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Further Characterization of the Interaction of Histidine-Rich Glycoprotein with Heparin: Evidence for the Binding of Two Molecules of Histidine-Rich Glycoprotein by High Molecular Weight Heparin and for the Involvement of Histidine Residues in Heparin Binding†

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ABSTRACT: Rabbit histidine-rich glycoprotein (HRG, 94 kDa) binds heparin with high affinity (apparent K_d 60–110 nM). Eosin Y (1 equiv) bound to HRG was used as a reporter group to monitor associations of HRG with heparins of molecular mass 10, 17.5, and 30 kDa. The stoichiometries of the heparin–HRG complexes were determined by fluorescence and absorbance measurements as well as by analytical ultracentrifugation. Two types of complex form: complexes of 1 heparin:1 HRG and of 1 heparin:2 HRG. The 1:2 complex formation requires a minimum heparin chain length since 17.5-kDa but not 10-kDa heparin binds two HRG molecules. The formation of the 1:2 complexes of the larger heparin fractions is enhanced by divalent copper or zinc (1–10 equiv) bound to HRG. However, metal is not required for complex formation since all sizes of heparin examined interact tightly with HRG in the presence of ethylenediaminetetraacetic acid. Between 0.1 and 0.3 M ionic strength, both 1:1 and 1:2 complexes of heparin with HRG are progressively destabilized. No heparin–HRG complex is found at ionic strengths of 0.5 M. Between pH 8.5 and pH 6.5 both 1:2 and 1:1 complexes are found with 17.5-kDa heparin, but at pH 5.5 only 1:1 complexes are formed. The heparin–HRG interaction is progressively decreased by modification of the histidine residues of HRG, whereas modification of 22 of the 33 lysine residues of HRG has little effect. Supporting the role of histidine in heparin binding, a histidine–proline–glycine-rich peptide (molecular mass 28 kDa) derived from HRG and intact HRG binds to heparin–Sepharose at pH 6.8, but only HRG binds to the affinity medium at pH 7.4.

Histidine-rich glycoprotein (HRG) is a serum protein known to interact with heparin (Koide et al., 1982), plasminogen (Lijnen et al., 1980), thrombospondin (Leung et al., 1984), fibrinogen and fibrin (Leung, 1986), metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}) (Guthans & Morgan, 1982), and iron porphyrins (Morgan, 1978, 1981). Despite these known associations in vitro, the physiological function of HRG remains unclear. The interaction of human HRG with the anticoagulant heparin (a single-stranded negatively charged polysaccharide) has been the subject of several studies. The heparin binding activity of HRG has been examined indirectly by monitoring the formation of platelet aggregates (Kindness et al., 1984) and the inhibition of proteases by antithrombin III (Lijnen et al., 1983; Lane et al., 1986) and heparin cofactor II (Tollefsen & Pestka, 1985). HRG was found to bind heparin with similar affinity to antithrombin III and platelet factor 4 (Tollefsen & Pestka, 1985) and to bind dermatan sulfate only weakly. In addition, HRG binds heparin of molecular weight larger than 5400 better than smaller forms and has no preference for high-affinity heparin (Lane et al., 1986). In view of the known metal-binding ability of HRG,

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an interesting observation in former studies (Lijnen et al., 1983; Tollefsen & Pestka, 1985; Lane et al., 1986) was that ethylenediaminetetraacetic acid (EDTA) was reported to prevent the formation of the human HRG–heparin complex. However, major questions remain as to the stoichiometry of the heparin–HRG complex, the mechanism of binding, and the location of the heparin binding site(s) on the HRG molecule.

In the present study we have used rabbit HRG and molecular weight fractionated heparin to study heparin–HRG complex formation by direct physical methods. Rabbit HRG (M_r 94K, 11.2 mol % histidine) demonstrates all the interactions of its human congener; i.e., it binds the same set of ligands including plasminogen[1] and heparin (this work). In addition, the N-terminal amino acid sequence of rabbit HRG[1] is homologous to that of human HRG (Koide et al., 1986b) and has a domain that is rich in histidine, proline, and glycine (Morgan, 1985). Koide et al. (1986a) suggested that because of sequence homology, the heparin binding site of HRG might resemble that of antithrombin III. However, this possibility remains to be shown directly, and the large histidine content of HRG and consequent potential for ionic interactions with heparin raise the additional possibility that HRG also binds heparin via histidine residues in its histidine-rich domain.

