Histidine-Proline-rich Glycoprotein as a Plasma pH Sensor

MODULATION OF ITS INTERACTION WITH GLYCOSAMINGLYCANS BY pH AND METALS*†

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The middle domain of plasma histidine-proline-rich glycoprotein (HPRG) contains unusual tandem pentapeptide repeats (consensus G(H/P)(H/P)PH) and binds heparin and transition metals. Unlike other proteins that interact with heparin via lysine or arginine residues, HPRG relies exclusively on histidine residues for this interaction. To assess the consequences of this unusual requirement, we have studied the interaction between human plasma HPRG and immobilized glycosaminoglycans (GAGs) using resonant mirror biosensor techniques. HPRG binding to immobilized heparin was strikingly pH-sensitive, producing a titration curve with a midpoint at pH 6.8. There was little binding of HPRG to heparin at physiological pH in the absence of metals, but the interaction was promoted by nanomolar concentrations of free zinc and copper, and its pH dependence was shifted toward alkaline pH by zinc. The affinity of HPRG for various GAGs measured in a competition assay decreased in the following order: heparin > dermatan sulfate > heparan sulfate > chondroitin sulfate A. Binding of HPRG to immobilized dermatan sulfate had a midpoint at pH 6.5, was less influenced by zinc, and exhibited cooperativity. Importantly, plasminogen interacted specifically with GAG-bound HPRG. We propose that HPRG is a physiological pH sensor, interacting with negatively charged GAGs on cell surfaces only when it acquires a net positive charge by protonation and/or metal binding. This provides a mechanism to regulate the function of HPRG (the local pH) and rationalizes the role of its unique, conserved histidine-proline-rich domain. Thus, under conditions of local acidosis (e.g. ischemia or hypoxia), HPRG can co-immobilize plasminogen at the cell surface as well as compete for heparin with other proteins such as antithrombin.

HPRG1 is a relatively abundant plasma glycoprotein (125 μg/ml or about 1.5 μM) (1). Its two N-terminal cystatin modules identify it as a member of the cystatin superfamily, along with kininogen and fetuin (2). Based on its interactions with various ligands, HPRG has been proposed to be a regulator of coagulation and fibrinolytic systems, including heparin (3), plasminogen (4), and fibrinogen (5); cells, e.g. T-cells (6), macrophages (7), and platelets (8); and small ligands, such as transition metal ions and heme (9). No hypothesis of the function of HPRG has been able to rationalize these numerous interactions or explain how the activity of HPRG may be regulated in vivo.

The most noteworthy feature of HPRG is a very high content of histidine, 13 mol %, concentrated in the central His-Pro-rich domain which contains a pentapeptide consensus sequence (GHHPH) repeated in tandem 12 times in human HPRG (10) and the sequences GHPHp and GPPHP repeated 18 times in the rabbit protein (11). Thus, the ionic charge of HPRG is exquisitely sensitive to pH in the range of pH 6–7, and an electrophoretic titration curve of rabbit HPRG showed that protonation of histidine side chains spans the physiological pH range, with the maximum change occurring between pH 6.5 and 7 (11).

Considering these observations, surprisingly little attention has been given to the effect of pH on the structure and function of HPRG. Histidine residues in the His-Pro-rich domain of HPRG mediate interactions with heparin (12), transition metals, and heme (13). Binding of HPRG to heparin and the subsequent neutralization of heparin’s anticoagulant effect have obvious pharmacological interest. An analysis of proteins from normal human plasma that bound to immobilized heparin indicated that HPRG was the only protein likely to inhibit anti-coagulant activity by direct competition with antithrombin (14), although several plasma proteins display this ability in purified systems. Interaction of heparin with heparin-binding proteins is largely electrostatic but requires lysine or arginine side chains in other known cases. It is thus noteworthy that histidine residues are responsible for HPRG binding to heparin, and this leads to the immediate prediction that protonation of histidine side chains at low pH or binding of transition metals such as zinc, which also bestows positive charge on the His-Pro-rich domain, will strengthen heparin binding to HPRG. Indeed, inhibition by HPRG of the heparin-catalyzed thrombin-antithrombin reaction is modulated by pH (15) or zinc (16), and the stoichiometry of the heparin-HPRG interaction is shifted from 1:1 to 1:2 at low pH or in the presence of zinc and copper (12).

