred sites are apolar relative to solvent or to the other RB binding sites present in HRG. The second class of sites are contained in the histidine-rich domain.

Upon addition of mesoheme, RB is displaced from HRG, showing that heme is bound even to the preferred RB sites with greater affinity. Analysis of the fluorescence and absorbance spectral data leads to the conclusion that two "preferred" heme binding sites exist with a $K_d \sim 1$ $\mu$M.

A 15-kDa peptide of HRG retains two tight sites for RB and one for heme. Copper(II) displaces heme from this peptide and also quenches its intrinsic fluorescence. Therefore, Cu(II) can occupy the preferred RB and heme binding sites.

## ACKNOWLEDGMENTS

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**Registry No.** RB, 11121-48-5; copper, 7440-50-8; mesoheme, 18040-04-5; eosin Y, 17372-87-1.

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