Solution-Phase Equilibrium Binding Interaction of Human Protein S with C4b-Binding Protein†

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ABSTRACT: Solution-phase equilibrium binding studies of human protein S (HPS) and C4b-binding protein (C4BP) were undertaken using purified components. Free C4BP was measured in solutions at equilibrium by using HPS immobilized on a solid phase, coupled with an antibody detection system. Disruption of the solution-phase equilibrium was minimized by using a brief (15 min) exposure to the solid-phase HPS. These studies yielded an equilibrium dissociation constant (K_d) $\approx 6 \times 10^{-10}$ M and a stoichiometry of approximately 1.7 molecules of HPS bound to each molecule of C4BP. This K_d is between 27-fold and 930-fold lower than previously published values obtained by using solid-phase and nonequilibrium methods. Equilibrium was achieved in solutions containing low nanomolar concentrations of both HPS and C4BP in ≤ 1 h at 37 °C, suggesting a rapid association rate constant for the interaction. Thrombin cleavage of HPS had no effect on the observed binding parameters. The binding interaction between HPS and C4BP appears to be partly calcium dependent, since in the presence of EDTA the K_d was increased to about 6×10^{-9} M, with no change in the stoichiometry. This high-affinity binding interaction between HPS and C4BP, whose K_d is more than 500-fold lower than the proteins' plasma concentrations, heightens the apparent physiologic importance of complex formation.

Vitamin K dependent protein S, a coagulation system cofactor, and C4b-binding protein, a complement system regulator, both circulate in human plasma at about 0.35 μM (Fair & Revak, 1984; Villiers et al., 1981). Protein S is a cofactor for activated protein C (APC),[1] facilitating its proteolytic inactivation of two key procoagulant cofactors—factors Va and VIIIa (Walker, 1980; Walker et al., 1987). These cofactors are required for significant catalytic activity of two central procoagulant complexes of the hemostatic system, the prothrombinase and Xase complexes (Mann et al., 1990). Within the assembled prothrombinase complex (factors Va and Xa, calcium ions, and a phospholipid surface), factor Va is protected from APC proteolysis (Nesheim et al., 1982). Human protein S exerts its influence, at least in part, by nullifying this protective effect (Solymoss et al., 1988). Bovine protein S facilitates the binding of APC to phospholipid surfaces (Walker, 1981), a mechanism that may explain its enhancement of factor Va inactivation by APC. Human protein S is a single-chain glycoprotein of 635 amino acids that exhibits amino-terminal sequence homology to vitamin K dependent coagulation proteins (Lundwall et al., 1986) and carboxyl-terminal homology to steroid-binding proteins (Baker et al., 1987; Gershagen et al., 1987). Thrombin cleavage of HPS results in a disulfide-linked two-chain molecule with no cofactor activity (Suzuki et al., 1983; Solymoss et al., 1988).

Complement component C4b-binding protein accelerates the proteolysis of C4 by complement factor I (Fujita et al., 1978) and inhibits C3 convertase of the classical complement pathway (Gigli et al., 1979). Unfractionated C4BP is a heterogeneous population of molecules composed of six or seven identical 70-kDa α-chains and zero or one unique 45-kDa β-chain (Hillarp & Dahlbäck, 1989), all covalently linked by disulfide bonds near their carboxyl termini. The majority of C4BP in plasma consists of seven α-chains (Scharfstein et al., 1978; Nagasawa & Stroud, 1980; Dahlbäck et al., 1983) and a single β-chain (Hillarp & Dahlbäck, 1988). The presence of the β-chain appears to be required for binding to HPS (Hillarp & Dahlbäck, 1987, 1988; Suzuki & Nishioka, 1988). When HPS is bound to C4BP, the cofactor functions of the former are abrogated (Dahlbäck, 1986), while those of the latter are unimpaired (Dahlbäck & Hildebrand, 1983). The site on protein S of C4BP binding likely involves the carboxyl-terminal half of the molecule, and peptide competition studies have implicated residues 605–614 of the mature protein in the interaction (Walker, 1989).

Previously reported values for the K_d of the HPS–C4BP interaction in purified systems are 0.9×10^{-7} M (Dahlbäck, 1983a), 0.5×10^{-7} M (Dahlbäck, 1986), 0.16×10^{-7} M (Nishioka & Suzuki, 1990), and 5.6×10^{-7} M (Malm et al., 1990). A K_d of 0.7×10^{-7} M was reported for the interaction in plasma (Dahlbäck, 1983a). Because of the fairly weak interaction these numbers implied and the high (~0.14–0.21 μM) plasma levels of each free and bound form of HPS and C4BP (Dahlbäck, 1983a), the physiologic importance of the interaction between these two proteins was not obvious. A noteworthy shortcoming of previous studies was their application of solution-phase thermodynamics to partly solid-phase equilibria and to nonequilibrium solution-phase systems. In the present study, a technique originally used to determine true solution-phase equilibrium binding constants of antibody–antigen association/dissociation reactions (Friguet et al., 1985) has been modified and applied to the HPS–C4BP interaction. The reaction has been examined in the presence and absence of calcium ions, in the presence of EDTA, and by using thrombin-cleaved HPS. These studies demonstrate a much

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[1] Abbreviations: HPS, human protein S; C4BP, human C4b-binding protein; BHPS, biotinylated HPS; APC, human activated protein C; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; IgG, immunoglobulin G; K_d, equilibrium dissociation constant; BSA, bovine serum albumin; SE, standard error (95% confidence interval); r, correlation coefficient.

