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Purification of human C4b-binding protein and formation of its complex with vitamin K-dependent protein S

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C4b-binding protein was purified from human plasma in high yield by a simple procedure involving barium citrate adsorption and two subsequent chromatographic steps. Approx. 80% of plasma C4b-binding protein was adsorbed on the barium citrate, presumably because of its complex-formation with vitamin K-dependent protein S. The purified C4b-binding protein had a molecular weight of 570000, as determined by ultracentrifugation, and was composed of about eight subunits (M_r approx. 70000). Uncomplexed plasma C4b-binding protein was purified from the supernatant after barium citrate adsorption. On sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in non-reducing conditions and on agarose-gel electrophoresis it appeared as a doublet, indicating two forms differing slightly from each other in molecular weight and net charge. The protein band with the higher molecular weight in the doublet corresponded to the C4b-binding protein purified from the barium citrate eluate. Complex-formation between protein S and C4b-binding protein was studied in plasma, and in a system with purified components, by an agarose-gel electrophoresis technique. Protein S was found to form a 1:1 complex with the higher-molecular-weight form of C4b-binding protein, whereas the lower-molecular-weight form of C4b-binding protein did not bind protein S. The K_D for the C4b-binding protein–protein S interaction in a system with purified components was approx. 0.9×10^{-7} M. Rates of association and dissociation at 37°C were low, namely about 1×10^3 M$^{-1}\cdot$s^{-1} and 1.8×10^{-4}–4.5×10^{-4} s^{-1} respectively, In human plasma free protein S and free higher-molecular-weight C4b-binding protein were in equilibrium with the C4b-binding protein–protein S complex. Approx. 40% of both proteins existed as free proteins. From equilibrium data in plasma a K_D of about 0.7×10^{-7} M was calculated for the C4b-binding protein–protein S interaction.

It has been reported that the vitamin K-dependent protein S in plasma exists in two forms, i.e. as free protein, and in complex with the complement component C4b-binding protein (C4bp) (Dahlbäck & Stenflo, 1981). C4bp is an important cofactor in the degradation of fluid-phase C4b by the enzyme Factor I (previously named C3b inactivator) (Scharfstein *et al.*, 1978; Fujita *et al.*, 1978; Nagasawa *et al.*, 1980), whereas it is redundant in the degradation of surface-bound C4b (Gigli *et al.*, 1979; Iida & Nussenzweig, 1981). C4bp also accelerates decay-dissociation of the classical C3 convertase, C42. Scharfstein *et al.* (1979) showed that C4bp was incorporated into immunocomplexes *in vitro* and *in vivo*. Human C4bp exists in two forms, antigenically identical but of slightly different molecular weight (540000 and 590000) and net charge (Scharfstein *et al.*, 1978). Fujita & Nussenzweig (1979) separated the two forms of C4bp (referred to as C4bp-high and C4bp-low) and showed that both forms served as cofactors in the

The nomenclature used for the complement proteins is that recommended by the World Health Organisation (1968, 1981), together with the following further abbreviations: C4bp, C4b-binding protein; C4bp-high and C4bp-low, the two forms of C4bp with slightly different molecular weights; C4bp(s), C4bp purified from a barium citrate eluate; C4bp(Ba-sup), C4bp purified from the supernatant after barium citrate adsorption. All other abbreviations are the same as in the preceding paper (Dahlbäck, 1983).

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cleavage of C4b in solution. Both forms of C4bp are composed of an unknown number of disulphide-linked subunits (M_r approx. 70000) with a single amino acid sequence (Dahlbäck & Stenflo, 1981; Reid & Gagnon, 1982).

The problems reported in the purification of human C4bp have been the difficulty of separating the protein from contaminating immunoglobulin M, and the occurrence of complex-formation between C4bp and C4b during manipulation (Scharfstein *et al.*, 1978; Nagasawa & Stroud, 1980; Villiers *et al.*, 1981). The present paper reports a simple purification procedure for human C4bp. Furthermore, complex-formation between vitamin K-dependent protein S and C4bp, both in plasma and in a system with purified components, is described.

Materials and methods

Materials

Sephacryl S-300, Sepharose CL-6B, agarose A and B (used for the C4bp electroimmunoassay) and molecular-weight markers for gel filtration were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Ultrogel AcA-34 was supplied by LKB Produkter, Bromma, Sweden. Heparin–Sepharose was provided by Kabi, Stockholm, Sweden. All other materials were the same as reported in the preceding paper (Dahlbäck, 1983).

Human vitamin K-dependent protein S was purified as described in the preceding paper (Dahlbäck, 1983).

Purification of C4b-binding protein

Chromatography and centrifugation were run at 4°C, all manipulations being performed on an ice bath. Freshly frozen plasma was obtained from the blood bank and, after it had been thawed at 37°C, the following proteinase inhibitors were added: benzamidine hydrochloride (10mM), di-isopropyl phosphorofluoridate (1mM), phenylmethanesulphonyl fluoride (1mM) and soya-bean trypsin inhibitor (50mg/l). The C4bp purified from the barium citrate eluate is referred to below as C4bp(s), and the C4bp purified from the supernatant plasma after barium citrate adsorption as C4bp(Ba-sup).

