Interaction of Histidine−Proline-Rich Glycoprotein with Plasminogen: Effect of Ligands, pH, Ionic Strength, and Chemical Modification†

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ABSTRACT: The association of plasma histidine−proline-rich glycoprotein (HPRG) with plasminogen (PLG) was examined using a sucrose density gradient assay in order to evaluate the effects of several relevant conditions on complex formation. Addition of PLG shifts the S-value of ^{125}I-labeled HPRG from 4.8S to 6.8S, providing the first direct evidence that HPRG associates with the zymogen form of plasmin in solution. Complex formation is not sensitive to pH in the range of pH 6.5−8.5, but is abolished at high ionic strength (1 M NaCl). No species differences were found, as either rabbit or human HPRG bound readily to rabbit or human PLG. Of the ligands of HPRG tested, mesoheme (20 μM) but not heparin (M_r 10 000, 10 μM) inhibits the formation of the HPRG−PLG complex. Modification of lysine residues of HPRG or competitive binding by lysine and anti-fibrinolytic agents containing primary amino groups also inhibits association. Conversely, modification of arginine or histidine residues of HPRG has no effect on complex formation. These results indicate that HPRG has independent binding sites for heparin and PLG and confirm that one or more lysine residues of HPRG are involved in its recognition by PLG. The protein−protein interaction was also quantitatively characterized at thermodynamic equilibrium by analytical ultracentrifugation. The stoichiometry and dissociation constant (K_D) of the complex were determined from the equilibrium distribution of fluorescein−isothiocyanate-labeled PLG in the presence of HPRG. The experimental data were analyzed by nonlinear least-squares curve fitting and indicated that a heterodimer is formed. The binding is characterized by a K_D of 0.75 μM, and heme (30 μM) and ϵ-aminocaproic acid (5 mM) decrease the apparent affinity of the interaction by 12- and 4-fold, respectively. The temperature dependence of the association was also examined from 4 to 25 °C. A van't Hoff plot of the data was linear and yielded a small negative change in enthalpy (−7.6 kcal/mol) and a positive change in entropy (2.7 cal/mol·deg), suggesting that electrostatic forces play a prominent role in the interaction of HPRG with PLG.

Histidine−proline-rich glycoprotein[1,2] (HPRG) is a non-enzymatic plasma glycoprotein known to interact with a number of biologically important ligands *in vitro*. These include metal ions (Guthans & Morgan, 1992; Larsen *et al.*, 1992), heme (Morgan, 1978, 1981), plasmin (Lijnen *et al.*, 1980), thrombospondin (Leung *et al.*, 1984), fibrinogen and fibrin (Leung, 1986), and heparin (Koide *et al.*, 1982; Lijnen *et al.*, 1983; Tollefsen & Pestka, 1985; Lane *et al.*, 1986; Burch *et al.*, 1987; Peterson *et al.*, 1987). The serum concentration of HPRG in healthy human adults is *ca.* 125 μg/mL (Morgan *et al.*, 1978), but it is increased substantially in patients with cardiovascular disorders like myocardial infarction and hereditary thrombophilia (Morgan *et al.*, 1978; Smith *et al.*, 1985; Engesser *et al.*, 1988). Moreover, plasminogen (PLG) is activated more rapidly to plasmin by tissue-type plasminogen activator when bound to HPRG than when it is free in solution (W. T. Morgan, unpublished results).

Rabbit HPRG (M_r 80 000) exhibits the same activities as its human congener, *i.e.*, it binds the same set of ligands, including PLG (this work) and heparin (Burch *et al.*, 1987; Peterson *et al.*, 1987). As in human HPRG (Koide & Odani, 1987), the N-terminal amino acid sequence of rabbit HPRG has high homology with the cystatin superfamily and contains the single central domain that is rich in histidine and proline (Morgan, 1985; W. T. Morgan *et al.*, unpublished results). However, major questions remain regarding the mechanisms of binding and the locations of binding sites on the HPRG molecule.

Previous reports have examined the association of HPRG with plasmin using measurements of plasmin activity to monitor the interaction (Lijnen *et al.*, 1980) or the cleavage of HPRG by plasmin (Smith *et al.*, 1985). Since HPRG *in vivo* primarily contacts the zymogen form of plasmin, it is important to obtain basic information on the HPRG−PLG

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[1] Histidine−proline-rich glycoprotein is used rather than the former name histidine-rich glycoprotein since the protein has a central domain rich in both proline and histidine. Therefore, the revised term more accurately reflects this unusual property of the protein.

[2] Abbreviations: HPRG, histidine−proline-rich glycoprotein; DTT, dithiothreitol; EACA, ϵ-aminocaproic acid; heme, iron−protoporphyrin IX; mesoheme, iron−mesoporphyrin IX; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; FITC, fluorescein isothiocyanate; DMSO, dimethyl sulfoxide; S, Svedberg unit; DEP, diethyl pyrocarbonate; EDTA, ethylenediaminetetraacetic acid; PLG, plasminogen; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; TCA, trichloroacetic acid; PBS, 0.01 M sodium phosphate and 0.15 M NaCl, pH 7.4; RMS, root mean square error.

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interaction. Although this interaction has been studied in whole plasma using a modified immunoelectrophoretic assay (Kluft & Los, 1988), no direct quantitative data are available on the interaction between these proteins in solution. In the present study, sedimentation equilibrium and sucrose density gradient centrifugation were used to define the formation and affinity of the HPRG−PLG complex. Chemical modification of arginine, histidine, and lysine residues of HPRG was tested for its effect on complex formation as well. In this report, we have also analyzed the effects of pH, ionic strength, and several ligands of HPRG on the interaction with PLG, providing the first study on the structural determinants and regulation of this protein association.