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tighter, more rapid binding interaction between HPS and C4BP than has been reported previously. In addition, the extinction coefficient of C4BP purified from barium citrate precipitates of human plasma has been experimentally determined by two independent methods.

Experimental Procedures

Proteins. All proteins except antibodies and BSA were of human origin. Protein S was purified from fresh-frozen pooled plasma by procedures described elsewhere (Solymoss et al., 1988) and was a gift of Dr. Paula Tracy, University of Vermont College of Medicine. C4BP either was a gift of Dr. Frederick Walker, American Red Cross, Farmington, CT, or was purified from plasma by immunoaffinity chromatography of redissolved 30% ammonium sulfate precipitate of barium citrate adsorbed plasma, using a monoclonal antibody provided by Dr. William Church, University of Vermont College of Medicine. Following elution from the antibody column with 3 M sodium thiocyanate, the C4BP was dialyzed into Tris–saline (0.02 M Tris-HCl, pH 7.6, 0.15 M NaCl), made 6 M in guanidine hydrochloride (Heico), and applied to a 1-m Sephacryl S-300 column (Pharmacia-LKB) equilibrated with the same buffer. The first peak contained C4BP free of HPS and was dialyzed into Tris–saline, concentrated by using Centriprep concentrators (Amicon), and stored in 50% glycerol at −20 °C. The purity of all protein preparations was confirmed with SDS–PAGE, before and after disulfide bond reduction with 2-mercaptoethanol, visualized with silver nitrate (Morrissey, 1981). α-Thrombin was a gift of Dr. Sriram Krishnaswamy, University of Vermont College of Medicine. Protein S was biotinylated with sulfosuccinimidyl 6-(biotinamido)hexanoate (Pierce) in 50 mM sodium bicarbonate buffer, pH 8.5, according to manufacturer's instructions.

The molecular weight and extinction coefficient ($E^{0.1\%}_{280nm}$) used to determine the concentration of HPS were 70 690 (Lundwall et al., 1986) and 0.95 (DiScipio & Davie, 1979), respectively. The extinction coefficient of C4BP purified as described above was determined by far-ultraviolet spectrophotometry (van Iersel et al., 1985) to be 1.34 and by quantitative amino acid analysis to be 1.37. For the latter method, protein concentration was calculated by a summation of amino acid yields relative to a norleucine standard, with individual amino acid yields being compared to the overall amino acid composition of C4BP containing seven α-chains (Chung et al., 1985) and one β-chain (Hillarp & Dahlbäck, 1990). An average value of 1.36 was used in absorbance/concentration calculations. The molecular weight of C4BP used in calculations was 570 000 (Dahlbäck, 1983a). Absorbance values used for protein quantification were corrected for Rayleigh scattering by using a single absorbance reading at 320 nm and a multiplier of 1.7.

Thrombin Proteolysis of HPS. α-Thrombin was incubated with HPS at a molar ratio of 1:25, in Tris–saline containing 2 mM EDTA, at 37 °C for 2 h. The absorbance of the reaction mixture was monitored at 320 nm at 15-min intervals as a check for light scattering that would result from dramatic self-association or aggregation of HPS. No significant change in absorbance was observed. Thrombin was either removed from the mixture by passage over a sulfopropyl–Sephadex column or inhibited by addition of D-phenylalanylprolylarginyl chloromethyl ketone to a concentration of 2 μM. The reaction was judged complete by the apparent total conversion of single-chain HPS to the two-chain form evident after disulfide bond reduction, SDS–PAGE, and silver staining.

Equilibrium Binding Assay. The general outline and logic of the method were as originally described by Friguet et al. (1985). A schematic illustration of the method used in the present study is shown in Figure 1. Differences in data analysis and specific details of the assay as applied to the HPS–C4BP binding equilibrium are described below. Polystyrene flat-bottom 96-well ELISA plates (Corning no. 25801) were coated with 130 μL/well of HPS at 2 μg/mL in 50 mM sodium carbonate/bicarbonate buffer, pH 9.6, overnight at 4 °C. The HPS was removed and the wells were filled with 200 μL/well of assay buffer (0.02 M Tris-HCl, pH 7.6, 0.5 M NaCl), containing 1% (w/v) BSA (FisherBiotech, tissue culture grade) and 0.01% (w/v) thimerosal, and incubated for at least 2 h at 4 °C prior to use. Plates were sealed with parafilm and stored for up to 2 weeks at 4 °C. These HPS-coated plates were used for detection of free C4BP in solutions at equilibrium, as described below.