Barium citrate adsorption, elution and initial chromatography on DEAE-Sephacel were performed as described in the preceding paper (Dahlbäck, 1983). C4bp(s) was recovered in the first major protein peak eluted from the DEAE-Sephacel column, separate from the peak containing free protein S. The fractions containing C4bp(s) were pooled (see the preceding paper, Dahlbäck, 1983) and dialysed against 50mM-Tris/HCl buffer, pH7.5, containing 1mM-benzamidine hydrochloride. A small amount of precipitated material was removed by centrifugation at 10000*g* for 10min. The supernatant solution was made 2mM with respect to $CaCl_2$, and applied to a column (2.5cm × 18.5cm) packed with heparin–Sepharose in 50mM-Tris/HCl buffer, pH7.5, containing 1mM-benzamidine hydrochloride and 2mM-$CaCl_2$ (Fig. 1). The column was eluted at 70ml/h, and six fractions/h were collected. After application of the protein solution the column was washed for 2h with equilibration buffer. The C4bp was then eluted with a linear gradient of NaCl (0–0.7M) in starting buffer, 300ml in each vessel. The serum amyloid P-component (the major contaminant in the material applied) was retained on the column after the gradient. It was eluted with 50mM-Tris/HCl buffer, pH7.5, containing 1M-NaCl and 10mM-EDTA. Fractions containing C4bp(s) were pooled as indicated in Fig. 1, dialysed against 50mM-Tris/HCl buffer, pH8.0, containing 0.15M-NaCl and 10mM-EDTA, and stored at −70°C.

To minimize the protein S content in the purified C4bp, the preparation was passed over a column (2.5cm × 90cm) with Sepharose CL-6B in 50mM-Tris/HCl buffer, pH7.5, containing 1M-NaCl and 10mM-EDTA at 4°C at a rate of 20ml/h, and

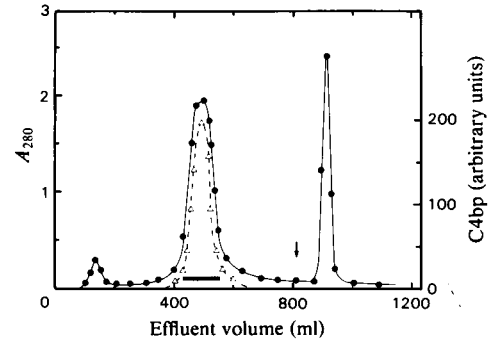

Fig. 1. *Heparin–Sepharose chromatography of the C4pb(s) pool from the initial chromatography on DEAE-Sephacel*

The column (2.5cm × 18.5cm) was equilibrated in 50mM-Tris/HCl buffer, pH7.5, containing 2mM-$CaCl_2$ and 1mM-benzamidine hydrochloride, C4bp(s) was eluted with a linear gradient of NaCl (0–0.7M), 300ml in each vessel. The gradient was started at an effluent volume of 200ml. Chromatography was run at 70ml/h, and 11.7ml fractions were collected. The fractions were monitored immunochemically with an antiserum against C4bp. The arrow indicates elution of serum amyloid P-component with 50mM-Tris/HCl buffer, pH7.5, containing 1M-NaCl and 10mM-EDTA. ●, A_{280}; △, C4bp(s). Fractions were pooled as indicated by the horizontal bar.

four fractions/h were collected. Before application, the sample was concentrated by the addition of solid $(NH_4)_2SO_4$ to 50% saturation. After the mixture had been stirred at room temperature for 1h, the precipitate was collected and dissolved in the column equilibration buffer. No major purification was achieved by this gel-filtration chromatography, and the amount of dissociated protein S was less than 1mg per 120mg of applied material. The fractions containing C4bp were pooled, dialysed against 50mM-Tris/HCl buffer, pH7.5, containing 0.15M-NaCl and 10mM-EDTA, and stored at −70°C. This preparation was used for the C4bp–protein S binding experiments, amino acid analysis and ultracentrifugation study.

Uncomplexed C4bp was purified from 3.4 litres of the supernatant plasma after barium citrate adsorption. An equal volume of distilled water was added to lower the ionic strength. The diluted plasma was mixed with pre-swollen QAE-Sephadex A-50 (150ml/l of starting plasma) equilibrated in 50mM-Tris/HCl buffer, pH8.0, containing 0.1M-NaCl, 1mM-benzamidine hydrochloride and 2mM-$CaCl_2$. After the mixture had been stirred gently for 1h, the non-adsorbed proteins were washed away on a glass-filter funnel with 2 litres of equilibrium buffer. The QAE-Sephadex was then packed in a column (9cm × 20cm) and washed overnight with 2 litres of equilibration buffer. The QAE-Sephadex was then resuspended and layered on top of 370ml of fresh QAE-Sephadex in equilibration buffer packed in a column (5cm × 50cm). The final height of the column was 50cm. The adsorbed proteins were eluted with a linear gradient of NaCl (0.1–0.7M) in 10mM-Tris/HCl buffer, pH8.6, containing 1mM-benzamidine hydrochloride and 2mM-$CaCl_2$, 1.5 litres in each chamber. The column was run at 30ml/h, and three fractions/h were collected. The position of C4bp was monitored immunochemically, the bulk of it, having the same elution volume as C3, being recovered in the first major protein peak. The fractions containing C4bp were pooled (285ml) and made 5% (w/v) with respect to poly(ethylene glycol) by the addition of solid poly(ethylene glycol) with a mean molecular weight of 6000. The solution was stirred for 1h and the precipitate, collected by centrifugation at 10000*g* for 10min, was suspended in 50ml of ice-cold 20mM-sodium phosphate buffer, pH7.2, containing 1mM-benzamidine hydrochloride, 1mM-di-isopropyl phosphorofluoridate and 1mM-phenylmethanesulphonyl fluoride. The undissolved precipitate, containing C4bp, was collected by centrifugation at 10000*g* for 10min and dissolved in 7ml of 50mM-Tris/HCl buffer, pH8.0, containing 1M-NaCl and 10mM-EDTA. The dissolved protein was applied to a column (2.5cm × 95cm) packed with Sephacryl S-300 in 50mM-Tris/HCl buffer, pH8.0, containing 1M-NaCl and 10mM-EDTA. The column was eluted at 26ml/h, and six fractions/h were collected. C4bp was eluted as a symmetrical peak close to the void volume. Fractions containing C4bp were pooled, dialysed against 50mM-Tris/HCl buffer, pH8.0, containing 0.15M-NaCl and 10mM-EDTA, and stored at −70°C.