MATERIALS AND METHODS

Proteins. Both rabbit and human HPRGs were isolated by ion exchange chromatography as previously described (Morgan, 1981). After purification the proteins were lyophilized and stored at 4 °C. Isolated HPRG species were found to run as single bands in sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), confirming protein purity. The purified material had the characteristic UV absorbance spectrum of HPRG (Morgan, 1981), and an extinction coefficient at 278 nm of 3.5×10^4 M^{-1} cm^{-1} was used to determine the concentrations of HPRG solutions. PLG was prepared from fresh frozen human serum (Community Blood Bank, Kansas City, MO) or from rabbit plasma (Pel-Freeze, Rogers, AR) by affinity chromatography on a lysine−Sepharose column (Pharmacia) according to the method of Castellino and Powell (1981). Plasminogen obtained by this affinity procedure was used for binding experiments without further purification. This procedure gave a homogeneous band of protein when examined by SDS−PAGE. PLG concentrations were determined spectrophotometrically using an extinction coefficient of 1.5×10^5 M^{-1} cm^{-1} at 280 nm (Robbins & Summaria, 1970). Following conversion to plasmin with urokinase (Morris *et al.*, 1981) (Calbiochem), the PLG preparations showed 70−80% activation by active-site titration using the chromogenic substrate *p*-nitrophenyl *p'*-guanidinobenzoate (Sigma). All absorbance measurements, except in analytical ultracentrifugation, were obtained with an SLM-Aminco spectrophotometer (Milton Roy, Rochester, NY).

To obtain plasmin-clipped HPRG, HPRG was incubated with plasmin immobilized on Sepharose-4B (Pharmacia) for 2 h with stirring at room temperature in 0.1 M sodium phosphate (pH 7.4). Thirty milligrams of PLG was first coupled to 3 mL of CNBr-activated Sepharose-4B, according to the manufacturer's protocol, and then activated to plasmin by urokinase. The effectiveness of the proteolysis of HPRG was assessed using SDS−PAGE since the procedure gives rise to a nicked protein with three disulfide-linked polypeptides containing structurally different domains, which can be released under reducing conditions (Morgan, 1985).

Chemical Modification. The modification of histidine residues on HPRG was accomplished with diethyl pyrocarbonate (DEP) (Morgan, 1981); 2−10 μL of a 0.2 M DEP solution in ethanol was added to 2.0 mL of a 4−5 μM solution of HPRG in 10 mM phosphate (pH 7.4) at 4 °C. The reaction was complete within 1 h, and the difference absorption spectrum (modified HPRG versus unmodified HPRG) from 270 to 230 nm was recorded. The concentration of modified histidine was ascertained using a change in extinction at 240 nm of 3.2 mM^{-1} cm^{-1} (Miles, 1977). The percent modification was calculated on the basis of the total number of histidine residues in rabbit HPRG (W. T. Morgan, unpublished data). HPRG treated similarly but without the modification agent was used in control experiments. Pyridoxylation of lysine residues was carried out according to the method of Pecon and Blackburn (1984), and *O*-methylisourea hydrogen sulfate was used to guanidinate lysine residues (Kimmel, 1967). The reaction containing 2 mL of 0.3 M *O*-methylisourea, 10 mg/mL HPRG, and 10 mM CAPS buffer (pH 10.0), was incubated for 3 days at 4 °C, and the pH was checked periodically. Modification was stopped by the addition of an equal volume of 1 M phosphate buffer (pH 7.4). The mixture was then dialyzed against 5 mM phosphate buffer (pH 7.4) 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 the homoarginine peak were monitored using a Glenco modular amino acid analyzer with ninhydrin postcolumn derivatization. HPRG treated similarly but without the modification agent was used in control experiments. Arginyl residues of HPRG were modified by butadione as described by Church *et al.* (1986). Briefly, HPRG was dissolved in borate buffer (50 mM and 0.1% PEG 6000, pH 8.0) and 10 mM butadione was added in a minimal volume. This was allowed to react for 1 h at room temperature in the dark, after which the reaction was halted by the addition of ice-cold 10 mM L-arginine in the same buffer. The modified HPRG was dialyzed against buffer for 2 h, and the spectrum from 330 to 240 nm was recorded. Aliquots of the modified HPRG were snap-frozen in liquid nitrogen and stored at −70 °C. The number of surface-exposed arginyl residues modified was estimated using phenanthrenequinone (Smith & MacQuarrie, 1978). HPRG treated similarly but without the modification agent was used in control experiments.

Protein Labeling. To prepare the FITC derivative of PLG, the protein (1 mg) in 50 mM sodium borate (pH 9.2), containing 0.2 M NaCl, was mixed with 20 μL of FITC (5 mg/mL in DMSO). The reaction was carried out for 2 h at room temperature (Holmes & Fowlkes, 1992). Unbound FITC was then removed by dialysis against PBS. The FITC/protein ratio was estimated to be 4 mol of the chromophore per mole of PLG according to Holmes and Fowlkes (1992), using extinction coefficients of 1.5×10^5 M^{-1} cm^{-1} at 280 nm for PLG and 0.7×10^5 M^{-1} cm^{-1} at 495 nm for FITC. Radioiodinated HPRG or PLG used in sucrose density gradient experiments was prepared by incubating HPRG or PLG (2 mg in 1 mL) with three Iodo-beads (Pierce Chemical Co., Rockford, IL) and 10 μCi of carrier-free ^{125}I (ICN, Irvine, CA) for 15 min on ice followed by 2 equiv of unlabeled iodine for another 15 min. Unbound reagent was removed by dialysis, and >95% of the ^{125}I was precipitable with 10% TCA.