Rabbit HRG has two thermodynamically preferred binding sites for fluorescein derivatives such as eosin Y and rose bengal (Burch & Morgan, 1985). We show here that eosin Y bound to HRG can be used as a reporter group to monitor directly the interactions of HRG with heparin since binding of heparin to eosin–HRG induces changes in the absorbance and fluorescence spectra of the protein-bound dye. In addition, analytical ultracentrifugation of HRG–heparin complexes was used to substantiate the stoichiometry of the complexes formed between the protein and the mucopolysaccharide. The effects of pH, ionic strength, EDTA, metals, and chemical modification of histidine and lysine residues of HRG on its ability to form complexes with heparin were also examined to define further the heparin–HRG interaction. Finally, chromatography on heparin–Sepharose of a His-Pro-Gly-rich peptide isolated from HRG after limited proteolysis (Morgan, 1985) was used to test whether the histidine-rich domain of HRG can bind heparin.

Materials and Methods

Rabbit HRG was isolated as previously described by ion-exchange chromatography (Morgan, 1981), and the protein migrated as a single band (apparent M_r 94K) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), confirming protein purity. The characteristic absorbance spectrum of HRG was used to quantitate HRG solutions (E_{278} of 50 mM^{-1} cm^{-1}). HRG was digested with plasmin, and peptides were isolated and characterized as previously described (Morgan, 1985). Due to differences in electrophoresis techniques, the current estimates of peptide molecular weights differ slightly from those previously reported.

Histidine modification with diethyl pyrocarbonate (Sigma) was accomplished by addition of 2–10 μL of a 0.2 M diethyl pyrocarbonate solution in ethanol to 2.0 mL of a 4.5 μM solution of HRG (10 mM phosphate, pH 7.4) at 4 °C. The reaction was complete within 1 h, and the difference absorption spectrum (modified HRG versus unmodified HRG) from 270 to 230 nm was recorded. The concentration of modified histidine was ascertained with an E_{240} of 3.2 mM^{-1} cm^{-1} (Miles, 1977). The percent modification was then calculated by assuming 77 total histidine residues in HRG.

O-Methylisourea hydrogen sulfate was used to guanidinate lysine residues (Kimmel, 1967). The reaction mixture was 2 mL of 0.3 M *O*-methylisourea, 10 mg/mL HRG, and 10 mM EDTA at pH 10. The reaction was allowed to proceed for 3 days at 4 °C, the pH checked occasionally, and modification stopped by addition of an equal volume of 1 M phosphate buffer, pH 5. The modified protein solution was then dialyzed against 5 mM phosphate, pH 7.5, and lyophilized. The extent of modification was determined by amino acid analysis of the protein after hydrolysis in 6 M HCl for 24 h at 110 °C in vacuo. The decrease in the lysine peak and the appearance of a homoarginine peak were monitored with a Glenco modular amino acid analyzer. A total of 23 out of 33 lysines were modified.

Unless stated otherwise, the buffer used was 10 mM in sodium phosphate, pH 7.4, at a total ionic strength of 0.10 M (NaCl) containing 1 mM Na_2EDTA. Higher ionic strengths were obtained by the appropriate addition of NaCl.

Solutions of eosin Y (Aldrich, 85% purity) were prepared by dissolution of the weighed solid in water in a volumetric flask. This technique allowed determination of an extinction coefficient at 516 nm (98 mM^{-1} cm^{-1}) by serial dilution in μ = 0.10 M, 10 mM phosphate, pH 7.4. Eosin Y stock solutions (0.3 mM) were stable for months when kept at 4 °C and protected from light.

Samples of heparin (Sigma) [5 g in 50 mL of 0.02 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)–0.15 M NaCl, pH 7.4] were chromatographed on tandem Sephacryl S-300 columns (each 2.5 × 100 cm) equilibrated with the same buffer. The concentration of heparin in the eluate was quantitated with Azure A (Jacque et al., 1949). Individual fractions were used directly or further fractionated on an affinity column of antithrombin III coupled to activated CH-Sepharose (Nesheim, 1983). Heparin with high affinity for antithrombin III was eluted with 2 M NaCl in 0.02 M Tris-HCl, pH 7.4. The molecular weight of heparin fractions was determined by sedimentation equilibrium ultracentrifugation (Yphantis, 1964) in 0.02 M Tris-HCl–2.0 M NaCl, pH 7.0, with a partial specific volume of 0.47 mL g^{-1} (Lasker & Stivala, 1966). Three major pools consisting of 10-kDa, 17.5-kDa, and 30-kDa heparin fragments and one of high-affinity heparin were studied. Stock solutions (0.2 mM) were stored frozen at −20 °C.