Here we characterize directly and quantitatively the modulation by pH and transition metals of HPRG-glycosaminoglycan (GAG) interactions, and we propose a new paradigm of the function of HPRG based on these data. We suggest that the unique, conserved His-Pro-rich domain is a pH sensor, providing a plausible mechanism for regulating HPRG’s activity and an approach to understanding its physiological role. In this model, when the local pH drops (e.g. in ischemia or hypoxia) histidine side chains of HPRG are protonated, the His-Pro-rich domain is deprotonated, which weakens heparin binding and decreases HPRG’s ability to interact with other proteins.
strongly to negatively charged GAGs on cell surfaces. As a consequence ligands of HPRG such as plasmogen can be co-immobilized at the cell surface, and other GAG-binding proteins like antithrombin may be displaced.

EXPERIMENTAL PROCEDURES

Reagents—Porcine intestinal mucosa heparin, dermanatan sulfate, heparan sulfate, and chondroitin sulfates A and C were purchased from Sigma, and low molecular mass (5 kDa) heparin was from Calbiochem. Bovine serum albumin (BSA)-heparin conjugate (Sigma) contained an average of 3.4 m mol of heparin/mmol of BSA coupled to the amino groups of the protein via the reducing ends. Bovine aorta derrmanatan sulfate proteoglycan (DSPG) was purchased from Becton Dickinson. Other commercial reagents were the highest quality available, and all buffers and stock solutions were prepared with water treated with a Barnstead Nanopure system.

Human HPRG was isolated from human plasma (Kansas City Community Blood Center) by metal-chelate affinity chromatography on nickel-nitritoltriacetate (NTA) agarose. Briefly, 10 volumes of human plasma containing 5 mM imidazole were applied to 1 volume of Ni-NTA agarose and washed sequentially with 1 volume each of 20 and 80 mM imidazole buffer, pH 7.5, containing 0.5 mM sodium chloride, followed by 1 volume of 25 mM sodium acetate, pH 4.7, containing 0.15 mM sodium chloride. HPRG was then eluted with 250 mM imidazole, pH 7.5. In the absence of the low pH wash, human HPRG preparations exhibited low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash, human HPRG preparations exhibited absence of the low pH wash. HPRG containing fractions were then dialyzed against 150 mM sodium chloride was coupled to amino-silane cuvettes via its reducing ends by reductive amination in the presence of 50 mM sodium borohydride as suggested by the manufacturer. Coupling of the ligand to the amino-silane cuvettes, extensively dialyzed against 5 mM sodium phosphate, pH 7.2, and frozen in aliquots at −90°C. Rabbit HPRG was isolated as described (17).

Anti-rabbit HPRG antibodies were purified by passing the IgG fraction of polyclonal goat antiserum through an affinity column of rabbit HPRG immobilized on Affigel-15 (Bio-Rad; 8 mg HPRG/ml gel), followed by elution with 0.1M glycine, pH 2.5, and immediate neutralization with Tris base. Plasminogen and antithrombin were purified by affinity chromatography on lysine-Sepharose (18) and heparin-agarose (19), respectively.