Solution Reagents. All buffer components were of reagent grade. Mesoheme[3] was purchased from Porphyrin Products, Inc. (Logan, UT) and dissolved in DMSO (Aldrich Chemical

[3] The heme analog, mesoheme, was used throughout this study due to its greater stability and solubility than the naturally occurring iron−protoporphyrin. No differences in the binding of heme and mesoheme to HPRG have been observed.

Co., Milwaukee WI), and concentrations were estimated spectrophotometrically using an extinction coefficient of 1.7 $\times 10^5$ M^{-1} cm^{-1} at 394 nm (Brown & Lantzke, 1969). The amount of DMSO in the mesoheme/protein solutions was less than 5%. EACA, flufenamic acid, and tranexamic acid were from Sigma and were prepared at a concentration of 50 mM in PBS. Solutions of $ZnCl_2$ (Baker) were prepared in 10 mM HCl, and the buffer used in this case was 0.1 M HEPES (pH 7.4) containing 0.15 M NaCl. Heparin of defined molecular weight (M_r 10 000) was provided by Dr. M. N. Blackburn, SmithKline-Beecham Research Center. Chondroitin sulfate (bovine trachea, Sigma), dextran sulfate (8 KDa, Sigma), and λ-, κ-, and τ-carrageenans (supplied by Dr. G. Kindness, University of Dundee, Dundee, Scotland) were weighed out and dissolved in PBS.

Analytical Ultracentrifugation. Samples were loaded into charcoal-filled Epon six-channel Yphantis cells (12 mm thick) and placed into an An-60 four-hole titanium analytical rotor. High-speed sedimentation equilibrium experiments were performed in a Beckman Model XL-A analytical ultracentrifuge at 10 000 rpm and 20 °C for 24 h. In separate experiments, it was found that 24 h was sufficient to attain sedimentation equilibrium, *i.e.*, the time when successive scans taken at different time intervals were invariant. The FITC−protein concentration gradient in the cell was determined by UV absorption at 495 nm by consecutive automated optical scans acquired at each 0.001 cm of radial spacing (Giebeler, 1992). All protein samples were prepared just before use in PBS. Several analytical runs were also made to examine the temperature dependence of the equilibrium. In this case, a single sample containing HPRG and PLG was analyzed over a temperature range from 25 to 4 °C. After equilibrium and data acquisition, the temperature was decreased, and the rotor speed at the new temperature was increased to 30 000 rpm for 1 h and then decreased to 10 000 rpm until a new equilibrium distribution was obtained. In order to assess parameters of chemical interest, such as association constants and stoichiometries, nonlinear regression analysis of the data using appropriate mathematical models was used. All curve-fitting routines in this study employed a nonlinear least-squares algorithm based on the Marquardt−Levenberg method (Johnson & Faunt, 1992).

For gradient experiments, solutions containing 5−30% sucrose gradients in a total volume of 5 mL were prepared. The concentration of sucrose in the solutions used to prepare density gradients was measured using a Bausch & Lomb refractometer and adjusted to the desired concentration before use. The samples in a total volume of 100 μL were prepared by gently mixing 1 μM HPRG and 10 μM PLG, radioiodinated or unlabeled, as noted in the figure legends. After 30 min incubation on ice, samples were layered onto the sucrose gradient and centrifuged in a Beckman VTi 80 rotor for 3.04 $\times 10^{11}$ $\omega^2 t$. To determine the effect of ligands and treatments on HPRG, the test substance was first mixed with HPRG and incubated for 15 min on ice before PLG was added. The S values were estimated using radioiodinated protein standards (RNase, 1.9S; carbonic anhydrase, 3.2S; ovalbumin, 3.5S; bovine serum albumin, 4.4S; transferrin, 4.9S; and glucose oxidase, 7.9S) run simultaneously in parallel gradients. The gradients were fractionated by puncturing the bottom of the centrifuge tube and collecting 10-drop fractions, which yielded 32−33 fractions in each experiment. Fractions were counted on a Beckman 7500 γ-spectrometer with a standard counting program for ^{125}I. Unless stated otherwise, the buffer used in all experiments was PBS. Higher ionic strengths were obtained by increasing the NaCl concentration.

Data Analysis. At sedimentation equilibrium, the total protein concentration in an ideal heterogeneous system at the radial position, r, is given by the sum of the concentrations of individual components and their reversible complex forms:

$$c_r = \sum_{i=1}^{n} c_{0,i} \exp[\sigma_i M_i (r^2 - r_0^2)/2] + e \qquad (1)$$

where $c_{0,i}$ refers to the concentration of the ith protein at the radial reference position, r_0 (at the base of the cell) M_i is the molecular weight of the ith component; and e is a base-line error term. The parameter σ_i is the reduced molecular weight, and it is defined as

$$\sigma_i = (1 - \bar{v}_i \varrho)\omega^2/RT \qquad (2)$$

where R is the gas constant, T is the absolute temperature, $\bar{v}_i$ is the partial specific volume of the ith species, ϱ is the solution density, and ω is the angular velocity.