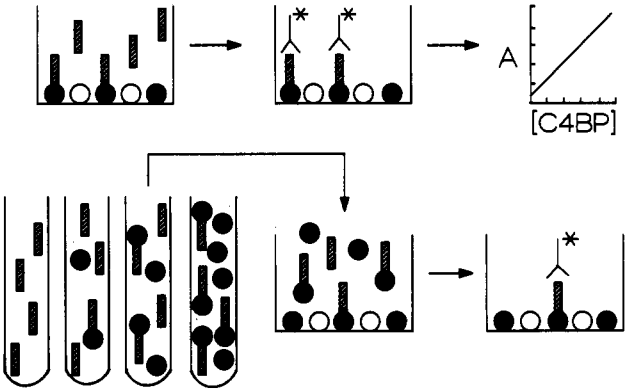

FIGURE 1: Schematic description of the solution-phase equilibrium binding assay. Closed circles represent HPS molecules capable of binding C4BP. Open circles represent HPS not able to bind C4BP because of immobilization on the microtiter well. Hatched rectangles represent C4BP molecules. Inverted "Y"s with asterisks represent the double antibody/enzyme-linked detection system described under Experimental Procedures. Proteins are not drawn to scale. The upper panel illustrates calibration of HPS-coated ELISA plate microtiter wells by incubation with several known concentrations of C4BP. For clarity, only a single well incubated with one C4BP concentration is illustrated. The result (top right) establishes a linear correspondence between enzymatically generated color, measured as absorbance (A), and C4BP concentration in solution. The lower panel illustrates the equilibrium incubations, consisting of varied HPS concentrations at two different C4BP concentrations. For clarity, only one fixed C4BP and four HPS concentrations are shown. A portion of each equilibrium mixture is transferred to microtiter wells of a calibrated ELISA plate (transfer of only one is illustrated), and the concentration of free C4BP is determined on the basis of the standard curve (top right of upper panel). Plate incubation, washing, and color detection are described in detail under Experimental Procedures. The rate of color development in the microtiter well is proportional to the concentration of free C4BP in the solution-phase equilibrium mixture.

Equilibrium solutions were incubated in MiniSorp tubes (Nunc) for 3 h at 37 °C unless otherwise indicated and were prepared in ≥500 μL of Tris–saline containing 1% (w/v) BSA. These solutions covered a range of HPS concentrations at two fixed C4BP concentrations and included a set of serially diluted C4BP solutions for standard curve construction. The remaining steps of the assay were performed at room temperature. Duplicate 100-μL aliquots from each equilibrium solution were pipetted to a plate prepared as described above that was washed two times with 175 μL/well of assay buffer. After 15 min, the plate was washed three times with wash buffer (assay buffer supplemented with 0.01% (w/v) thimerosal and 0.05% (v/v) Tween-20) and received 100 μL/well of 3.8 μg/mL anti-C4BP monoclonal antibody in the same buffer supplemented with 1% (w/v) BSA. A second 15-min incubation and wash was followed by the addition of 100 μL/well of peroxidase conjugated goat anti-mouse IgG

(Jackson Immunoresearch Laboratories, Inc.) diluted 1:2750. A final 15-min incubation and wash was culminated in the addition of 200 μL/well of substrate buffer (0.1 M sodium citrate, pH 5.0, 0.8 mg/mL *o*-phenylenediamine dihydrochloride, 0.015% hydrogen peroxide). Beginning immediately after substrate addition, the rate of color development was determined by measurement of absorbance at 450 nm in a V_{max} plate reader (Molecular Devices Corporation) at 20-s intervals for 7–10 min. Color development was stopped by the addition of 50 μL/well of 4 N sulfuric acid and an end-point absorbance measured at 490 nm. The rate of color development was extracted from the kinetic data by the V_{max} software. A standard curve of serially diluted C4BP was included in every assay to confirm a linear correspondence between free C4BP concentration in solution and ELISA signal.

A control assay to measure the maximum possible disruption of the solution-phase HPS–C4BP equilibrium during the plate incubation was done as follows. After a standard equilibrium incubation and initial 15-min plate incubation, 75 μL from several of the incubations was transferred to a second ELISA plate and assayed in direct comparison with 75 μL from the corresponding original incubation mixtures. The ratio of the ELISA signals generated with and without the initial plate incubation provided an estimate of the fraction of free C4BP captured by solid-phase HPS during contact with the plate.

Data Analysis. Standard curves for each plate were subjected to regression analysis and found to have linear correlation coefficients ≥0.995. This linear dependence of absorbance upon C4BP concentration in solution validates the relationship between free and total concentration of C4BP (C_F, C_T) and the absorbance obtained in the presence and absence of HPS (A, A_0): $C_F/C_T = A/A_0$, as previously described (Friguet et al., 1985). This equation can be rearranged to relate the fraction of C4BP bound (b) to absorbance, to give $b = (A_0 - A)/A_0$. A blank absorbance value resulting from the incubation of C4BP in microtiter wells *not* coated with HPS was subtracted from both A and A_0 values. Data analysis was performed on the average absorbance values from duplicate wells.