Preparation of Immobilized GAG Surfaces—GAGs were coupled to hydrophobic, flat amino-silane cuvettes (Affinity Sensors, Paramus, NJ). Before coupling, these cuvettes exhibited no specific binding of HPRG under any experimental condition used in this paper, in contrast to virgin carboxyl-dextran cuvettes which bound HPRG nonspecifically below pH 6.3 and therefore were not used. Heparin-BSA or DSPG at 0.2 mg/ml in 15 mM sodium phosphate, pH 7.3, 150 mM sodium chloride were allowed to react for 30 min with cuvettes previously activated with polymeric glutaraldehyde as suggested by the manufacturer. Coupling using the lysine side chains of the protein moiety optimized immobilization since the GAG moiety was not affected and ligand heterogeneity was reduced. The binding capacity of GAG surfaces for HPRG varied with pH. For instance, although there was a detectable HPRG binding to heparin-BSA at pH 8, maximal binding of an estimated 1.0–1.8 molecules of HPRG per heparin chain was achieved at pH 5.3 (the lower value assumes no heparin contribution to the refractive index of the conjugate and the higher value assumes an equal contribution of heparin and albumin).

Dermatan sulfate at 4 mg/ml in 50 mM sodium phosphate, pH 7.5, 150 mM sodium chloride was coupled to amino-silane cuvettes via its reducing ends by reductive amination in the presence of 50 mM sodium cyanoborohydride. This surface yielded results similar to those obtained with immobilized DSPG although its maximum binding capacity for HPRG was somewhat lower.

Binding Experiments—Binding data were collected with a thermally stabilized Usys resonant mirror biosensor (Affinity Sensors). All experiments were carried out at 23°C and maximum stirring rate in Pipes-buffered saline (PBS), 10 mM Pipes, 140 mM sodium chloride, 0.005% Tween 20. The buffer also contained 0.2 mM NTA except when other chelators were used as described in the text or figure legends. The various metal salts were added to yield the calculated free metal concentration noted (see below). Pipes was chosen since it can be used in the pH range 5.5–7.5 and does not interact appreciably with transition metals. After each binding/dissociation cycle, the GAG surface was regenerated by a brief wash (5–15 s) with 0.1 M sodium borate, pH 9.5, 1 M sodium chloride, 0.2 mM EDTA. Further demonstrating the specificity of interaction, excess soluble heparin completely inhibited binding of HPRG to immobilized heparin. Most experiments were carried out using both human and rabbit HPRG, and no significant differences were found. The results shown are those obtained with the human protein, unless otherwise noted.

Chelators were used to buffer the metal ion concentration throughout this work since, in the presence of a metal buffer, the free metal concentration can be (a) controlled to prevent undesirable side effects; (b) varied over a broad range by changing the chelator and the metal: chelator ratio; (c) calculated from the stability constants; and (d) made less dependent on the presence of HPRG since the metal ion is in large excess and mostly complexed with the chelator. Free metal ion concentrations were calculated as described (20), using the stability constants and pK_a values from the same reference. In the presence of an excess of ligands like NTA or citrate that form 1:1 complexes with metals, pM = −log M = log β − log a_L − log([M]/([L]_0 − [M]_p)), where pM is the negative log of the free metal ion concentration; β is the stability constant of the metal-chelator complex; a_L is [L]_0/[L]_p + 1 = exp(pK_a) − pH and accounts for ligand protonation, and the subscript p denotes the total concentration of metal or ligand used. A pH-independent metal buffer system (20) was prepared by adding an excess of calcium chloride (1 mM) to 0.2 mM NTA, and under these conditions: [M]/[M]_0 = β_CM = [Ca^2+] / ([Ca^2+]/[L]_p + [L]_p).

Generally in biosensor experiments the ligand "A" is in great excess over the immobilized ligand "B" so that the integrated form of the HPRG binding isotherm (assuming no transport limitation and homogeneous binding sites) can be described by the integrated rate equation as follows: R t = R_0 (1 − exp(−k_d t)), where R t is the baseline-corrected biosensor response, R_0 is the response at equilibrium, and k_d is the pseudo-first order off-rate constant (21). Since k_d = k_a [A] + k_p, the linear plot of k_d versus [A] has the association and dissociation rate constants, k_a and k_p, as the slope and the y intercept, respectively. The equilibrium constant (K_e) can be obtained as the ratio k_a/k_p, or by fitting the equilibrium response "R" to a binding isotherm: r = R_max / (K_e + [A]), where R_max represents the total binding sites.