Assuming a one-site binding model for the interaction between HPRG and PLG, the molar equilibrium association constant, K, for the formation of HPRG−PLG complexes, and the equilibrium constant, K', based on the absorbance (represented by A) concentration scale, are given by

$$K = [\text{HPRG−PLG}]/[\text{HPRG}][\text{PLG}] \qquad (3a)$$

Since [HPRG] = [PLG], then

$$K' = A_{\text{HPRG−PLG}}/A_{\text{PLG}}^2 \qquad (3b)$$

and thus

$$K = K' \epsilon_{\text{PLG}} \qquad (4)$$

where ϵ_{PLG} is the molar absorption extinction coefficient of FITC-labeled plasminogen at 495 nm. This coefficient was calculated to be 3.2 $\times 10^5$ M^{-1} cm^{-1} by analyzing several solutions of labeled PLG of known concentrations with a Lambert−Beer plot. Using the absorbance concentration scale, eq 1 may be written as follows:

$$A_r = A_{0,\text{PLG}} \exp[\sigma_{\text{PLG}} M_{\text{PLG}} (r^2 - r_0^2)/2] + A_{0,\text{HPRG−PLG}} \exp[\sigma_{\text{HPRG−PLG}} M_{\text{HPRG−PLG}} (r^2 - r_0^2)/2] + e \qquad (5)$$

Substitution from eq 3b yields

$$A_r = A_{0,\text{PLG}} \exp[\sigma_{\text{PLG}} M_{\text{PLG}} (r^2 - r_0^2)/^2] + K' A_{0,\text{PLG}}^2 \times \exp[\sigma_{\text{HPRG−PLG}} M_{\text{HPRG−PLG}} (r^2 - r_0^2)/2] + e \qquad (6)$$

This equation is the function used for the nonlinear least-squares fitting of the data, with K', $A_{0,\text{PLG}}$, and e as fitting parameters.

The least-squares fit of a model of this type, with highly interdependent parameters, presents several difficulties unless constraints are used to avoid convergence in local minima with negative values for the equilibrium constant. This problem may be avoided in part by fitting the natural

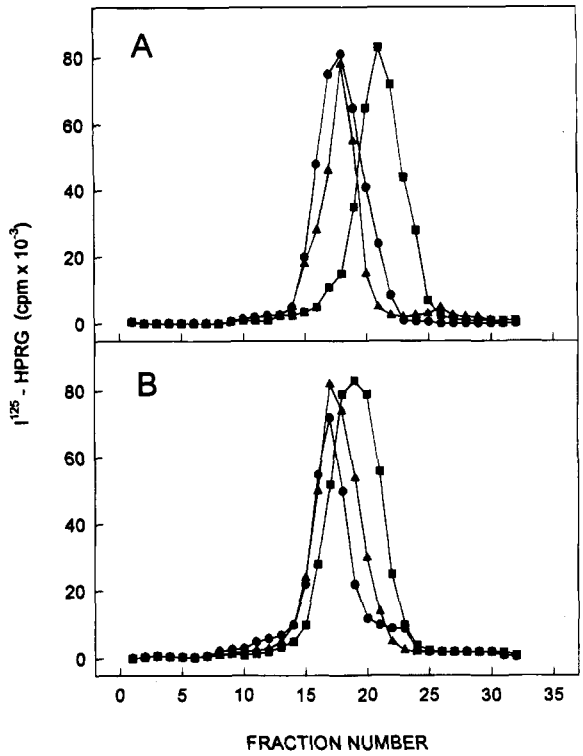


FIGURE 1: Sucrose density gradient centrifugation of HPRG in the presence of PLG. HPRG was used at 1 μM and PLG at 10 μM; 5–30% sucrose gradients were employed. Rabbit [^{125}I]HPRG (■) was centrifuged in the presence of human (●) or rabbit PLG (▲) (Panel A). The interaction was also examined using human ^{125}I-HPRG (■) mixed with human (●) or rabbit PLG (▲) (panel B). In similar experiments, radiolabeled human and rabbit PLG (1 μM) were tested for binding to human and rabbit HPRG (10 μM), and analogous results were obtained (data not shown).

logarithms of K' and the concentrations (Johnson *et al.*, 1981), and this type of transformation was used in all fitting procedures. The minimum root mean square (RMS) error and the minimum systematic deviation of the data points from the fitting lines (residuals) for each distribution were basically the two criteria for discriminating among other possible models.

The equilibrium constants obtained under different temperature conditions were analyzed according to the van't Hoff relation:

$$\ln K = \Delta S°/R - (1/T)(\Delta H°/R) \quad (7)$$

$\Delta H°$, $\Delta S°$, and $\Delta G°$ are the standard enthalpy, entropy, and free energy changes, respectively. Using eq 7, the enthalpy and entropy changes of the association may be obtained from the slope and intercept, respectively, of a plot of ln K versus $1/T$ (van't Hoff plot), provided it is linear. The analysis of the relative magnitudes of these thermodynamic parameters allows assessment of the type of interaction involved in the association (Ross & Subramanian, 1981).

RESULTS AND DISCUSSION

Sucrose gradient centrifugation was used as a reproducible, convenient, qualitative means to examine the association between HPRG and PLG. Figure 1A shows that binding to PLG increases the S-value of [^{125}I]HPRG from 4.8S to 6.8S for the HPRG–PLG complex, providing the first direct evidence for an association between HPRG and PLG in a solution of defined composition. There is no discernible difference with regard to species, since rabbit HPRG, like human HPRG, binds to either human or rabbit PLG (Figure 1B). The radioactivity profile of human [^{125}I]HPRG, both alone and when bound to rabbit or human PLG, consistently had both a leading and a trailing edge, likely due to some dimerization and breakdown of the human HPRG. Human HPRG is extremely sensitive to proteolysis (Smith *et al.*, 1986), but sedimentation profiles obtained with rabbit HPRG showed less degradation and dimerization; thus, the rabbit congener was used in subsequent experiments.