The isolated proteins were quantified spectrophotometrically by using the following values for $A^{1\%}_{1cm}$ at 280nm: protein S, 9.5 (DiScipio & Davie, 1979); C4bp, 9.3 (Villiers *et al.*, 1981).

Electrophoretic and immunochemical methods

Agarose-gel electrophoresis was run at pH8.6 in 75mM-sodium barbital buffer containing 2mM-EDTA on the flexible polyester film Gelbond TM as described by Jeppsson *et al.* (1979). After the gels had been fixed, dried and stained, the distribution of ^{125}I-labelled protein S was determined by radioautography. Quantitative data were obtained by cutting out the labelled protein bands and measuring the radioactivity in a γ-radiation counter. SDS/polyacrylamide-slab-gel electrophoresis was performed as described by Blobel & Dobberstein (1975), with 5–15%-gradient gels and the buffer system of Maziel (1971). Molecular weights were assessed by comparison with those of standard proteins.

The concentrations of C4bp in pooled human citrated plasma, and in barium citrate-adsorbed plasma, were determined by electroimmunoassay (Laurell, 1972). A mixture of medium-electroendosmosis and high-electroendosmosis agarose (1:1 mixture of Pharmacia agarose A and agarose B) was used, and electrophoresis was run in 75mM-sodium barbital buffer, pH8.6, containing 400i.u. of heparin/ml and 2mM-EDTA. The samples were diluted in a similar buffer but containing 10mM-EDTA. Under these conditions no conversion of C4 into C4b was observed during electrophoresis, and thus no complex-formation between C4b and C4bp occurred. Pure C4bp(s) was used as standard. The standard was dissolved in human barium citrate-adsorbed plasma that was totally depleted of C4bp after immunoadsorption with the immunoglobulin fraction of a C4bp antiserum coupled to CNBr-activated Sepharose (Cuatrecasas, 1970). The C4bp antiserum was raised in rabbits by using purified C4bp(s). A rabbit antiserum against C4 was available at the laboratory.

Complex-formation between protein S and C4bp in plasma and in a system with purified components

The ratio of free to complexed protein S in plasma was determined by the addition of a trace amount of ^{125}I-labelled protein S to pooled human citrated plasma. The volume of the added ^{125}I-labelled

protein S was 1% (v/v) and the final concentration of ^{125}I-labelled protein S was less than 0.2 μg/ml. Parallel mixtures were incubated at 37°C for between 5 min and 16 h, and samples were then subjected to agarose-gel electrophoresis in the presence of 2 mM-EDTA. Free and complexed protein S were effectively separated during electrophoresis. The gels were subjected to radioautography, and quantitative data on protein S distribution between the two forms were obtained by cutting out the labelled protein bands and measuring the radioactivity in a γ-radiation counter.

The rate of complex-formation and the equilibrium binding between purified protein S and C4bp were studied essentially by the technique described above. Protein S (final concn. 2.5–100 μg/ml) with a trace amount of ^{125}I-labelled protein S was added to parallel incubation mixtures with C4bp(s) (50–300 μg/ml) or C4bp(Ba-sup) (300 μg/ml) in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl and 10 mg of bovine serum albumin/ml. At intervals samples of the reaction mixtures were subjected to agarose-gel electrophoresis in the presence of EDTA. The ratio of free to complexed ^{125}I-labelled protein S was determined as described above. The second-order equation (1) was used to calculate the rate constant of association (k_{+1}):

$$k_{+1} = 2.303(1/t)\cdot[1/(a-b)]\cdot\log[b(a-x)/a(b-x)] \quad (1)$$

where a is the concentration of protein S, b is the concentration of C4bp (1 protein S-binding site per C4bp was assumed in calculation) and x is the concentration of C4bp–protein S complex formed during the time t.

The rate of dissociation of protein S from an isolated C4bp–protein S complex was measured at different temperatures (4°C, 22°C and 37°C) by using the agarose-gel-electrophoresis separation technique. The C4bp–protein S complex used was isolated on a column (0.9 cm × 30 cm) packed with Ultrogel AcA-34 in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl and 10 mg of bovine serum albumin/ml. Chromatography was run at 4°C; the flow rate was 3 ml/h, and 0.75 ml fractions were collected. A 0.5 ml portion of a mixture of C4bp(s) (0.5 mg/ml) and protein S (0.1 mg/ml, with a ^{125}I-labelled protein S tracer) was applied to the column. Before application, the C4bp/protein S mixture was incubated for 4 h at 37°C to reach equilibrium. The isolated C4bp–protein S complex was used within 1 h of its elution from the column. An excess of unlabelled protein S (final concn. 0.4 mg/ml) was added to the isolated C4bp–protein S complex, and at intervals samples were withdrawn, kept on an ice bath and subjected to agarose-gel electrophoresis within 1 h. The distribution of radioactivity was measured as described above. The rate constant of dissociation was calculated from eqn. (2):

$$k_{-1} = 0.693/t_{\frac{1}{2}} \quad (2)$$

where $t_{\frac{1}{2}}$ is the half-time of dissociation.

Other methods

The ^{125}I-labelling technique and the methods to determine the amino acid composition and the content of γ-carboxyglutamic acid residues are given in the preceding paper (Dahlbäck, 1983). Automated Edman degradation was performed with a Beckman sequencer (model 890C), with a modified Quadrol programme (Edman & Begg, 1967). The phenylthiohydantoin derivatives of amino acids were identified by means of high-pressure liquid chromatography and t.l.c. performed as described by Fernlund *et al.* (1978).