Fluorescence measurements were made with a Perkin-Elmer 650-40 fluorescence spectrophotometer in ratio mode using the auto zero feature. Excitation and emission slit widths were set at 5 nm. Titrations of the quenching of HRG tryptophan fluorescence (3–5 μM) with eosin Y were performed with an excitation wavelength of 290 nm, the emission being monitored from 310 to 380 nm (emission maximum at 342 nm). Titrations of HRG–eosin Y complex with heparin used an excitation wavelength of 516 nm, and emission was monitored from 520 to 560 nm (emission maximum at 542 nm). A Hamilton repeating dispenser was used to deliver 2 μL of titrant per addition, and the total volume changes were less than 5%. Absorbance spectral titrations of the HRG–eosin Y complex with heparin were carried out on a Cary 219 spectrophotometer.

Sucrose gradients (5 mL, 35%–20%, Beckman heat-seal tubes) were prepared by sequential freezing of sucrose layers (Luthe, 1983). The ^{125}I-HRG used in the gradient experiments was prepared by incubating HRG (2 mg in 1 mL) with three Pierce iodo-beads and carrier-free ^{125}I for 60 min at ambient

[1] W. T. Morgan, unpublished observations.

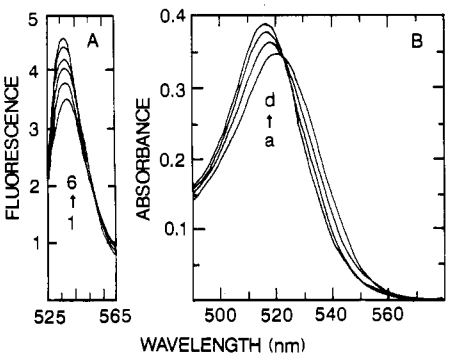

FIGURE 1: Fluorescence and absorbance spectra of eosin Y–HRG complex as a function of added heparin. Panel A illustrates the changes in the emission spectrum of 4.7 μM eosin Y–HRG (excitation wavelength = 516 nm) upon the addition of 10-kDa heparin: (1) no heparin, (2) 0.19 equiv of heparin, (3) 0.37 equiv of heparin, (4) 0.56 equiv of heparin, (5) 0.83 equiv of heparin, and (6) 1.09 equiv of heparin. Panel B shows the absorbance changes of 5.2 μM eosin Y–HRG complex upon addition of 10-kDa heparin: (a) no heparin, (b) 0.34 equiv of heparin, (c) 0.67 equiv of heparin, and (d) 0.99 equiv of heparin. For both panels, no further changes are observed upon adding more heparin. The buffer is 10 mM phosphate, pH 7.4, μ = 0.10 M, and 1 mM Na_2EDTA.

temperature. Non-protein-bound ^{125}I was removed by passage through a column of Sephadex G-25. The ligand–HRG solutions (200 μL; 3–5 μM) were applied to the top of the gradient, and the gradients were spun in a Beckman VTi-80 vertical rotor in a Beckman L8-80 ultracentrifuge for 2 h at 80 000 rpm at 4 °C. The gradients were fractionated from a puncture in the bottom of the tube with a peristaltic pump and a Gilson fraction collector set to advance at every seventh drop. This yielded a total of 32–33 fractions for every gradient tube. The fractions were then counted on a Beckman 7500 γ counter with a standard ^{125}I counting program.

HRG and the peptide (50–100 μg) were chromatographed on 2-mL columns of heparin–Sepharose (Pharmacia) at 30 mL/h in 20 mM sodium phosphate buffers at the pH and conductivity values noted in the legend to Figure 7. Buffers at the same pH but higher conductivity (0.5 M) were used to stepwise elute bound protein or peptide, and 1-mL fractions were collected. Absorbance at 220 nm was used to monitor the eluted material.