RESULTS

The Effect of pH on HPRG Binding to Immobilized Heparin.—The amount of HPRG bound to equilibrium to immobilized heparin at physiological ionic strength was markedly influenced by pH (Fig. 1A). A plot of the amount of HPRG bound at equilibrium versus pH resembles an acid-base titration curve with human HPRG having a pK_a of 6.8, near that of histidine (Fig. 1B). HPRG treated with excess diethylpyrocarbonate (DEPC) to chemically modify histidine residues (22) does not bind, supporting the importance of histidine in the interaction with heparin (12). Binding of heparin-BSA to immobilized HPRG also showed marked pH dependence with a mid-point at pH 6.5 (not shown). In contrast, binding of anti-thrombin to heparin was much less dependent on pH (Fig. 1B) and exhibited a lower maximum binding, probably due to the preference of antithrombin for a specific pentasaccharide sequence, whereas HPRG is more indiscriminate. To examine HPRG binding to heparin under conditions more like those found in vivo, rabbit serum (diluted 20-fold) was added to the immobilized heparin cuvette at different pH values, followed by affinity purified anti-rabbit HPRG IgG (which does not bind in the absence of serum) (Fig. 2). An increase in signal due to the specific binding of IgG to HPRG already bound to heparin is observed at pH 6.7 and to a limited extent at, but not above, pH 7.0, in good agreement with the results obtained with the purified system (Fig. 1B).

The Effect of Transition Metals on HPRG Binding to Immobilized Heparin.—The influence of various transition metal ions on HPRG binding to heparin in the presence of immobilized buffer at physiological pH is depicted in Fig. 3A. Transition metals bound by HPRG augment the affinity of the protein for heparin at physiological pH. Zinc and copper, 10–20 μM in plasma, are more effective than nickel or cobalt, whereas metals not bound by HPRG (9) like manganese, calcium, and magnesium (not shown) do not promote HPRG binding to heparin. The effects of metals on the binding of HPRG to heparin were

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also assessed in the presence of citrate or NTA (Fig. 3B), since the formation of a 1:1 metal-chelator complex simplifies the computation of free metal concentration and since the amount of protein bound to heparin at equilibrium for human HPRG (filled circles), rabbit HPRG (open circles), DEPC-treated rabbit HPRG (squares), and antithrombin (triangles). Binding was measured using a resonant mirror biosensor as described under “Experimental Procedures.” The units refer to the change in refractive index due to binding which is recorded in arc seconds.

**Fig. 1.** Effect of pH on the interaction between HPRG and immobilized heparin. A, binding of 0.1 µM HPRG to immobilized heparin-BSA in PIBS-NTA at different pH values. B, pH dependence of the amount of protein bound to heparin at equilibrium for human HPRG (filled circles), rabbit HPRG (open circles), DEPC-treated rabbit HPRG (squares), and antithrombin (triangles). Binding was measured using a resonant mirror biosensor as described under “Experimental Procedures.” The units refer to the change in refractive index due to binding which is recorded in arc seconds.

**Fig. 2.** Binding of rabbit serum proteins to immobilized heparin at different pH values. Serum was diluted 20-fold in PIBS-NTA, and then affinity purified anti-rabbit HPRG IgG was added at the time indicated by the arrow. Binding to immobilized heparin-BSA was measured as described in Fig. 1.

**Fig. 3.** Effect of transition metals on the interaction between HPRG and immobilized heparin. A, binding of 0.1 µM HPRG to immobilized heparin-BSA was measured in PIBS, pH 7.3, containing 0.2 mM iminodiacetate and divalent transition metals as shown. B, binding of 0.1 µM HPRG to immobilized heparin in the presence of divalent transition metals was measured in PIBS, pH 7.3, containing 1 mM citrate (open symbols), 0.2 mM NTA (filled symbols), or 0.2 mM NTA with 1 mM calcium chloride (dotted symbols). Binding was assessed as in Fig. 1.