Table 1: Summary of the Qualitative Effects of Ligands of HPRG and Solution Conditions on the HPRG–PLG Association[a]

solution component	concentration	HPRG S-value shift[b]	HPRG–PLG complex[a]
$ZnCl_2$	50 μM	↑	+, ↑
	100 μM	↑	+, ↑
mesoheme	10 μM	↑	+, ↑
	20 μM	↑	–
	30 μM	↑	–
heparin	10 μM	↑	+, ↑
EDTA	2 mM		+
pH	6.5	↑	+
	7.5		+
	8.5	↓	+
NaCl	100 mM		+
	500 mM	↓	+
	1000 mM	↓	-

[a] The gradient profiles obtained in all cases were reproducible within one fraction. Therefore, a shift in radioactive peak position of two or more fractions, either up or down, was defined as indicating a change in the sedimentation of HPRG. On the basis of the standard proteins employed, this represents an apparent change in S-value of more than 1S unit. [b] An arrow in this column indicates that the addition of the ligand or the condition alone affected the sedimentation of HPRG. An up arrow (↑) indicates an increase in S-value, and a down arrow (↓) indicates a decrease in S-value. These samples were run in parallel with gradients containing only HPRG as controls. [c] A + in this column indicates that a complex formed between HPRG and PLG, and a – indicates that a complex did not form, on the basis of the lack of shift in S-value compared to parallel gradients with HPRG or HPRG–ligands as controls. An up arrow (↑) indicates that the observed S-values of HPRG–ligand–PLG were larger than those with HPRG–PLG alone and that sufficient ligand binds to the HPRG–PLG complex to increase its sedimentation rate.

The binding of HPRG to PLG is saturable, as demonstrated by the inhibition of binding of radiolabeled HPRG to PLG produced by an excess of unlabeled HPRG, and a 1:1 stoichiometry of binding between HPRG and PLG is suggested since the complex sediments at 6.8S over a 10-fold molar ratio of HPRG–PLG (data not shown). The effects of several ligands that are bound by HPRG on the S-value of HPRG itself and on the formation of the HPRG–PLG complex were also examined. Heme is bound in high amounts by HPRG *in vitro* (Morgan, 1981) and possibly also *in vivo* under conditions of extensive hemolysis when the hemopexin system becomes saturated. Mesoheme increases the S-value of HPRG alone, but it also abolishes HPRG–PLG complex formation at low levels (Table 1). HPRG also is able to bind up to 20 metal ions per mole of protein in its His–Pro-rich central domain (Morgan, 1985). Zn^{2+} (50–100 μM) causes an increase in sedimentation of both HPRG alone and the HPRG–Zn^{2+}–PLG complex, but protein association is not inhibited. Although sucrose density gradient studies cannot define the mechanism of inhibition, these results confirm that the binding of small ligands by

Table 2: Summary of the Qualitative Effects of Antifibrinolytic Agents, Exogenous Amino Acids, Chemical Modification, and Plasmin Treatment of HPRG on the HPRG–PLG Complex

treatment	condition	HPRG S-value shift[a]	HPRG–PLG complex[b]
antifibrinolytic agent	flufenamic acid (5 mM)	−	+
	tranexamic acid (5mM)	−	−
	EACA (5 mM)	−	−
lysine modification[c]	*O*-methylisourea	−	−
	pyridoxylation	−	−
histidine modification[d]	diethyl pyrocarbonate	−	+
arginine modification[e]	butadione	−	+
plasmin digestion[f]		↓	+
exogenous amino acids	lysine (5 mM)	−	−
	histidine (5 mM)	−	+

[a] A down arrow (↓) in this column indicates that the treatment alone produced a decrease in the S-value of HPRG, and a − indicates that no shift was observed. Gradients run in parallel with HPRG alone were used as controls. See footnote *a* in Table 1 for further definition of the symbols used. [b] A + in this column indicates that a complex formed between HPRG and PLG after the addition of the specified component or modification of HPRG as indicated by an increase in S-value, and a − indicates that no complex formed. [c] Over 85% of the 33 lysine residues of HPRG were modified by O-methylisourea (Burch & Morgan, 1985). [d] Over 85% of the 54 histidine residues of HPRG were modified by DEP (Morgan, 1981). [e] About 16 of 23 total arginine residues of HPRG were modified by butadione, consistent with the number of residues estimated to be easily accesible to phenanthrenequinone. [f] HPRG was digested with plasmin as previously described (Morgan, 1985).

HPRG can influence subsequent binding of its macromolecular ligands and suggest that these interactions may be physiologically relevant under conditions of metal or heme overload.

The observations of Lijnen *et al.* (1980) on the plasmin–HPRG interaction were extended by showing that low molecular weight antifibrinolytic agents like lysine, tranexamic acid, and EACA were all inhibitors of the HPRG–PLG association. In contrast, flufenamic acid, which lacks an amino group, had no effect on the association (Table 2). Moreover, modification of lysine residues on HPRG also causes inhibition of protein association (Table 2). On the other hand, extensive modification of the arginine or histidine residues on HPRG does not inhibit formation of the HPRG–PLG complex (Figure 2 and Table 2). Interestingly, modification of arginines does not block the binding of heparin to arginine-modified HPRG (Figure 2B). This contrasts with antithrombin III, an anticoagulant serum protein with sequence homology at the N-terminal portion with HPRG, and provides additional evidence that the heparin binding site of HPRG is different from that of antithrombin (Burch *et al.*, 1987). Both the competition and modification experiments showed that one or more lysine residues of HPRG are involved in the association between HPRG and PLG. In this regard, HPRG has several lysine residues (Lys 40, Lys 84, Lys 445 and Lys 525 in the human protein) that are conserved in the human (Koide & Odani, 1987), rabbit (W. T. Morgan, unpublished), and bovine proteins (Sørensen *et al.*, 1993), and increasing evidence indicates that the C-terminal lysine of HPRG is an important determinant for the interaction (D.-B. Borza and W. T. Morgan, unpublished).