Results

Previous studies have shown that HRG has two thermodynamically preferred sites for the binding of Fe(III) mesoporphyrin IX and halogenated fluorescein derivatives such as rose bengal and eosin Y (Burch & Morgan, 1985). The binding of eosin Y to HRG can be readily quantitated by changes in the spectral properties of the dye and by quenching of protein tryptophan fluorescence upon interaction of the dye with HRG. Titration of the protein with eosin Y yields two binding sites with a K_d of 0.5 μM. Eosin Y bound to HRG can be used as a reporter group to monitor interactions of HRG with heparin, similar to the use of 8-anilinonaphthalenesulfonate (ANS) to examine the heparin–antithrombin III association (Einarsson, 1978). Figure 1A illustrates that an increase of approximately 25% in the fluorescence emission intensity of eosin Y occurs as 1 equiv of 10-kDa heparin binds to the equimolar eosin Y-HRG complex in the presence of EDTA. Figure 1B shows that changes in absorbance spectra with isosbestic behavior of the eosin Y–HRG complex in the presence of EDTA also occur upon the addition of 10-kDa heparin. That eosin Y remains bound to HRG throughout the titration with heparin is shown by the absence of the fluorescence and absorbance properties

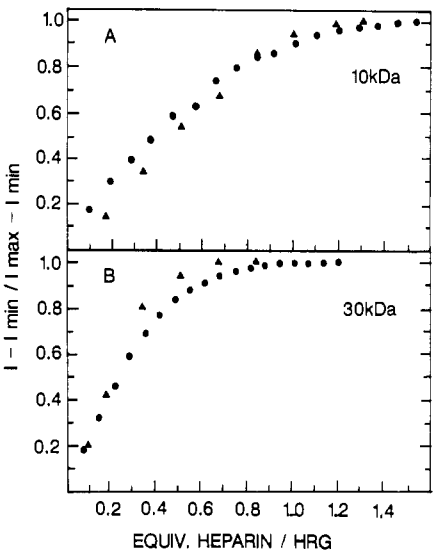

FIGURE 2: Fluorescence and absorbance titrations of eosin Y–HRG with heparin. Shown are additions of 10-kDa heparin (panel A) and of 30-kDa heparin (panel B) to eosin–HRG (3.5 μM). Circles represent fluorescence data and triangles absorbance data, and the ordinate is a normalized intensity axis. Titration with 17.5-kDa heparin (not shown) is nearly congruent to that observed for 30-kDa heparin. The buffer is 10 mM phosphate, pH 7.4, μ = 0.10 M, and 1 mM Na_2EDTA.

Table I: Stoichiometries and K_d Values for Heparin–HRG Complexes[a]

heparin (kDa)	stoichiometry (equiv of heparin/HRG)	K_d (nM)
10	1.0	60
17.5	0.5	60
30	0.5	110

[a] Results from fluorescence and absorbance measurements were gathered at ambient temperature, pH 7.4, μ = 0.10 M in 1 mM Na_2-EDTA. The stoichiometry and apparent K_d values were obtained from the data with the equation of Stinson and Holbrook (1973): $K_d/(1 - a) = [L_0]/a - [P_0]$. In this equation, a is the fraction of ligand bound, $[P_0]$ is the total concentration of ligand binding sites, and $[L_0]$ is the total concentration of eosin Y added; the slope of a plot of $1/(1 - a)$ versus $[L_0]/a$ is $1/K_d$, and the x intercept is $[P_0]$. Similar results were obtained in the absence of EDTA (not shown).

characteristic of free eosin and by the continued quenching of the intrinsic tryptophan fluorescence of HRG by bound eosin Y (data not shown).

Changes in absorbance and fluorescence of eosin Y cease after 1 equiv of the 10-kDa heparin has been added (Figure 2A). Interestingly, very similar spectral changes occur upon addition of 17.5-kDa or 30-kDa heparin to eosin Y–HRG, but these changes are complete at different stoichiometries (Figure 2B, Table I). The stoichiometries for the complex of 17.5-kDa or 30-kDa heparin with HRG are 0.5 heparin per HRG (i.e., one heparin per two HRG) and indicate that two HRG molecules can interact simultaneously with one molecule of the longer chain size heparins. Similar increases in binding stoichiometry with increasing heparin chain size are also seen with the interaction of heparin with antithrombin III or thrombin (Nesheim et al., 1986; Pletcher et al., 1986).