The Stokes radii of C4bp(s) and of C4bp(Ba-sup) were determined by gel-filtration chromatography on a column (0.9 cm × 97 cm) of Sepharose CL-6B in 10 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl and 5 mM-EDTA. Chromatography was run at room temperature for 3.4 ml/h, and 1 ml fractions were collected. The following standard proteins were used (Stokes radii given in parentheses): thyroglobulin (8.50 nm), ferritin (6.10 nm), catalase (5.22 nm) and fructose bisphosphate aldolase (4.81 nm). $K_{av.}$ values were calculated as described by Laurent & Killander (1964). A calibration curve was drawn on semi-logarithmic graph paper by plotting the $K_{av.}$ values (on the linear scale) against the corresponding Stokes radius (on the logarithmic scale).

The molecular weight of C4bp(s) was determined by sedimentation equilibrium in a Beckman model E analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). The rapid long-column meniscus-depletion method (Chervenka, 1970) was used, the centrifuge being run at 8–10°C at a speed of 5600 rev./min. The solvent was 50 mM-Tris/HCl buffer, pH 7.5, containing 1 M-NaCl and 10 mM-EDTA, and the initial protein concentration was 1.5 g/l. A partial specific volume of 0.72 ml/g was calculated from the amino acid and carbohydrate composition of the protein (Nagasawa & Stroud, 1980; Reid & Gagnon, 1982), by using published partial specific volumes of amino acid and sugar residues (Cohn & Edsall, 1943; Gibbons, 1972).

Results

Purification of C4b-binding protein

C4bp(s) was purified from freshly frozen human plasma by barium citrate adsorption and elution, followed by two successive chromatographic steps. The purification procedure is summarized in Table 1. Approx. 75–80% of the C4bp in plasma was

Table 1. *Purification of C4bp from the barium citrate elutate*

For full experimental details see the text. The protein concentration was measured from the absorbance at 280nm, assuming $A^{1\%}_{1cm} = 10$. C4bp was determined by electroimmunoassay.

Fraction	Volume (ml)	Protein (mg)	C4bp (arbitrary units)	Recovery (%)	Purification (fold)
Plasma	5000	330000	1000	100	1
Barium citrate eluate	930	7440	750	75	33
DEAE-Sephacel	230	797	400	40	166
Heparin–Sepharose	124	179	180	18	332

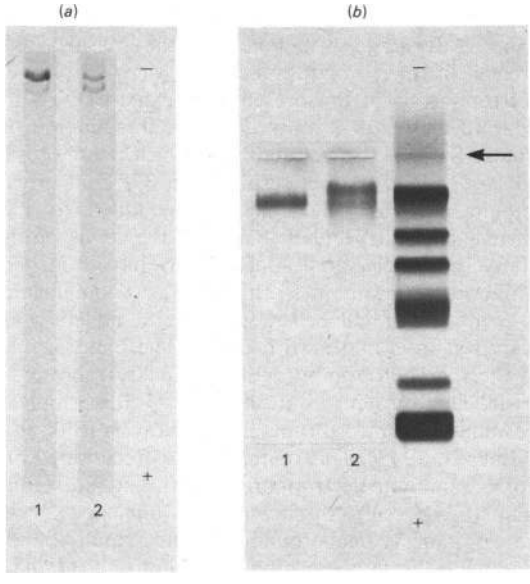

Fig. 2. *Electrophoretic analysis of purified C4bp*
(*a*) SDS/5%-polyacrylamide-slab-gel electrophoresis of C4bp(s) (lane 1) and C4bp(Ba-sup) (lane 2). The samples 1 and 2 were unreduced. Approx. 10–15 μg of protein was applied per gel. (*b*) Agarose-gel electrophoresis of (1) C4bp(s) and (2) C4bp(Ba-sup). The electrophoresis was run in the presence of 2mM-EDTA. The arrow indicates the application slot. Approx. 10 μg of protein was applied per track. A normal plasma sample is shown as a reference.

adsorbed on barium citrate. This step proved to be very efficient in the purification of C4bp(s). Both C4 and C1 were recovered in the supernatant after barium citrate adsorption, and thus complex-formation with C4b during the purification procedure was avoided, When analysed by SDS/5%-polyacrylamide-slab-gel electrophoresis, purified C4bp(s) appeared as a high-molecular-weight protein (Fig. 2*a*). A small amount of the purified material (less than 10%) migrated slightly faster, indicating a somewhat lower molecular weight. This material was present in small amounts (less than 10%) in most C4bp(s) preparations. After reduction, a band of protein with apparent M_r 70000 was observed (result not shown), and no high-molecular-weight material was noticed. On agarose-gel electrophoresis C4bp(s) appeared homogeneous, and migrated at the same rate as fibrinogen (Fig. 2*b*). The amino acid composition of C4pb(s) is given in Table 2, together with those on record. Purified C4bp(s) reacted with a monospecific antiserum against protein S (result not shown), indicating the presence of protein S in the purified material. The content of γ-carboxyglutamic acid residues of different C4bp(s) preparations varied between 0.3 and 1 residue/molecule of C4bp(s) subunit when calculated relative to aspartic acid in base hydrolysates. The C4bp(s) preparation used for studies of the complex-formation with protein S, Stokes radius, amino acid composition and molecular-weight deter-