Although there is little change in HPRG or HPRG–PLG complex S-values from pH 6.5 to 8.5, the HPRG–PLG complex is sensitive to high ionic strengths (Table 1). This also supports the importance to binding of ionic interactions through lysines; however, the conformational change in PLG caused by chloride ion might also play a role (Gaffney *et al.*, 1988). Since plasmin-digested HPRG retains its ability to interact with PLG even after reduction with DTT (Table 2), a stable domain or conformation of HPRG appears to contain the essential lysine residue(s) and perhaps other residues important for the HPRG–PLG association.

Figure 2C shows that HPRG can bind PLG and heparin simultaneously, since the ternary mixture showed a higher

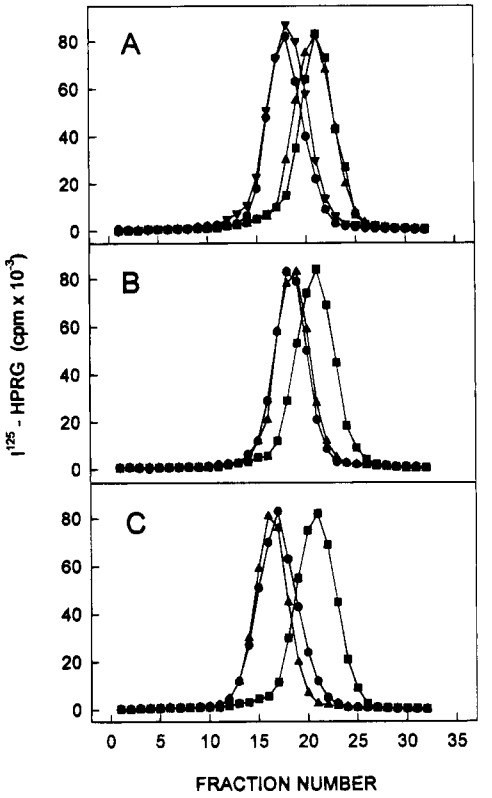


FIGURE 2: Effect of heparin and modification of arginine residues of HPRG on the HPRG–PLG interaction. Panel A shows the sucrose gradient profiles of control [^{125}I]HPRG (■), arginyl-modified HPRG (▲), arginyl-modified HPRG with 10 μM PLG (●), and unmodified HPRG with 10 μM PLG (▼). Panel B depicts arginyl-modified HPRG (■), arginyl-modified HPRG with 10 μM heparin (●), and control (unmodified) HPRG with 10 μM heparin (▲). Panel C shows arginyl-modified HPRG alone (■), with both 10 μM PLG and 10 μM heparin (●), and unmodified HPRG with both 10 μM PLG and 10 μM heparin (▲). The HPRG concentration was 1 μM, the heparin M_r was 10 000, and the sucrose gradients were 15–30%.

S-value than the HPRG–PLG binary complex. This demonstrates that the binding sites for PLG and heparin on HPRG are independent and that there is no apparent steric hindrance between them. Moreover, a discrete proteolytic fragment of HPRG is able to interact with PLG (D.-B. Borza and W. T. Morgan, unpublished results), providing evidence that an independent single domain contains specific lysine(s) suf-

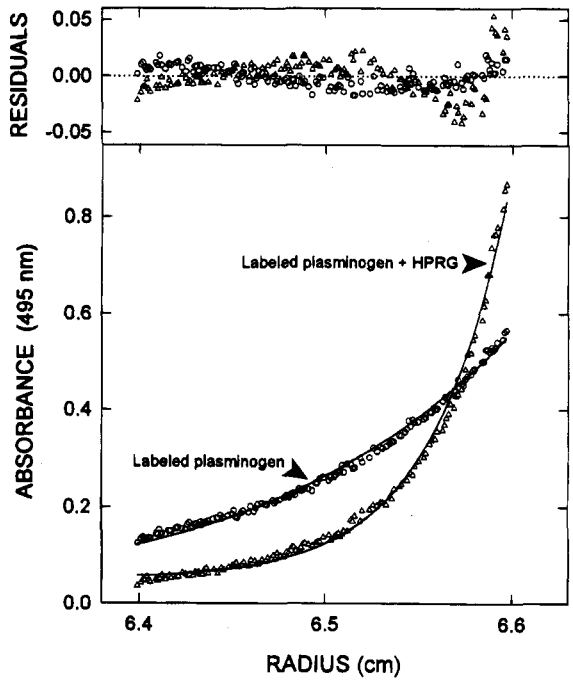


FIGURE 3: Sedimentation equilibrium centrifugation of PLG and PLG in the presence of HPRG. FITC-labeled PLG, at an initial concentration of 2 μM in PBS, was centrifuged in a Beckman Model XL-A at 10 000 rpm and 20 °C for 24 h, and the protein concentration was monitored at 495 nm. The distributions of PLG alone (○) and of PLG in the presence of unlabeled HPRG (△) are shown. The solid lines depict the least-squares best fit M_r values of 99 300 for labeled PLG and 210 000 for the complex. The top panel shows the plots of the residuals for PLG and PLG in the presence of HPRG (○ and △, respectively).