The K_d values obtained in the presence of 1 mM EDTA from these absorbance and fluorescence titrations (Table I) are somewhat larger than the value of 7 nM reported for the human HRG–heparin complex (Lijnen et al., 1983). However, Lijnen and co-workers (Lijnen et al., 1983) like Tollefsen and Pestka (1985) observed *no* interaction between heparin and human HRG in the presence of EDTA, in direct opposition to these findings with the rabbit protein. In control experiments, similar K_d values were found in the absence of EDTA,

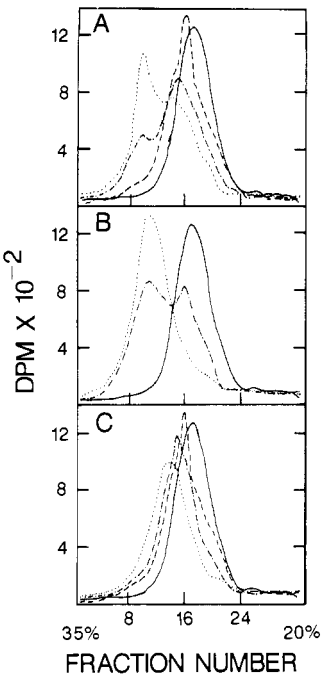

FIGURE 3: Analytical centrifugation using sucrose gradients (20–35%) of heparin–^{125}I-HRG. The HRG concentrations were between 3.5 and 5.5 μM in each experiment. Solutions and gradients were prepared in 10 mM phosphate, pH 7.4, μ = 0.10 M, containing 1 mM Na_2-EDTA. Panel A is the ^{125}I-HRG profile of gradients with HRG alone (solid line), HRG + 1 equiv of 10-kDa heparin (dashed line), HRG + 1 equiv of 17.5-kDa heparin (dash–dot line), and HRG + 1 equiv of 30-kDa heparin (dotted line). Panel B shows the radioisotope distribution of the gradient with ^{125}I-HRG alone (solid line), HRG + 0.5 equiv of 17.5-kDa heparin (dash–dot line), and HRG + 0.5 equiv of 30-kDa heparin (dotted line). Panel C is the radioisotope profile of sucrose gradients with HRG alone (solid line), HRG + 10 equiv of 10-kDa heparin (dashed line), HRG + 10 equiv of 17.5-kDa heparin (dash–dot line), and HRG + 10 equiv of 30-kDa heparin (dotted line). Standards of β-amylase (9.4 S) and human plasminogen (4.6 S) peaked at fractions 7 and 16. Rabbit HRG (4.3 S) runs at highest concentration in fraction 17 in this gradient.

eosin did not influence the association of HRG with heparin on sucrose gradients (see below), and heparin did not block subsequent eosin binding, indicating that eosin does not mask a high-affinity heparin binding site.

To confirm that 1 heparin:1 HRG and 1 heparin:2 HRG complexes are formed between HRG and the 17.5-kDa and 30-kDa heparin and that EDTA does not prevent complex formation, analytical density gradient velocity ultracentrifugation with sucrose gradients was performed. Figure 3A illustrates the distribution of ^{125}I-HRG alone (4.3 S) and with an equimolar amount of 10, 17.5, or 30-kDa heparin added. The 10-kDa heparin forms only a 1 heparin:1 HRG (4.6 S) complex with HRG. Clearly, the 17.5-kDa and 30-kDa heparins form both 1 heparin:1 HRG complexes (5.2 S and 5.7 S, respectively) and 1 heparin:2 HRG (7.9 S) complexes with HRG. As the heparin chain length becomes longer, the template surface for the binding of HRG increases, and formation of the larger complex becomes more likely; hence, the 10-kDa heparin shows no 1:2 complex formation and the 30-kDa heparin participates in the greatest amount of 1:2 complex formation.

The equilibrium between 1:1 and 1:2 complexes can be shifted by decreasing or increasing the amount of heparin present (see eq 1). Figure 3B shows the expected shift toward

$$\text{1:1 heparin:HRG} \underset{-\text{heparin}}{\overset{+\text{heparin}}{\rightleftharpoons}} \text{1:2 heparin:HRG} \qquad (1)$$