**Fig. 4.** Binding of rabbit serum proteins to immobilized heparin at different pH values. Serum was diluted 20-fold in PIBS-NTA, and then affinity purified anti-rabbit HPRG IgG was added at the time indicated by the arrow. Binding to immobilized heparin-BSA was measured as described in Fig. 1.

Kinetic and thermodynamic parameters for the HPRG-heparin interaction were determined at experimental pH and free Zn(II) concentrations yielding equal HPRG binding in the different buffers were within 0.3 log units (a factor of 2), the corresponding total zinc concentrations differed by more than 60-fold (between 1.2 and 75 µM). It is also notable that the concentration of free metal required to produce half-maximal binding of HPRG to heparin is about 1 nM for copper and 10 nM for zinc, well below the previously published affinity of HPRG for these metals, 10 µM (23). We attribute this difference to the electrostatic concentration of divalent cations in the vicinity of the negatively charged heparin surface.

Comparison between pH- and Metal-mediated HPRG Binding to Heparin—Treatment of HPRG with excess DEPC abolishes both the pH- and zinc-dependent interactions, indicating that histidine residues are necessary for both. In fact, the two binding modes appear to be facets of the same phenomenon, as suggested by experiments carried out at various pH and zinc concentrations (Fig. 4). At pH 6.5, when most of the histidine residues are protonated, binding of HPRG to heparin is maximal, and the presence of zinc has no further effect. Conversely, at pH 7.4 most of the histidine residues are unprotonated, and binding to heparin is directly dependent on zinc concentration. At intermediate pH values, both protonation of histidine and binding of zinc each contribute to the net positive charge of HPRG, and the overall effect is a shift of the binding curves to more alkaline pH which is proportional to the metal concentration.

Kinetic and thermodynamic parameters for the HPRG-heparin interaction were determined at experimental pH and free Zn(II) values that yielded the same amount of bound HPRG,
method (Fig. 5) that simplifies the experimental design and requires smaller amounts of protein (24). Similar affinities were measured by both equilibrium methods (Table I), and the $K_d$ values from pH-induced (37–44 nM) and metal-induced (45–53 nM) binding experiments were not significantly different. These results are in excellent agreement with $K_d$ values previously determined by fluorescence titrations, 53–57 nM (15) and 60 nM (12).

The pH-induced binding of HPRG to heparin appeared faster than the metal-induced binding (Fig. 5B), but the former reaction rates cannot be accurately measured because of mass transport limitations, which produce abnormally low values of $k_d$ and kinetically derived $K_d$ (25). The plot for zinc-dependent binding of HPRG to heparin is linear, yielding $k_a$ and $k_d$ values of $4.5 \times 10^5$ M$^{-1}$ s$^{-1}$ and $2.5 \times 10^{-3}$ s$^{-1}$, respectively. The $K_d$ values obtained by kinetic and thermodynamic means were in agreement and are very close to those obtained for the interaction between heparin and acidic fibroblast growth factor (26). In neither case could $k_d$ be measured directly from the dissociation phase due to rebinding.

Inhibition of HPRG Binding to Heparin by Other GAGs—The relative affinities of HPRG for various GAGs were assessed by competition experiments, in which HPRG was added to solutions containing increasing concentrations of GAGs in PIBS buffer at pH 6.8 (Fig. 6). The concentration of GAG at which 50% of HPRG binding to immobilized heparin is inhibited is proportional to the affinity of HPRG for the added GAG. It can be shown that the relative amount of HPRG bound at equilibrium at pH 6.8 (Inset, Fig. 6) or at pH 7.3 in the presence of 12.6 nM free zinc (squares), assuming binding to a single class of sites. Progress curves were analyzed with the IAsys FASTFit software provided by the manufacturer, assuming a single exponential association. The fit was not significantly improved by using a double exponential association model. Binding units are as in Fig. 1.