Table 2. *Amino acid composition of C4bp*

Amino acid	Amino acid composition (residues/100 residues) Present work	Nagasawa & Stroud (1980)	Reid & Gagnon (1982)
Asp	7.59	7.93	7.87
Thr*	6.47	5.62	6.38
Ser*	9.98	7.28	7.96
Glu	10.60	13.65	10.99
Pro	6.21	7.65	7.62
CyS	4.74	4.89	6.17
Gly	6.68	7.84	7.05
Ala	4.15	3.96	3.39
Val†	6.04	5.07	5.97
Met	0.73	0.74	0.87
Ile†	4.23	4.15	4.38
Leu	6.34	5.99	6.11
Tyr	3.79	4.06	4.35
Phe	3.26	3.41	3.08
Lys	5.65	5.25	5.71
His	2.5	2.58	2.81
Arg	4.31	4.24	5.23
Trp	Not determined	5.62	3.95

* Extrapolated to zero-time hydrolysis.
† 72h hydrolysis value.

mination by ultracentrifugation contained approx. 0.38 γ-carboxyglutamic acid residue/C4bp subunit. This indicates a ratio between protein S and C4bp subunit in the purified material of 1:26 (or less than 5%), assuming that protein S contained 10 γ-carboxyglutamic acid residues/molecule (DiScipio & Davie, 1979).

Approx. 20–25% of plasma C4bp remained in the supernatant after barium citrate adsorption. This material, C4bp(Ba-sup), was purified with a yield of approx. 28%. The purified material represents approx. 7% of the total amount of C4bp in the starting plasma. Inhibitors of proteolytic enzymes were added throughout the purification to minimize the risk of conversion of C4 into C4b with subsequent complex-formation with C4bp. On SDS/5%-polyacrylamide gels the purified material appeared as a closely spaced doublet (Fig. 2*a*). Different preparations showed different ratios between the two protein bands, and, in the first two preparations, only the slightly-faster-migrating band was observed, as previously reported (Dahlbäck & Stenflo, 1981). The upper band in the doublet had the same migration rate as the dominant C4bp(s) band. After reduction of C4bp(Ba-sup), a dominant band with M_r 70000 was observed on the gels (result not shown). When analysed by agarose-gel electrophoresis, C4bp(Ba-sup) appeared as a closely spaced doublet (Fig. 2*b*). The faster-migrating protein band in the doublet, representing approx. 40% of the material applied, appeared to have the same migration rate as C4bp(s). A 6 mg portion of reduced and carboxymethylated C4bp(Ba-sup) was subjected to automated Edman degradation. A single *N*-terminal sequence was found, Asn-Cys-Gly-Pro-Pro-Pro-Thr-Leu-Ser-Phe-Ala-Ala, which is identical with that of C4bp(s) (Dahlbäck & Stenflo, 1981), and also with that reported by Reid & Gagnon (1982). The structural background for the heterogeneous appearance of C4bp(Ba-sup) is unknown. Both forms of C4bp(Ba-sup) formed complexes with purified C4b (reported in the following paper, Dahlbäck & Hildebrand, 1983), whereas only the form that migrated as C4bp(s) on SDS/polyacrylamide and agarose gels was able to form a complex with protein S (see below). The purified C4bp(Ba-sup) did not react with a monospecific protein S antiserum (result not shown), indicating the absence of protein S from the purified material. The purified C4bp(Ba-sup) contained a trace amount of immunoglobulin M detected by electroimmunoassay.

The molecular weight of C4bp(s) was determined by sedimentation equilibrium in 1 M-NaCl, pH 7.5. An initial experiment at physiological ionic strength gave evidence of appreciable aggregation of the protein. At the higher ionic strength, however, the logarithm of fringe displacement, plotted against the square of the distance from the centre of rotation, was linear from a displacement of about 100 μm to 2500 μm at the cell bottom. This corresponds to a protein concentration range of about 85 mg/l to 2.1 g/l. A molecular weight of 570000 was obtained. Error in this value is estimated to be about 10%. Stokes radii of C4bp(s) and C4bp(Ba-sup) were found to be approx. 11.5 nm and 11.0 nm respectively, which, within permissible experimental error, are identical.

C4b-binding protein–protein S complex in plasma

As discussed above, protein S in plasma exists in two forms: as free protein and in complex with C4bp. Advantage was taken of the marked difference in their migration rates (on agarose-gel electrophoresis in the presence of 2 mM-EDTA) to separate them. As discussed below, dissociation of protein S from the complex during electrophoresis did not influence the results. At 37°C a trace amount of ^{125}I-labelled protein S added to the plasma was distributed between the two forms relatively rapidly and appeared to have reached equilibrium after about 1–2 h (Fig. 3). At equilibrium 60% of the ^{125}I-labelled protein S added was recovered in complex with C4bp. The band visible on the radioautography with β_1-mobility represents ^{125}I-labelled protein S in a C4bp–C4b complex, presumably formed during electrophoresis (Sjöholm & Laurell, 1973). This material was precipitated on crossed immunoelectrophoresis with an antiserum against C4 (result not shown). It is unclear what is the structural background of the small band, occupying the same position as albumin, seen late in the incubation. As discussed in the preceding paper (Dahlbäck, 1983), part of the purified protein S appeared as a disulphide-linked two-polypeptide-chain molecule. An equivalent of this form of protein S presumably does not exist in plasma. However, essentially similar results were obtained when ^{125}I-labelled protein S containing only the two-chain form was used (results not shown). The concentration of free protein S in a mixed human citrated plasma pool was approx. 10 mg/l (see the preceding paper, Dahlbäck, 1983). Thus the concentration of protein S in the C4bp–protein S complex was approx. 15 mg/l, and the total protein S content in plasma was 25 mg/l. The concentration of C4bp in the same plasma pool was estimated to be 200 mg/l. On a molar basis the ratio between total protein S and C4bp concentrations in plasma was calculated to be 0.8:1, by using 85000 and 570000 as molecular weights for protein S and C4bp respectively.