Data representing the fraction of C4BP bound, systematically varied HPS, and fixed C4BP concentrations were analyzed with a nonlinear least-squares computer program written and kindly provided by Dr. Sriram Krishnaswamy, University of Vermont College of Medicine. The program employs the Marquardt algorithm to iteratively fit data to the quadratic binding equation derived from the equilibrium expression nC4BP + HPS ⇄ C4BP$_n$HPS, a derivation analogous to that employed for fluorophore binding to phospholipid (Krishnaswamy et al., 1986). Noninteracting, equivalent binding sites are assumed. The quality of the fit was assessed by the standard errors (95% confidence interval) of the extracted K_d and stoichiometry (n), the root-mean-squared deviation, and the distribution of residuals to the fitted curves.

Confirmation of a Reversible Equilibrium. To verify that the HPS–C4BP interaction did indeed represent a reversible equilibrium binding interaction under our experimental conditions, equilibrium solutions of HPS and C4BP were prepared under conditions favoring participation of nearly all C4BP in complexes (14-fold molar excess of HPS). Similar solutions were prepared with saturating concentrations of biotinylated HPS. To these solutions was added either biotinylated or unlabeled HPS to double the total HPS concentration. After 24 h at 37 °C, the solutions were transferred to ELISA plates coated with a monoclonal antibody to C4BP, incubated for 1 h, washed, incubated for 30 min with streptavidin–alkaline phosphatase conjugate, and developed with 5 mg/mL *p*-nitrophenyl phosphate in a 0.1 M bicarbonate buffer, pH 9.8, containing 1 mM $MgCl_2$. The rate of color development was measured at 405 nm and interpreted as a semiquantitative measure of biotinylated HPS specifically incorporated into a complex with C4BP.

Table I: Reversibility of the HPS–C4BP Interaction under Experimental Conditions of the Equilibrium Binding Assay

protein components[a]		signal
1st incubation	2nd incubation	(mA_{405}/min[b])
BHPS		3 ± 1
C4BP + HPS		0.3 ± 0.2
C4BP + HPS	BHPS	71 ± 12
C4BP + BHPS		182 ± 33
C4BP + BHPS	HPS	72 ± 8
C4BP + BHPS + HPS		77 ± 6

[a] Buffer conditions were as described for the equilibrium binding assay. First incubation solutions contained 7 nM C4BP and 100 nM of the indicated protein S species, at 37 °C for 2 h. The second incubation was initiated by bringing one-half of the indicated first incubation solutions to 100 nM HPS or BHPS and continued for 24 h at 37 °C. All incubation solutions were measured simultaneously on a single ELISA plate following the second incubation. [b] Errors represent the standard deviation of three measurements.

Results

Confirmation of Reversibility. The results of the experiment done to confirm reversibility of the HPS–C4BP binding interaction are summarized in Table I. These results are consistent with an association/dissociation interaction between HPS and C4BP and are in agreement with earlier work (Dahlbäck, 1983a). Under conditions of saturating HPS, in which nearly all C4BP would be in a complex, additional biotinylated HPS could replace unlabeled HPS in the complex. Biotinylated and unlabeled HPS appear to compete equally well for C4BP binding sites: when they are added simultaneously and allowed to come to equilibrium with C4BP, they produce a signal equivalent to that produced by incubation solutions in which they are added sequentially.

Experimental and Theoretical Basis for the Equilibrium Binding Assay. The validity of applying solution-phase thermodynamic equations to protein–protein binding data depends upon the existence of a true solution-phase equilibrium (Scheinberg, 1982). The equilibrium system depicted in Figure 1 employs solutions of pure proteins in a BSA-containing buffer. Neither protein participating in the equilibrium binding interaction is on a solid phase or is chemically modified. Furthermore, free C4BP is measured directly in the equilibrium solution, obviating the separation of free and bound species. The only opportunity for disequilibrium occurs during the exposure of the solution-phase equilibrium mixtures to the solid-phase detection system. The possibility of equilibrium disruption during this plate incubation, that is, of solid-phase HPS causing increased solution-phase free C4BP concentrations, has been minimized by limiting it to 15 min. Over the entire range of C4BP concentrations employed in the assay, in both the presence and the absence of HPS, approximately 10% of the total signal was captured during the 15-min exposure to the microtiter plate. Under these conditions, then, any readjustment of the solution-phase equilibrium would have to result from the removal from the solution of 10% of the total C4BP. Hence, the assay permits minimal, if any, perturbation of the solution-phase equilibrium by solid-phase HPS, even in the unlikely event that every C4BP molecule captured on the plate results in immediate compensatory dissociation of a solution-phase complex.