formation of the 1 heparin:2 HRG complex with reduced

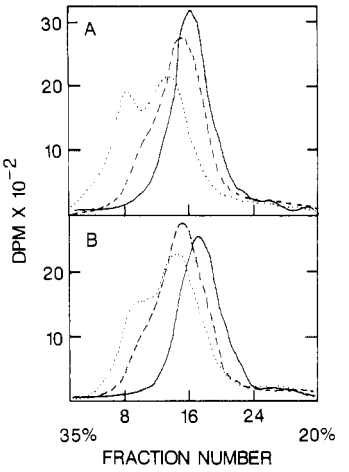

FIGURE 4: Influence of Cu(II) and Zn(II) on the binding of 1 equiv of 17.5-kDa heparin to HRG. Sucrose gradients were run as usual in 10 mM phosphate, pH 7.4, μ = 0.15 M. EDTA was not present in these experiments. Panel A displays ^{125}I-HRG + 10 equiv of Cu(II) (solid line), HRG + 1 equiv of 17.5-kDa heparin (dashed line), and HRG + 10 equiv of Cu(II) + 1 equiv of 17.5-kDa heparin (dotted line). Panel B shows ^{125}I-HRG alone (solid line), HRG + 1 equiv of 17.5-kDa heparin (dashed line), and HRG + 10 equiv of Zn(II) + 1 equiv of 17.5-kDa heparin (dotted line).

amounts of heparin. In this case with 1 mol of heparin to 2 mol of HRG, 30-kDa heparin forms solely a 1:2 complex, whereas the 17.5-kDa heparin shows a shift in equilibrium toward the 1:2 complex as judged by the relative proportions of the 1:2 and 1:1 complex peaks. The 10-kDa heparin did not form 1:2 complexes under any circumstance. Figure 3C illustrates the pattern of complex formation under conditions of 10 mol of heparin to 1 mol of HRG. It is clear that only 1:1 complexes are formed with increasing s values as the heparin molecular weight increases: HRG alone, 4.3 S; 10-kDa heparin–HRG, 4.6 S; 17.5-kDa heparin–HRG, 5.2 S; 30-kDa heparin–HRG, 5.7 S.

Similar results were obtained whether EDTA was present or absent, again demonstrating that metals are not required for heparin binding. This has also been confirmed by recent results showing that HRG effectively competes with antithrombin III for heparin in the presence of EDTA (Peterson et al., 1987). Moreover, the stoichiometry of the heparin–HRG complex decreases from 1:1 to 1:2 with increasing heparin chain length, a result obtained also for the interaction of heparin with thrombin and antithrombin III (Nesheim et al., 1986; Pletcher et al., 1986).

The results of sucrose gradient ultracentrifugation in the presence of metal (Figure 4) indicate that, although metals are not required for heparin binding by HRG, metals do exert effects on the binding equilibria. Addition of 10 mol of divalent Cu or Zn, known to bind to HRG with high affinity via histidine residues (Morgan, 1981), to a 17.5-kDa heparin–HRG mixture before centrifugation shifts the 1:1–1:2 complex equilibrium toward formation of the 1:2 complex. Even 1 equiv of Zn(II) or Cu(II) slightly shifts the equilibrium toward 1:2 complex (not shown). This effect of metal may be exerted by metal-induced conformation charges in HRG or by metals coordinating with histidine residues of HRG important for binding. Cu(II) binding to HRG in the absence of heparin causes conformational changes in HRG as demonstrated by circular dichroism,[2] and the sedimentation coefficient also changes with Cu(II) addition [4.3 S, HRG

[2] B. M. Muhoberac, M. K. Burch, and W. T. Morgan, submitted for publication.

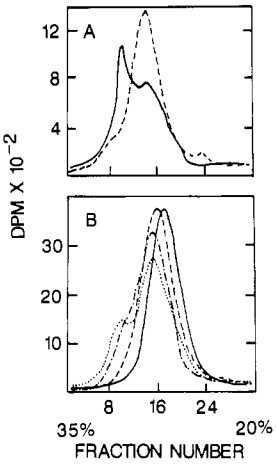

FIGURE 5: Effect of pH and ionic strength on the binding of heparin to HRG. The sucrose gradients and ^{125}I-HRG–heparin solutions were as in Figure 3A. The concentration of 30-kDa heparin and of HRG was 5 μM. In panel A, gradients in 10 mM phosphate, μ = 0.10 M, at pH 7.4 (solid line) and pH 6.4 (dashed line) were used. Panel B shows the effect of ionic strength on the binding of 1 equiv of 17.5-kDa heparin to ^{125}I-HRG. Shown are 0.10 M ionic strength (dotted line), 0.15 M ionic strength (dash–dot line), and 0.30 M ionic strength (dashed line). ^{125}I-HRG alone (solid line) is included for comparison. The buffer was 10 mM phosphate, pH 7.4, containing 1 mM Na_2-EDTA.

alone; 4.6 S, HRG with 10 equiv of Cu(II)]. The effects of Zn(II) on the heparin–HRG complexes are not as pronounced perhaps because of differences in conformational effects induced in HRG between Cu(II) and Zn(II)[2] or because of the lower affinity of HRG for Zn(II) (Morgan, 1981). The low affinity of heparin for Cu(II) and Zn(II) (K_d near 2 mM; Woodhead et al., 1983) would preclude an effect of metal–heparin binding under the present conditions.