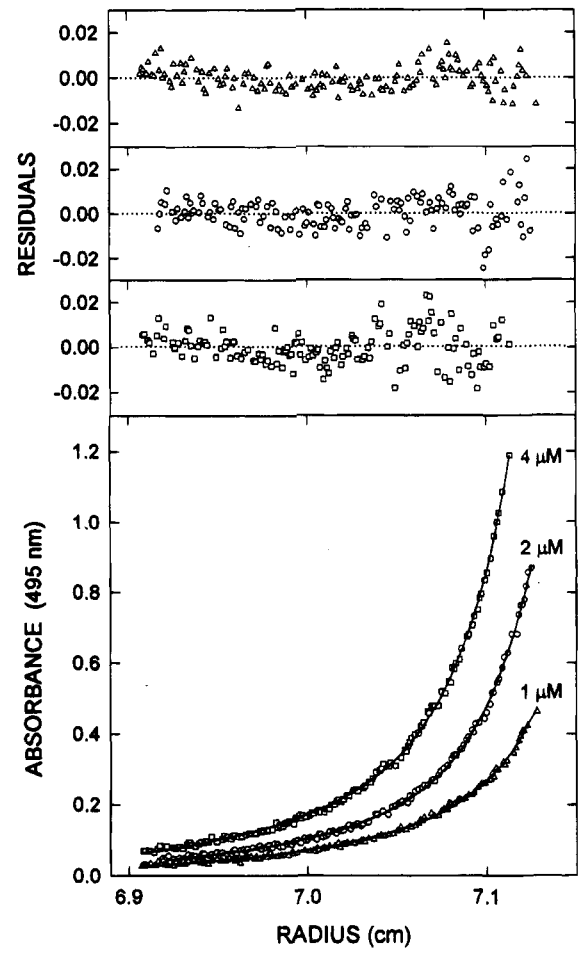


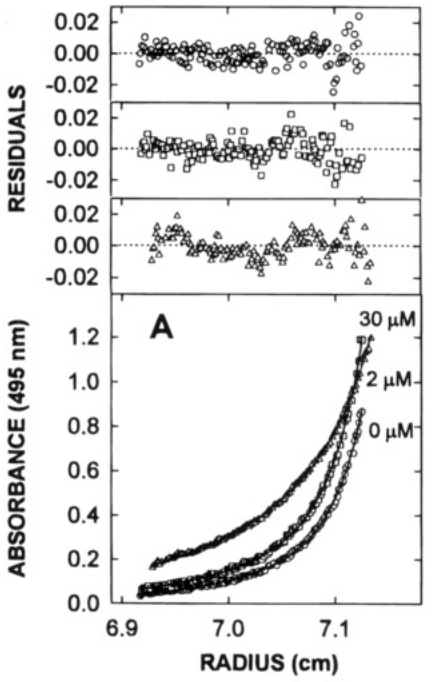
FIGURE 4: Affinity of the association between PLG and HPRG. Analytical ultracentrifugation was performed under the same conditions as in Figure 3, except that the equilibrium distributions of three different equimolar mixtures of FITC−PLG and HPRG were determined. The initial protein concentration of each was 1 (△), 2 (○) or 4 μM (□). The respective residuals of each least-squares fit are shown in the upper panel.

ficient for protein−protein recognition. Since the His−Pro-rich central domain and the C-terminal domain of HPRG are known to be regions which bind heparin (Burch *et al.*, 1987), these results also suggest that the N-terminal domain of HPRG is the PLG binding domain. However, the role of the C-terminal lysine must also be considered. Since a disulfide bridge connects the N- and C-terminal portions of the protein, *in vivo* both domains may participate in binding PLG.

Sedimentation equilibrium techniques were used to confirm and extend the sucrose gradient results and to enable quantitative analyses. At equilibrium, both FITC−PLG and the sample mixture containing unlabeled HPRG formed exponential gradients characteristic of each (Figure 3). The calculated M_r for labeled PLG, using a partial specific volume of 0.713 mL/g (Castellino & Powell, 1981), was 99 300 and increased to 210 000 when complexed with HPRG. No significant self-association of PLG was detected, and any nonideal effects were negligible. Addition of purified, unlabeled PLG returned the profile to that of FITC−PLG alone, providing additional evidence for protein integrity after chemical modification (data not shown). Labeled PLG was found to be not only a suitable means to monitor PLG in this mixture but also a useful approach to study "crowding" effects on associations. This is of particular interest since experiments at high concentrations, resembling the crowded molecular conditions encountered *in vivo,* can exert significant effects on molecular interactions that are not detectable in dilute solutions (Ralston, 1990).

Interacting systems can be studied using ultracentrifugal analyses either by running several initial concentrations simultaneously at a single rotor speed or by running one initial concentration at several rotor speeds (Roark, 1976). In this study, different equimolar concentrations of unlabeled HPRG and FITC-labeled PLG were analyzed after centrifugation for 24 h at 10 000 rpm by scanning the cell for absorption at 495 nm, the maximum for the derivatized PLG. Figure 4 shows a typical simultaneous fit of the mathematical model (eq 6) to three different concentration distributions of HPRG, PLG, and the complex of the two proteins obtained at equilibrium. The fitting procedure gave values of $A_{0,PLG}$ and e unique to each sample and values of K and the root mean square (RMS) error common to all three cells. A K_D of 0.75 ± 0.025 μM and an RMS of 0.006 absorbance unit were obtained. No differences were found when Lys−PLG, the proteolytic product of plasmin-cleaved PLG, was used instead of PLG or when "clipped" HPRG was used instead of undigested HPRG (data not shown). The plots of the distributions and of the residuals indicate that the fits are good, and no systematic error distribution is apparent. This agreement also supports the 1:1 stoichiometry of the HPRG−PLG complex, since variations in the mathematical model that allow for association stoichiometries other than 1:1 significantly increased the error of fitting.