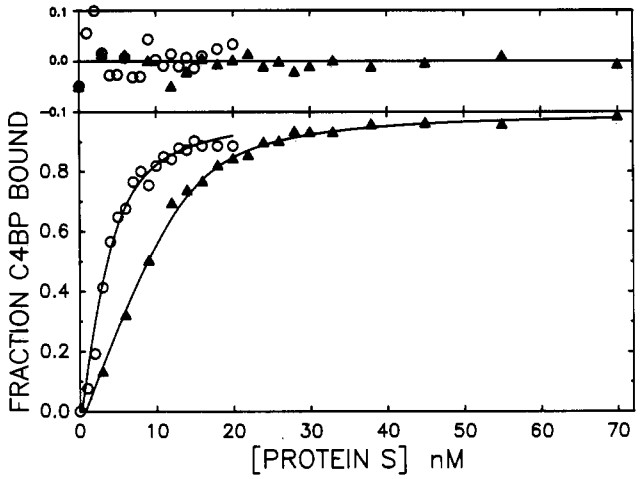

FIGURE 2: Equilibrium measurements of free C4BP in solution-phase mixtures of HPS and C4BP. Equilibrium incubations at 37 °C contained fixed C4BP concentrations of 2.1 nM (○) and 7.3 nM (▲) and systematically varied concentrations of HPS. Free C4BP was assayed and the fraction C4BP bound calculated as described under Experimental Procedures. The curves are drawn according to the fitted constants K_d = 0.72 ± 0.05 nM and stoichiometry = 0.52 ± 0.03 mol of C4BP combining/mol of HPS. The upper panel shows residuals to the fitted curves.

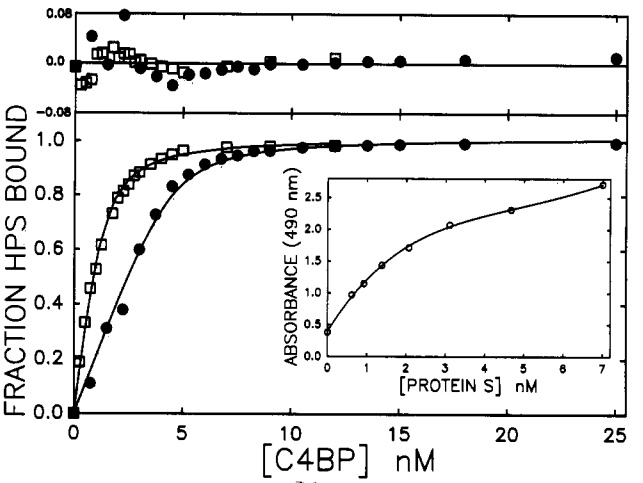

FIGURE 3: Equilibrium measurements of free HPS using immobilized C4BP: the reverse configured assay. The binding assay was performed in analogous fashion to the standard assay, as described under Experimental Procedures, except that HPS was held fixed at 2 nM (□) and 7 nM (●) and C4BP concentrations were systematically varied. A microtiter plate coated with C4BP was used to detect free HPS in the equilibrium solutions. The curves are drawn according to the fitted constants K_d = 0.43 ± 0.05 nM and stoichiometry = 1.6 ± 0.05 mol of HPS combining/mol of C4BP. The upper panel shows residuals to the fitted curves. The inset shows the standard curve data (○); the curve is the fourth-order polynomial fitted to the data.

Because solid-phase HPS is used for the detection of free C4BP in solution (Figure 1), two assumptions were made regarding the binding of solid-phase and solution-phase HPS to C4BP. First, that C4BP binds both solid-phase and solution-phase HPS at a single site (Dahlbäck, 1983a; Hillarp et al., 1989), so that C4BP captured by the immobilized HPS is a true measure of unliganded C4BP. This system differs from the antibody–antigen system, in which the antibody is bivalent and in which statistical corrections must be made to account for the number of ligand sites that are occupied (Stevens, 1987). The single-site assumption restricts interpretation of extracted stoichiometric information to HPS, the ligand species, in the standard assay as configured in Figure 1. (The reverse configured assay, in which C4BP is immobilized to detect free HPS in solution, is not restricted by this assumption. The equivalence of the stoichiometries extracted from the reverse assay data and the standard assay data supports the validity of this assumption for the standard assay. See under Discussion.) The second assumption is that immobilized HPS and solution-phase HPS bind C4BP in a topologically equivalent manner. Were this not the case, binding to the plate of C4BP in complex with HPS would occur, and plate-captured C4BP would not accurately reflect the concentration of unliganded C4BP in solution. Furthermore, binding of C4BP to solid-phase HPS would not be changed by solution-phase HPS, resulting in the fraction C4BP bound not approaching 1.0. Figures 2 and 4 show that detection of C4BP by solid-phase HPS is very sensitive to the concentration of solution-phase HPS and that the fraction C4BP bound does indeed approach 1.0. These observations support the assumption that solution phase and immobilized HPS compete for C4BP binding sites. Given the above assumptions, the physical heterogeneity HPS immobilized on polystyrene is unimportant, provided its overall ability to bind C4BP can be calibrated by using solutions of known C4BP concentration.