Complex-formation between purified protein S and C4b-binding protein

Complex-formation between purified protein S and C4bp(Ba-sup) or C4bp(s) was measured by

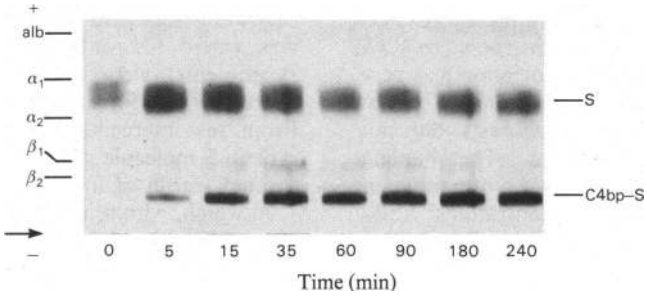

Fig. 3. *Equilibration of a ^{125}I-labelled protein S tracer between free and complexed protein S in plasma*
Trace amounts of ^{125}I-labelled protein S were added at intervals to equal samples of human citrated plasma. The samples were then incubated at 37°C before being submitted to agarose-gel electrophoresis run in the presence of 2mM-EDTA. Incubation times are given in the Figure. The gels were subjected to radioautography. The arrow indicates the application slots. The positions of free protein S (S) and C4bp–protein S complexes (C4bp–S) are shown to the right. The gel at zero time shows ^{125}I-labelled protein S before the addition to plasma. Key for markers: alb, albumin; α_1, α_1-position; α_2, α_2-position; β_1, β_1-position; β_2, β_2-position.

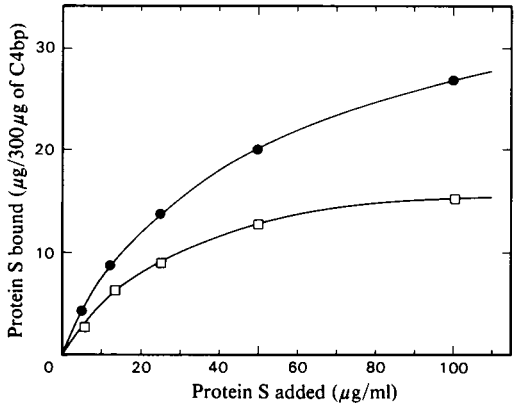

Fig. 4. *Binding of protein S to C4bp as a function of protein S concentration*
Protein S (final concentrations 5–100 μg/ml) with a ^{125}I-labelled tracer were added to parallel incubation mixtures of C4bp(s) (300 μg/ml) (●) or of C4bp(Ba-sup) (300 μg/ml) (□) in 50mM-Tris/HCl buffer, pH 7.5, containing 0.15M-NaCl and 10mg of bovine serum albumin/ml. After 4h incubation at 37°C, samples of the mixtures were subjected to agarose-gel electrophoresis, and the amounts of protein S bound to C4bp were determined as described in the text.

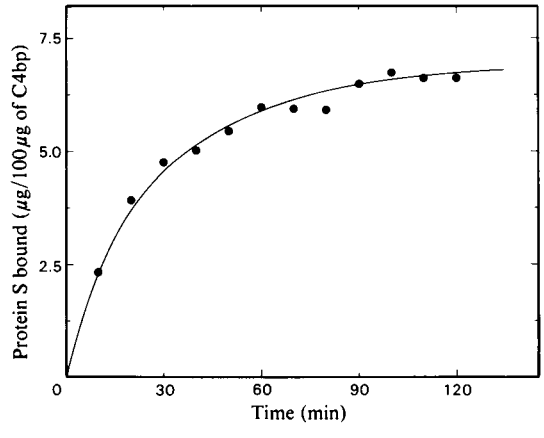

Fig. 5. *Rate of association between protein S and C4bp*
Protein S (25 μg/ml) with a ^{125}I-labelled tracer was added to a sample of C4bp(s) (100 μg/ml) and incubated at 37°C. The same buffer as indicated in Fig. 4 legend was used. Samples were withdrawn at intervals, kept on an ice bath and then analysed by agarose-gel electrophoresis.

using the agarose-gel-electrophoresis separation technique (Fig. 4). Protein S was found to form complexes both with C4bp(s) and with C4bp-(Ba-sup). However, C4bp(Ba-sup) bound a smaller amount of protein S, and the radiolabelled protein S was only recovered in the more anodally migrating of the two C4bp(Ba-sup) bands on agarose-gel electrophoresis, As previously discussed, this band corresponded to the higher-molecular-weight form of C4bp, and presumably only this form was able to form a complex with protein S. This was further investigated, by using a C4bp(Ba-sup) preparation containing only the lower-molecular-weight form, to which preparation no binding of protein S was observed (result not shown). At 37°C, the binding between protein S and C4bp(Ba-sup) or C4bp(s) was at equilibrium within 2h (see Fig. 5). To assure equilibrium even at the lower protein S and C4bp concentrations used, binding was measured after a