Sedimentation equilibrium experiments performed under similar conditions showed that the 15:1 mesoheme−HPRG



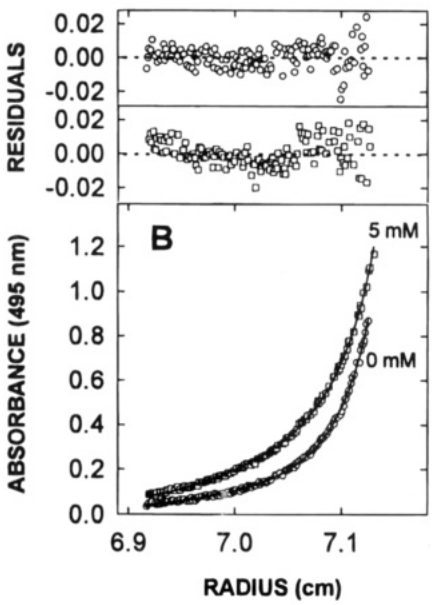


FIGURE 5: Quantitative analysis of the effects of mesoheme and ϵ-aminocaproic acid on the association between HPRG and PLG. All sedimentation conditions were as in Figure 3. Panel A shows the equilibrium distributions of three mixtures of HPRG with FITC−PLG (2 μM each) containing 0, 2, and 30 μM mesoheme (○, □, and △, respectively). Panel B shows the effect of 5 mM ϵ-aminocaproic acid (□) on the association. A control distribution is also shown (○). Residuals for each experiment are shown in the upper portion of each panel.

complex had a substantially reduced affinity for PLG since the apparent dissociation constant (K_D = 8.2 ± 0.05 μM) increased by about 12-fold (Figure 5A). Conversely, lower ratios of mesoheme to HPRG (Figure 5A and Table 1) and Zn^{2+} (data not shown) had no such inhibitory effect. Heme at low concentrations binds to HPRG at two thermodynamically preferred sites on the C-terminal domain of the protein (Burch & Morgan, 1985) and at a number of lower affinity sites located in the central His−Pro-rich region of the protein (Morgan, 1985). Thus, it appears that coordination of mesoheme at the central domain abolishes the protein−

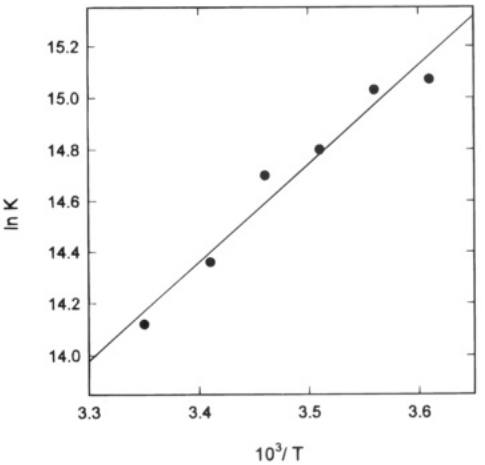


FIGURE 6: Temperature dependence of the equilibrium association constant for the HPRG−PLG complex. Six experiments like that shown in Figure 3 were performed over the range 4−25 °C. A van't Hoff plot of the data is shown in which the natural logarithm of the best fit estimates of the K_a values are plotted versus the reciprocal of the absolute temperature. The solid line is a linear regression of the data (r^2 = 0.960).

protein interaction, either sterically or perhaps by producing a conformational change in HPRG. The antifibrinolytic compound EACA also blocked the interaction between HPRG and FITC−PLG, probably in this case through a competitive mechanism involving the known lysine binding sites on PLG. The apparent K_D obtained in the presence of 5 mM EACA was 2.6 ± 0.04 μM (Figure 5B), representing a 4-fold lowering in affinity.

The temperature dependence of the equilibrium constant for complex formation is presented in Figure 6 in the form of a van't Hoff plot. A linear fit to the data (solid line in Figure 6) produced a change in enthalpy ($\Delta H°$) of −7.6 ± 0.78 kcal/mol and a change in entropy ($\Delta S°$) of 2.7 ± 0.13 cal/mol·deg. These values indicate that ionic interactions (Ross & Subramanian, 1981) are important in the formation of the HPRG−PLG complex. Since the HPRG−PLG complex was found to be resistant to relatively high NaCl concentrations (Table 1), this analysis suggests that PLG has buried residues in one or more "kringles", the triple-loop structures involved in lysine recognition, which are important for the association with HPRG.

In conclusion, sedimentation techniques were found to provide a convenient means to examine the specific interaction of HPRG with PLG under native conditions in solution. The evidence to date indicates that HPRG binds a number of blood plasma components, some of them simultaneously, *e.g.*, heparin and PLG. Upon binding to HPRG, the activity of the ligands may be modulated by HPRG, by the other ligand(s) in the complex, or by both. This supports a physiological role for HPRG as a modulating component in hemostasis, possibly by regulating the available concentrations of its ligands in plasma. Current research is aimed at defining the precise location and nature of ligand binding sites on HPRG, especially for fibrinogen.

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