A representative set of equilibrium binding curves for HPS and C4BP is shown in Figure 2. Measurements of either the rate of color development or the end-point color after stopping the reactions yielded equivalent fitted curves and binding constants. This was expected, since color development over the measurement time period was linear, as evaluated by the

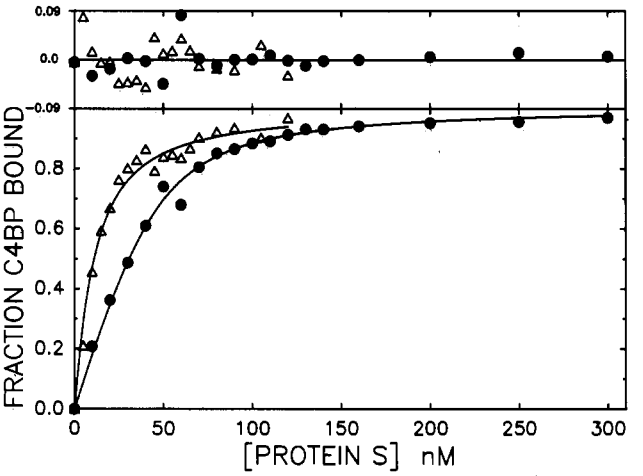

FIGURE 4: Equilibrium measurements in solution-phase mixtures of HPS and C4BP in the presence of EDTA. The binding assay was performed as described under Experimental Procedures, except that no $CaCl_2$ was added to any assay buffers, the equilibrium incubation buffer was supplemented with 4 mM EDTA, and the fixed C4BP concentrations were 3.1 nM (△) and 25.9 nM (●). The curves are drawn according to the fitted constants K_d = 4.9 ± 0.6 nM and stoichiometry = 0.58 ± 0.04 mol of C4BP combining/mol of HPS. The upper panel shows residuals to the fitted curves.

linear correlation coefficients of the absorbance versus time data for each well ($r \geq 0.990$). Because of enhanced color intensity and solubility of the peroxidase-generated product upon addition of sulfuric acid, the scatter of data points, especially at low free C4BP concentrations, was less for end-point than for kinetic measurements. Therefore all data shown are from end-point absorbance measurements made after the confirmation of linear color development. Absorbance values of intraassay duplicate microtiter wells typically differed from the mean by less than 5%. Interassay variability (one standard deviation from the mean) of extracted binding constants was 17% (K_d) and 33% (stoichiometry, mol of C4BP/mol of HPS), over seven assays done in 2 mM $CaCl_2$, using two different preparations of HPS and C4BP. Inclusion of 20 nM human coagulation factor X had no effect on the measured concen-

Table II: Binding Constants for HPS and C4BP Measured after Different Equilibrium Incubation Times

incubation at 37 °C[a] (h)	K_d ± SE[b] (nM)	stoichiometry ± SE[b] (mol of C4BP/mol of HPS)
1	0.78 ± 0.15	0.75 ± 0.06
2	0.58 ± 0.07	0.48 ± 0.02
3	0.72 ± 0.11	0.52 ± 0.03
25	0.78 ± 0.15	0.55 ± 0.04
44	0.56 ± 0.12	0.46 ± 0.03

[a] Assay conditions and data analysis are described under Experimental Procedures. [b] Standard errors (SE) represent 95% confidence intervals.

trations of free C4BP, providing evidence for the specificity of the assay system.

Because of the inherent symmetry of a binding interaction, the species held at fixed concentrations and detected in a free state in solution should be interchangeable with the varied species. That is, regardless of which protein is operationally defined as the ligand species, the extracted binding constants for their interaction should be the same. This was tested in the present system by coating the ELISA plate with C4BP and measuring free HPS in solutions of the two proteins at equilibrium (reverse configured assay, see Figure 3). Protein S captured by immobilized C4BP was detected with an HPS monoclonal antibody provided by Dr. William Church. Other assay details were unchanged. A lack of linear correspondence between color development and HPS concentration (Figure 3, inset) necessitated modification of the calculation of the fraction bound from that described under Experimental Procedures. In this case, the standard curve data were fitted by the least-squares method to a fourth-order polynomial subsequently used to calculate the fraction of HPS bound. The transformed data were then fitted to the quadratic binding equation by nonlinear least-squares regression analysis, as described under Experimental Procedures. The extracted binding constants (±SE) were K_d = 0.43 ± 0.05 nM and stoichiometry = 1.6 ± 0.05 mol of HPS combining/mol of C4BP. These values are not significantly different from those obtained by using the assay as diagramed in Figure 1 (see the following paragraph).

Equilibrium Binding Constants for the HPS–C4BP Interaction. The binding parameters of representative equilibrium assays are shown in Table III. Table II shows data from a series of assays performed after the indicated equilibrium incubation times. The similarity of these binding parameters suggests that equilibrium was achieved in ≤1 h at 37 °C. The binding constants calculated from the statistical summation of seven separate assays of equilibrium mixtures incubated 3 h at 37 °C were K_d = 0.6 ± 0.1 nM, stoichiometry (mol of C4BP/mol of HPS) = 0.6 ± 0.2 (values are ± one standard deviation from the mean). In the absence of added $CaCl_2$, the measured K_d (≈2 nM) is approximately 3-fold higher than in its presence, suggesting a partial dependence of the binding interaction upon calcium ions. In the absence of calcium and the presence of 4 mM EDTA, the K_d is further increased to 6 ± 2 nM (range of two assays was 4.9–7.8 nM). Because of the increased K_d in the presence of EDTA, both the spread in fixed concentrations of C4BP employed in the equilibrium phase of the assay and the upper range of varied HPS had to be increased in order to be able to resolve the two binding curves (Figure 4). While the concentrations of C4BP were increased as high as 26 nM in the presence of 4 mM EDTA, the standard curve still showed a linear correspondence of C4BP to ELISA signal under these conditions (r = 0.997). The binding constants for the interaction of C4BP with thrombin-cleaved HPS were not different from those with unmodified HPS (Table III).