4 h period of incubation. The separating agarose-gel electrophoresis was run on apparatus cooled to 4°C. At this temperature the rate of dissociation was very low (see Fig. 6), with a half-life of more than 10 h; thus dissociation during electrophoresis did not significantly influence the results. The binding between protein S and C4bp(s) was measured at several different C4bp(s) concentrations (50–300 μg/ml). In each experiment the C4bp(s) concentration was held constant, and that of protein S was varied (2.5–100 μg/ml). Data from different experiments were plotted in accordance with Scatchard (1949); a typical graph is shown in Fig. 7. From the intercepts on the abscissa approx. 0.9 protein S molecule per C4bp(s) molecule was found to bind (mean of four determinations; range 0.63–1.1), which, within acceptable experimental error, is compatible with the existence of 1 protein S-binding site per C4bp molecule. From the curve slopes a dissociation constant (K_D) of 0.87×10^{-7} M (mean of four experiments; range 0.71–1.14) was calculated. When data from the experiment with C4bp(Ba-sup) (Fig. 4) were plotted in accordance with Scatchard (1949), approx. 0.4 protein S molecule was found to bind per C4bp(Ba-sup) molecule, with a K_D of 1.3×10^{-7} M. As discussed, protein S bound only to the higher-molecular-weight form of C4bp, which represented approx. 40% of the C4bp in the preparation used. After correction of the binding data for this, a stoicheiometric ratio of approx. 1 : 1 between protein S and the higher-molecular-weight form of C4bp was calculated. This supports findings obtained with C4bp(s) and indicates that the small amount of protein S in the C4bp(s) preparation did not significantly affect the results. Important in this respect, too, was the observation that the small amount of protein S in the isolated C4bp(s) preparation was exchangeable. This was investigated by the addition of unlabelled protein S (0.4 mg/ml) to ^{125}I-labelled C4bp(s) (100 μg/ml). After 1 h at 37°C, the sample was analysed by the agarose-gel-electrophoresis technique (results not shown). In the band corresponding to free protein S, approx. 8% of the radioactivity applied was recovered; it was precipitated on crossed immunoelectrophoresis with an antiserum against protein S. In the control without added unlabelled protein S, approx. 2% of the radioactivity migrated as free protein S. Also important was the observation that both a protein S preparation containing only the two-polypeptide-chain form of protein S, and thrombin-modified protein S (see the preceding paper, Dahlbäck, 1983), were able to form complex with C4bp(s) (results not shown).

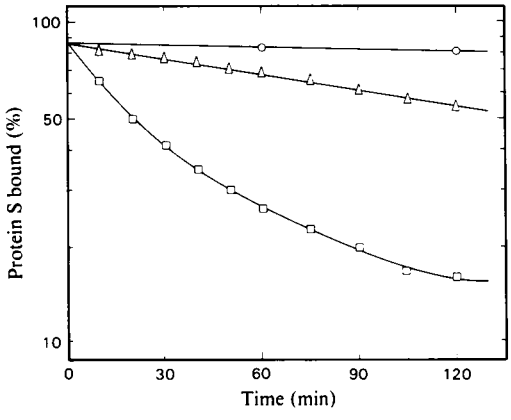

Fig. 6. *Dissociation rate of protein S from an isolated C4bp–protein S complex at different temperatures*
At zero time a molar excess of unlabelled protein S was added to the isolated C4bp–^{125}I-labelled protein S complex. Samples were withdrawn at intervals, kept on an ice bath and then analysed by agarose-gel electrophoresis. Incubation temperatures were 4°C (○), 22°C (△) and 37°C (□).

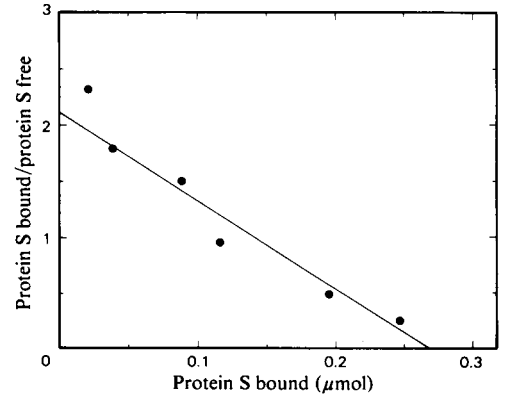

Fig. 7. *Scatchard plot of protein S binding to C4bp*
Increasing concentrations of protein S (2.5–100 μg/ml), with a ^{125}I-labelled protein S tracer, were incubated with C4bp(s) (200 μg/ml) at 37°C for 4 h and then analysed by agarose-gel electrophoresis.

The rates of association and dissociation of the C4bp–protein S complex were investigated (Figs. 5 and 6). The rate of association, at 37°C, was relatively low, and a rate constant of association (k_{+1}) of 7.1×10^3 M$^{-1} \cdot$ s^{-1} was calculated by using the data from the experiment in Fig. 5. Dissociation at 4°C was also slow (Fig. 6). The dissociation rate was about 5 and 40 times higher, at 22°C and 37°C respectively, than that observed at 4°C. The k_{-1} values at 4°C, 22°C and 37°C were 1.2×10^{-5} s^{-1}, 6.1×10^{-5} s^{-1} and 5×10^{-4}–1.8×10^{-4} s^{-1} respectively. The rate of dissociation, at 37°C, was not strictly first-order, as the non-linear semi-logarithmic

plot indicates. The k_{-1} value of 5×10^{-4} s^{-1} was calculated from the slope of the curve between zero time and 20min, whereas a value of $1.8 \times 10^{-4} s^{-1}$ was calculated from the slope between 40 and 90min. The apparent K_D at 37°C, obtained from $K_D = k_{-1}/k_{+1}$, was between 1.6×10^{-7} and 4.5×10^{-7}M, depending on the k_{-1} value used, which agrees relatively well with the K_D value measured from the equilibration experiments.