To further characterize the nature of the heparin–HRG complexes, the effects that pH, ionic strength, and chemical modification exert on these complexes were examined. Figure 5 illustrates the influence of pH and ionic strength on density gradient fractionation of HRG complexes with 30-kDa heparin. With equimolar concentrations of HRG and heparin, the amount of 1 heparin:2 HRG complex relative to the 1 heparin:1 HRG complex decreases as the pH is lowered from 7.4 to 6.4 (Figure 5A). At both pH values all of the HRG remains bound to heparin. The 17.5-kDa heparin also displays a decrease in the extent of 1:2 complex as the pH is lowered from 7.4 to 6.4, and the 10-kDa heparin shows no changes. Data gathered at pH 5.4 are identical with those observed at pH 6.4, and binding at pH 8.5 is similar to that at pH 7.4. The decrease in the amount of 1:2 complex formed at lower pH may correlate with the protonation of histidine residues, leading to repulsion between positively charged HRG molecules, or may be due to a pH-dependent change in the mode of interaction of HRG with heparin. Recent evidence (Peterson et al., 1987) indicates that the affinity of HRG for heparin is about twofold higher at pH 7.0 than at pH 7.4.

Figure 5B illustrates the sensitivity of the heparin–HRG interaction to ionic strength. Increasing the ionic strength from 0.10 to 0.15 M decreases the extent of 1:2 complex with 1 equiv of 17.5-kDa heparin reacting with HRG. Above 0.3 M ionic strength, the 1:1 complex appears to be destabilized, and the sedimentation peak shifts toward free HRG. At 0.5 M ionic strength (not shown), no heparin–HRG complex is formed. Experiments with "high-affinity" heparin (i.e., high affinity for antithrombin III) demonstrated that it binds to HRG but not at 0.5 M ionic strength (not shown). As noted before by others (Lijnen et al., 1983; Lane et al., 1986), there was no discernible difference between the interaction of high-affinity heparin and unfractionated heparin with HRG at 0.10 M ionic strength.

Table II: Effects of Histidine Modification of 10-kDa Heparin–HRG Complex Formation[a]

% histidine modification	equiv of heparin/HRG
0	1.0
43	0.7
74	0.5
100	0

[a] HRG was treated with DEP as noted under Materials and Methods and the extent of histidine modification determined by spectral changes (Miles, 1977). Heparin binding data were obtained by monitoring changes in the fluorescence of the HRG–eosin Y complex as a function of added heparin as described under Materials and Methods. No influence of histidine modification on the binding of eosin Y by HRG was detected, and the dye was confirmed to be bound to modified HRG prior to and during heparin titrations by monitoring the quenching of the intrinsic tryptophan fluorescence of HRG caused by the dye. Heparin causes a smaller decrease in this fluorescence than does eosin Y (Peterson et al., 1987).

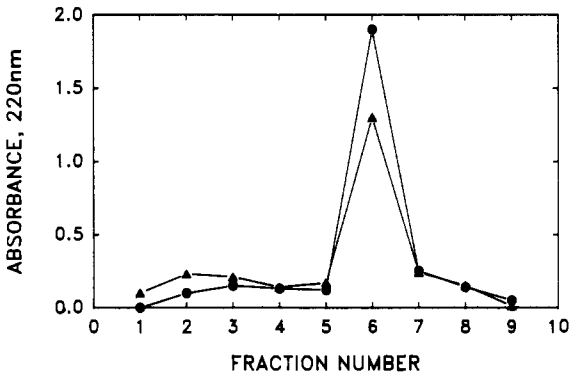

FIGURE 6: Heparin–Sepharose affinity chromatography. HRG (circles) and a 28-kDa histidine-rich peptide isolated from HRG (triangles) were passed over a 2-mL heparin–Sepharose column as described under Materials and Methods. Protein and peptide (50–100 μg per run) were monitored by their absorbance at 220 nm. After the column was washed with 20 mM phosphate buffer, pH 6.8, bound material was eluted in the same buffer containing 0.5 M NaCl. No additional material was eluted with 1 M NaCl. Both buffers contained 1 mM Na_2EDTA.