Table III: Binding Constants Determined by Using Different Solution-Phase Equilibrium Incubation Conditions

equilibrium conditions[a]	K_d ± SE (nM)	stoichiometry ± SE (mol of C4BP/mol of HPS)
standard (low)[b]	0.42 ± 0.12	0.67 ± 0.06
standard (high)	0.99 ± 0.11	0.78 ± 0.04
thrombin-cleaved HPS	0.69 ± 0.12	0.50 ± 0.03
no added $CaCl_2$	2.1 ± 0.6	0.60 ± 0.09
4 mM EDTA	7.8 ± 1.1	0.60 ± 0.06

[a] All equilibrium solutions were incubated for 3 h at 37 °C, under assay conditions described in detail under Experimental Procedures. [b] The data from the two standard assays shown represent the lowest and highest extracted K_ds of seven assays.

Discussion

The equilibrium binding interaction in solution of HPS and C4BP has been examined by using an ELISA-enabled technique to measure free C4BP and free HPS. This is an analogous application of the technique originally described by Friguet et al. (1985). To the best of our knowledge, this is the first reported use of this technique to determine the equilibrium binding parameters for two proteins, neither of which is an antibody. In addition to minor modifications of the method itself, nonlinear regression analysis has been used to extract dissociation constants and stoichiometries directly from measurements made on solutions at equilibrium. A control experiment to detect disruption of the solution-phase equilibrium during the incubation over solid-phase HPS evidenced only a small decrease in signal (≈10%) following the 15-min plate incubation. This result suggests little if any disturbance of the solution-phase equilibrium is occurring during its contact with the microtiter plate. If the solution-phase equilibrium was affected by solid-phase HPS, the ultimate outcome would be an underestimation of the fraction of C4BP bound and a corresponding overestimation of the K_d. For this reason, the extracted K_d can be viewed as an upper limit estimate. Advantages of this equilibrium binding assay are its rapidity, reproducibility, and requirement for small amounts of protein. Although not universally applicable, it should be useful for the determination of equilibrium binding constants in other nonantibody protein systems in which the dissociation rate constant is small compared to the total time required for the solid-phase detection steps of the assay (45 min).

In contrast to other investigators (Dahlbäck, 1983a, 1986; Nishioka & Suzuki, 1990; Malm et al., 1990), we find the binding of HPS to C4BP is a high-affinity interaction, with a $K_d \approx 0.6$ nM (Figure 2; Table III). Furthermore, at nanomolar concentrations of both proteins, equilibrium appears to be achieved in, at most, 1 h at 37 °C (Table II), indicating that the reported association rate constant of 10^3 M^{-1} s^{-1} (Dahlbäck, 1983a) is several orders of magnitude lower than the true value. The results of the present study also suggest that the binding interaction between HPS and C4BP is partly calcium ion dependent, since in the presence of 4 mM EDTA the K_d increases approximately 10-fold to about 6 nM (Figure 4). It is unlikely that this is an effect of EDTA unrelated to its calcium chelation, since without added calcium (and without EDTA) the K_d is some 3-fold higher than in the presence of calcium (Table III). The stoichiometry for the HPS–C4BP interaction remained unchanged under these various conditions (Table III). The observation that throm-

bin-cleaved HPS binds C4BP with the same binding constants as uncleaved HPS is in agreement with earlier reports (Dahlbäck, 1983a). This observation supports the concept that while thrombin cleavage of HPS may regulate the APC cofactor activity and factor Va deprotection activity of HPS in vivo, it has no influence on the C4BP binding of the molecule.

Because the K_d determined in the present study differed greatly from previously published values and because the validity of the employed data analysis depended upon the reaction being a freely reversible one, experimental confirmation of the reversibility of the interaction was sought. The results, summarized in Table I, demonstrated that biotin-labeled HPS could displace unlabeled HPS in complex with C4BP, and unlabeled HPS could likewise displace biotinylated HPS in a complex. These observations confirmed that the interaction of HPS with C4BP is in fact an association–dissociation reaction under the conditions of the equilibrium binding experiments.