The buffer was PIBS-NTA at pH 6.8 or PIBS-NTA at pH 7.3 in the presence of 12.6 nM free zinc. $K_a$ values were derived thermodynamically by analysis of individual association progress curves or of equilibrium titration data and kinetically from the ratio of dissociation to association rate constants.

![Figure 4](http://www.jbc.org/)

**Figure 4. Effect of zinc on the pH-dependent binding of HPRG to immobilized heparin.** Zinc chloride (0–50 μM) was added to a pH-independent metal buffer system (0.2 mM NTA, 1 mM calcium chloride) to produce concentrations of free zinc between 0 and 33 nM. Binding to immobilized heparin-BSA was carried out as in Fig. 1.

![Figure 5](http://www.jbc.org/)

**Figure 5. Kinetics and thermodynamics of the HPRG-immobilized heparin interaction.** A, sensorgram of an equilibrium titration of immobilized heparin-BSA with human HPRG in PIBS-NTA, pH 6.8. Inset, amounts of HPRG bound at equilibrium at pH 6.8 (circles) or pH 7.25 in the absence (triangles) or presence (squares) of 12.6 nM free zinc. The curves represent the nonlinear, least squares fits of experimental data to a binding isotherm. B, pseudo-first order on-rates for the HPRG-heparin interaction at pH 6.8 (circles) or at pH 7.3 in the presence of 12.6 nM free zinc (squares), assuming binding to a single class of sites. Progress curves were analyzed with the IAsys FASTFit software provided by the manufacturer, assuming a single exponential association. The fit was not significantly improved by using a double exponential association model. Binding units are as in Fig. 1.

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<tr>
<th>pH 6.8</th>
<th>pH 7.3 + Zn(II)</th>
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<tr>
<td>$K_a$ (nM), progress curves</td>
<td>37.5 ± 4.2</td>
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<td>$K_a$ (nM), titrations</td>
<td>44.4 ± 1.8</td>
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<td>$K_d$ (nM), $k_a/k_d$</td>
<td>5.1 ± 2.7</td>
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<td>$k_a$ (M$^{-1}$ s$^{-1}$), 10$^{-5}$</td>
<td>21.4 ± 0.8</td>
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<td>$k_d$ (s$^{-1}$, 10$^{-5}$)</td>
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affinities of 8 and 16 μM, respectively, whereas chondroitin sulfates A and C did not interact significantly with HPRG, despite having similar charge densities. The GAGs bound by HPRG (heparin, dermatan sulfate, and heparan sulfate) all
contain iduronic acid, a residue thought to enhance protein binding by increasing conformational flexibility of GAGs (27).

**HPRG Binding to Dermatan Sulfate**—In view of the strong pH and zinc dependence of HPRG binding to heparin, how these factors affect the interaction between HPRG and GAGs which occur on cell surfaces was of interest. Dermatan sulfate, the most effective competitor of HPRG binding to heparin tested, was immobilized onto amino silane cuvettes either by reductive amination via its reducing ends or via lysine side chains in dermatan sulfate proteoglycan (DSPG). As shown in Fig. 7A, the interaction between HPRG and DSPG was also pH-dependent, with the mid-points of the titration curve at pH 6.5 for human HPRG and at pH 6.3 for rabbit HPRG both being somewhat lower than seen for the HPRG-heparin interaction (pH 6.8, Fig. 1B). Binding of both human and rabbit HPRG to immobilized dermatan sulfate exhibited a mid-point at pH 6.5, and the titration curve was somewhat broader than for DSPG (data not shown). The acidic shift of the titration curves of HPRG binding to a dermatan sulfate surface relative to a heparin surface is not surprising in view of the electrostatic nature of the interaction: since dermatan sulfate has a lower negative charge density than heparin, a larger increase in the positive charge of HPRG is needed and is achieved at lower pH.