The importance of histidine residues in forming the heparin–HRG complex is suggested by the effects of metals and pH noted above as well as by the influence of ionic strength. The results of chemically modifying the histidine residues of HRG with diethyl pyrocarbonate provide further evidence on this point. Titrations with heparin (10 kDa) of HRG in which histidine was modified are summarized in Table II. It is evident that the extent of heparin binding is inversely proportional to the extent of histidine modification in HRG. Yet at 75% modification of the histidine residues of HRG, almost half of the heparin binding activity remains. At 100% histidine modification, shown previously not to detectably distort the conformation of HRG (Morgan, 1981), no heparin is bound. In contrast to antithrombin III which contains 1–2 easily modified lysines necessary for heparin binding (Pecon & Blackburn, 1984), modification of 22 of the 33 lysine residues of HRG with *O*-methylisourea did not influence heparin binding.

To further explore the role of the histidine residues of HRG in heparin binding and to initiate study of the location of heparin binding domains on HRG, affinity chromatography using heparin–Sepharose and HRG and the histidine-rich peptide isolated from it (Morgan, 1985) was employed. As shown in Figure 6, the 28-kDa His-Pro-Gly-rich peptide bound to the affinity medium at pH 6.8. Native HRG bound to the

column at pH 6.8 and also at pH 7.4 (not shown).

Discussion

The present results better define the HRG–heparin interaction and extend previous work in several ways. First, the interaction of rabbit HRG with heparin is shown here to be dependent on pH and ionic strength, to occur in the presence of 1 mM EDTA (i.e., not to require metal), and to not form a simple 1:1 complex under all conditions. While the influence of divalent metal ion may be a species-specific effect, the formation of 1 heparin:2 HRG complexes is not likely to be. Under physiological conditions, both the presence of metal ions and the chain length of heparin are likely to affect the relative amounts of 1:1 and 1:2 complexes formed with both species of HRG.

There are several lines of evidence that show that histidine residues of HRG are instrumental in forming and maintaining the HRG–heparin complex: (1) chemical modification of histidine abolishes the heparin interaction; (2) pH-dependent shifts in the 1:1–1:2 heparin–HRG equilibrium occur in the region of the pK_a of histidine (pH 6.4–7.4); (3) Cu(II) and Zn(II), which bind to histidine residues of HRG (Morgan, 1981), also influence the 1:1–1:2 heparin–HRG equilibrium; (4) a histidine-rich peptide derived from HRG (Morgan, 1985) binds to heparin–Sepharose and may represent a heparin-binding domain of HRG. In support of this, a histidine-rich protein from the malarial parasite *Plasmodium falciparum*[3] and a histidine-rich fragment of hemopexin[1] also interact tightly with heparin. Hydrogen bonding between protonated histidine and negatively charged sulfate and carboxylate groups of heparin is likely to be a principal mode of interaction and would explain the sensitivity of the binding of heparin by HRG to ionic strength and pH. The effects of metals like divalent Cu or Zn on the formation of the 1:2 heparin–HRG complex are likely to be exerted through the histidine-rich heparin-binding domain, probably by competition between heparin and metal for histidine.

The sequence homology between the N-terminal portions of human HRG and antithrombin III led Koide and coworkers to suggest that the heparin binding site of HRG would resemble that of antithrombin (Koide et al., 1986a,b). However, HRG interacts with heparin in a manner that differs in several respects from the interaction of antithrombin III with heparin. First, histidine residues of HRG are shown here to be important for its interaction with heparin. Second, extensive modification of the lysyl residues of HRG does not decrease heparin binding, whereas antithrombin III does not bind heparin with only one to two lysines modified (Pecon & Blackburn, 1984). Third, HRG does not show a preference for high-affinity heparin (Jordan et al., 1979), and the heparin–HRG interaction is more sensitive to pH and ionic strength than is the heparin–antithrombin III interaction (Peterson et al., 1987).

Major questions remain to be answered concerning the mechanism of the heparin–HRG interaction. For example, does HRG in fact contain two heparin binding sites, one resembling that of antithrombin as suggested by Koide et al. and one in its histidine-rich domain as indicated here? What structural attributes of heparin are important for binding to HRG? Experiments to further define the heparin binding of HRG are in progress.

[3] R. Howard, personal communication.

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