The stoichiometry for the HPS–C4BP interaction has been reported to be one molecule of HPS bound to each C4BP molecule (Dahlbäck, 1983a). This stoichiometry was determined by using an $E^{0.1\%}_{280nm}$ of 0.93 for C4BP quantification. The findings of the present study suggest a stoichiometry 1.7 ± 0.3 molecules of HPS bound to each molecule of C4BP. The most obvious explanation for this discrepancy is the choice of extinction coefficient for C4BP. The only published account of an experimentally determined value, $E^{0.1\%}_{280nm} = 0.93$ (Villiers et al., 1981), gave no details of method and employed a protocol for C4BP purification unlikely to remove bound HPS. This value is significantly different from the theoretical value of 1.41 calculated from the deduced amino acid sequence of the α-chain of C4BP (Perkins et al., 1986). Because this calculation was made before the existence of the β-chain was known and because it is significantly different from the reported experimental value, we employed two independent methods to experimentally determine the $E^{0.1\%}_{280nm}$ of C4BP adsorbed from plasma by barium citrate. Far-ultraviolet absorption spectrophotometry and quantitative amino acid analysis were in close agreement and yielded an average $E^{0.1\%}_{280nm}$ of 1.36. This coefficient is within 4% of the theoretically derived one and would change an apparent stoichiometry (mol of HPS/mol of C4BP) derived by using $E^{0.1\%}_{280nm} = 0.93$ of 1.0 to 1.46. This latter value is in line with the stoichiometry we observe. Other sources of error in protein quantification that could result in an erroneous calculated stoichiometry include the molecular weights of HPS and C4BP and the extinction coefficient of HPS. The cDNAs of both proteins have been cloned (Lundwall et al., 1986; Hoskins et al., 1987; Chung et al., 1985; Hillarp & Dahlbäck, 1990), and the molecular weights deduced from the cDNA code are in good agreement with those derived from physical measurements (DiScipio & Davie, 1979; Perkins et al., 1986). The extinction coefficient of HPS has been convincingly determined by differential refractometry in an analytical ultracentrifuge (DiScipio & Davie, 1979).

There are several possible explanations for our observed stoichiometry of HPS to C4BP being greater than 1:1. One phenomenon that could affect the stoichiometry is HPS self-association. Although a tendency of bovine protein S to form homodimers in the presence of calcium ions has been reported (Walker, 1986), this is unlikely to contribute to the stoichiometry observed in the present study, since this stoichiometry was unchanged in the presence of EDTA (Figure 4). Dimerization or self-association of HPS by some calcium ion independent mechanism cannot be ruled out. No evidence of large aggregation was observed by light scattering, as measured by absorbance at 320 nm, and when the extra precaution of centrifuging HPS solutions in a microfuge for 20 min prior to assay was taken, the results of the equilibrium binding assay were unchanged.

Another possible contributor to the high stoichiometry is that a fraction of the purified HPS used in these studies may be incapable of binding C4BP, due to denaturation, degradation, or other causes. There was no way of determining the specific binding activity—the bindability—of HPS preparations used in the present experiments, and it is conceivable that they were not 100%. Moreover, the present data cannot distinguish between the presence of unbindable HPS and self-association of HPS. Either situation, or a combination of both, would result in a stoichiometry (mol of HPS/mol of C4BP) of greater than 1, as is observed by measuring either free C4BP as diagrammed in Figure 1 (standard assay, $n \approx 1.7$, Figure 2, Table III) or free HPS (reverse configured assay, $n \approx 1.6$, Figure 3).

Steric constraints and the single-chain nature of HPS make it extremely unlikely that more than one C4BP molecule can bind to a single HPS molecule. Unlike the standard assay as configured in Figure 1, then, the reverse-configured assay requires no single ligand site assumption. That is, HPS captured by solid-phase C4BP in the reverse assay is certainly representative of the true concentration of free (unliganded) HPS. For this reason, the stoichiometry produced by the reverse assay pertains strictly to the number of HPS binding sites on C4BP or to the bindability of the HPS. The agreement between the observed stoichiometries and K_ds extracted from the standard assay data and the reverse-configured assay data provides evidence that the assumption of a single site on C4BP made for the standard assay is correct. The validity of this assumption renders unlikely a third explanation for the observed stoichiometry: that the heterogeneity of C4BP subunit composition includes molecules containing more than one β-chain, thereby capable of binding more than one HPS molecule.

The levels of circulating free HPS and HPS in complex with C4BP have been reported to be about 40% and 60% of total circulating HPS, respectively (Dahlbäck, 1983a,b). If the two proteins are in equilibrium described by the simple expression nC4BP + HPS $\rightleftarrows$ C4BP$_n$·HPS, then the low K_d we report (0.6 nM) would predict that nearly all circulating HPS would be bound to C4BP. Although most measurements of free and bound HPS and C4BP have been made in citrated plasma, the K_d of 6 nM we find in the presence of EDTA would also predict nearly complete participation of HPS in complexes, even in the absence of calcium. The explanation for these apparently contradictory observations is unclear. Further complicating the analysis in plasma is the establishment of C4BP as a positive acute phase reactant, whose circulating levels are highly variable and dependent on immediate physiological conditions (Funakoshi et al., 1988; Matsuguchi et al., 1989). In conclusion, the equilibrium expression above is almost certainly an oversimplification of the situation in plasma, where it is likely that other undetermined factors influence the binding equilibrium between HPS and C4BP.

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