The effect of zinc on the binding of HPRG to DSPG was also examined using a pH-independent metal buffer (Fig. 7A). Although zinc shifted the pH dependence of the interaction toward alkaline pH, the concentrations required for this effect were an order of magnitude higher than those seen with immobilized heparin (Fig. 4). For example, a free Zn(II) concentration of 250 nM was required to achieve half-maximal binding of human HPRG to DSPG in PiBS buffer at pH 6.8 (data not shown), i.e. 25-fold higher than that needed for heparin binding. Several of the following explanations for the reduced zinc influence are possible: first, since a larger positive charge is needed on HPRG to bind to DSPG, saturation of lower affinity zinc binding sites may be required; second, zinc coordinates with unprotonated histidine residues so that the affinity of HPRG for zinc decreases with pH; third, since the degree of saturation of HPRG with zinc may depend not only on the bulk concentration of free zinc but also on the electrostatically concentrated zinc near the negatively charged surface, the latter effect would be smaller with the less charged GAGs.

Interestingly, the binding of both human and rabbit HPRG to DSPG and to dermatan sulfate showed strong positive cooperativity (Fig. 7B), with a Hill coefficient of about 3.5. This effect was also apparent in the shape of the association curves, which increased almost linearly with time and precluded a Hill analysis. For instance, HPRG immobilized onto nickel chelate surface GAGs may in turn bind and co-immobilize other ligands. For instance, HPRG immobilized onto nickel chelate plates, but not soluble HPRG, strongly accelerates plasminogen activation by tissue plasminogen activator (28). Fig. 8A shows that plasminogen indeed specifically associates with heparin-bound HPRG in a manner that is completely and selectively inhibited by e-aminocaproic acid (an inhibitor of the plasminogen-HPRG interaction). These GAG-HPRG-
plasminogen "sandwiches" can form since the binding sites of HPRG for heparin (the His-Pro-rich domain) and plasminogen (the N/C domain) are different (28). Control experiments confirmed that plasminogen binding to the heparin surface is mediated by HPRG, since plasminogen did not bind to immobilized heparin-BSA at different pH values until equilibrium was reached. Glu-plasminogen (PLG, 1 μM) and ε-aminocaproic acid (ε-ACA, 2 mM) were sequentially added at the times indicated by the arrows. B, rabbit HPRG (upper trace) and reductively methylated HPRG (lower trace) were allowed to equilibrate on immobilized DSPG at pH 6.3, and then Glu-plasminogen aliquots (0.4 μM) were added at the times indicated by arrows. Specific dissociation of plasminogen from the sandwich was induced with 1 mM ε-aminocaproic acid. Binding units are as given in Fig. 1.

DISCUSSION

An immediate consequence of HPRG's binding to heparin or GAGs is competition with other heparin-binding proteins. Neutralization by HPRG of the anticoagulant activity of heparin by competition with antithrombin has long been suggested to be the primary, if not the sole, effect of the HPRG-heparin interaction, and this interaction has been extensively studied due to its pharmacological relevance. Heparin is used therapeutically in vivo in hypoxia or ischemia (32, 33) or one-half pH unit in inflammation (34) due to lactic acidosis. Moreover, postoperative complications are often accompanied by severe metabolic acidosis (35). We propose that HPRG functions mainly as a plasma pH sensor that binds to heparin or cell-surface GAGs only when the local pH decreases or local free metal concentration increases. HPRG is in a unique position among plasma proteins to perform this function due to its high content of histidine in the His-Pro-rich domain. Of the plasma proteins, only kininogen possesses a similar domain that is His-Gly-Lys-rich and that also mediates binding to negatively charged surfaces such as kaolin and acidic phospholipids. However, the permanently charged lysine residues make the kininogen domain much less dependent on pH than HPRG. HPRG is the first plasma protein to which a pH sensor role can be ascribed, although an analogous pH transducer role has been proposed for another histidine-rich protein, hisactophilin from Dictyostelium, whose interaction with actin and the negatively charged inner plasma membrane is regulated by the intracellular pH (36).

Zinc also alters the heparin-HPRG interaction (see above and Refs. 12 and 16). However, the effect is more difficult to evaluate. One uncertainty is how much zinc is endogenously bound to HPRG. The normal plasma concentration of free zinc, about 0.2 nM (37), is too low to promote HPRG binding to heparin at physiological pH, since 1–2 nM free zinc would be needed to affect HPRG-heparin interaction significantly at physiological pH, and the interaction between HPRG and GAGs such as dermatan sulfate is even less influenced by zinc. Although several pathophysiological processes affect local pH, the plasma concentration of available zinc is more stable and depends on the total concentrations of zinc and its ligands and on their affinities. The local concentration of zinc might increase upon platelet stimulation, since total zinc is enriched 30–60-fold relative to plasma in platelet α-granules (38). However, it seems most likely that zinc by itself does not regulate the interaction between HPRG and GAGs but rather acts synergistically with local acidosis to promote surface binding. For example, zinc shifts the pH dependence of HPRG binding to heparin toward physiological pH values so that smaller decreases in local pH would be required to achieve equal binding in the presence of zinc.

The interaction between HPRG and dermatan sulfate and the dependence on pH and zinc were also characterized quantitatively. Since heparin in vivo is normally confined inside mast cell granules, dermatan sulfate and heparan sulfate proteoglycans, natural constituents of the endothelial cell surface, are the most likely physiological binding partners for HPRG. Two studies of plasma proteins bound to cell-surface heparan sulfate proteoglycans (39) and immobilized dermatan sulfate (40) reported HPRG to be a major plasma ligand of these GAGs. However, elsewhere HPRG was found to be ineffective in in-
hibiting the interaction of antithrombin III with endothelial cell heparin-like compounds (41). This apparent discrepancy is readily explained by the present results showing that binding of HPRG to bovine aorta dermatan sulfate proteoglycan occurs only under acidic conditions or in the presence of nanomolar amounts of free zinc. It is possible that binding of HPRG to other cells such as macrophages, T-cells, or platelets is also mediated by cell-surface GAGs. In a recent report supporting this view, binding of HPRG to a T-cell line required the presence of zinc and was inhibited by heparin (42). Of special interest is the finding that HPRG can function as an adapter molecule and co-immobilize its ligands, e.g., plasminogen, on a heparin-coated surface. Since plasminogen activation by tissue plasminogen activator is stimulated by immobilized, but not soluble, HPRG (28), cell-surface GAGs may adsorb HPRG in vivo when the local pH is lowered, thereby stimulating plasminogen activation in the affected area. Preliminary results have indeed shown that plasminogen activation is accelerated in the presence of HPRG bound to immobilized GAGs at acidic pH. This reinforces the aforementioned analogy between HPRG and its cousin kininogen, which binds to negatively charged surfaces via its His-Gly-Lys-rich domain and co-localizes kallikrein and factor XI to enable efficient triggering by factor XII of the intrinsic coagulation pathway (47).

Finally, HPRG can be described as a facultative polycation since its His-Pro-rich domain acquires a positive charge upon protonation, and this may have important physiological consequences in addition to the already well described competition of antithrombin and heparin cofactor II for heparin. For example, cationic polyelectrolytes have numerous biological effects, including antimicrobial activity, enhancement of vascular permeability, modulation of leukocyte adherence, chemotaxis, and phagocytosis (43). In fact, polyhistidine itself is an opsonic agent (44) and a potent inducer of superoxide generation and respiratory burst in polymorphonuclear leukocytes (45, 46). It seems likely that many of the biological actions of HPRG are related to its polycationic nature and thus are regulated by pH and expressed only under conditions that produce local acidosis. HPRG could conceivably be used to target enzymes or other drugs to acidotic regions, local acidosis. HPRG could conceivably be used to target en-

REFERENCES