Discussion

The absorption of C4bp (presumably via protein S) on barium citrate proved a most efficacious step in the purification of the protein. The major advantage was the rapid separation of C4bp from the complement components C1 and C4, thus forestalling formation of C4b–C4bp complex, and from immunoglobulin M, which has been reported as a troublesome contaminant in the purification of C4bp (Scharfstein *et al.*, 1978; Nagasawa & Stroud, 1980; Villiers *et al.*, 1981). C4bp purified from the barium citrate eluate is presumably equivalent to the high-molecular-weight form of C4bp (C4bp-high) isolated by Fujita & Nussenzweig (1979). The low-molecular-weight form of C4bp (C4bp-low) (Fujita & Nussenzweig, 1979) was recovered in the supernatant plasma after barium citrate adsorption, together with some 'C4bp-high'. C4bp purified from barium citrate-adsorbed plasma contained no detectable protein S, whereas C4bp purified from the barium citrate eluent contained a trace of protein S even after gel-filtration chromatography in 1M-NaCl. Both C4bp-high and C4bp-low were able to form complexes with C4b (reported in the following paper, Dahlbäck & Hildebrand, 1983), in agreement with the results obtained by Fujita & Nussenzweig (1979). However, only C4bp-high was able to form a complex with protein S, indicating that protein S binds to a structural determinant absent from C4bp-low, and, furthermore, that protein S and C4b bind to different sites on the C4bp molecule. In agreement with this was the observation that the formation of protein S–C4bp complex remained unaffected by the presence of a molar excess of C4b (B. Dahlbäck, unpublished work).

Formation of C4bp–protein S complex was independent of the presence of the bivalent cations Ca^{2+} or Mg^{2+}, indicating that the vitamin K-dependent part of the protein S molecule was not involved in the C4bp–protein S interaction. In line with this, the thrombin modification of protein S, though affecting the affinity of protein S for Ca^{2+} (see the preceding paper, Dahlbäck, 1983), did not affect its binding to C4bp.

The molecular weight of C4bp-high was determined by ultracentrifugation to be 570000. The material used contained a trace amount of protein S, which did not apparently disturb the determination. The result agrees well with the reported M_r of 590000 estimated from SDS/polyacrylamide-gel electrophoresis in non-reducing conditions (Scharfstein *et al.*, 1978). The apparent M_r of the C4bp-high subunit was found to be approx. 70000, indicating that the molecule is composed of about eight subunits. The structural difference between C4bp-high and C4bp-low is unknown. The observation that protein S only binds to C4bp-high, whereas C4b binds similarly to the two forms (reported in the following paper, Dahlbäck & Hildebrand, 1983), indicates that the difference is not merely one of the number of subunits, as was suggested by Villiers *et al.* (1981). Nor would difference in the number of subunits satisfactorily account for the divergent net charges of the two C4bp forms, reported by Fujita & Nussenzweig (1979), and also observed in the present study.

The Stokes radius of C4bp was found to be approx. 11.5nm. From these data a frictional ratio ($f/f_{min.}$) of 2.1 was calculated. Despite relatively large inherent experimental error, the results indicate that the C4bp molecule is quite asymmetrical. The extremely asymmetrical fibrinogen molecule has been reported to have a frictional ratio ($f/f_{min.}$) of 2.34 (Doolittle, 1973).

In plasma and during purification procedures, free protein S and C4bp-high are in equilibrium with the C4bp–protein S complex in accordance with eqn. (3):

$$A + B \rightleftharpoons AB \qquad (3)$$

where A is free protein S, B is free C4bp-high and AB is C4bp–protein S complex. As reported in the preceding paper (Dahlbäck, 1983), the concentration of free protein S in plasma was found to be approx. 10mg/l (0.12μM), and the concentration of complexed protein S was approx. 15mg/l (0.18μM). On a molar basis this is equal to the concentration of C4bp–protein S complex, assuming a stoicheiometric ratio of 1:1 for protein S and C4bp in the complex. The total C4bp concentration in plasma was estimated to be approx. 200mg/l. However, only C4bp-high (accounting for approx. 80% of plasma C4bp) was able to form a complex with protein S. From this it follows that the total C4bp-high concentration was approx. 160mg/l (0.28μM) and that of free C4bp-high approx. 0.1μM. By using eqn. (4):

$$K_D = \frac{[A][B]}{[AB]} \qquad (4)$$

a K_D of approx. 0.7×10^{-7}M was calculated. In view of the inherent experimental error, this agrees surprisingly well with the K_D of approx. 0.9×10^{-7}M measured for the C4bp–protein S interaction at

equilibium in a system with purified components, and with the K_D of 1.6×10^{-7}–4.5×10^{-7}M calculated from the rate constants of dissociation and association.

Throughout the purification of C4pb(s), the concentration of C4bp-high was more than 10 times the K_D for formation of the C4bp–protein S complex, whereas, after the initial DEAE-Sephacel chromatography, concentrations of protein S in the C4bp(s) pools were low. The high C4bp concentration tended to drive the equilibrium in eqn. (3) to the right. Taken together with the low dissociation rate at 4°C, and the fact that the behaviour of free C4bp-high and C4bp–protein S complexes was essentially identical in both ion-exchange chromatography and gel-filtration chromatography, this explains the observed difficulty in isolating C4bp-high totally devoid of immunochemically detectable protein S. With regard to this, presumably all described purification procedures for human C4bp (Scharfstein *et al.*, 1978; Nagasawa & Stroud, 1980; Villiers *et al.*, 1981) result in preparations containing trace amounts of protein S: amounts detectable immunochemically, but too small to detect on ordinary SDS/polyacrylamide gels unless heavily overloaded (Dahlbäck & Stenflo, 1981). Considering the great difference in dissociation rates of protein S from the complex of different temperatures, a preferable final purification step for C4bp-high would be gel-filtration chromatography at a higher temperature than 4°C, e.g. at 37°C.

The functional significance of the formation of the C4bp–protein S complex is still unknown, It might be speculated that vitamin K-dependent protein S is important for the localization of C4bp–protein S complexes, C4b–C4bp–protein S complexes or immunocomplexes to cellular structures containing negatively charged phospholipid surfaces. The following paper (Dahlbäck & Hildebrand, 1983) reports the effect of formation of the C4bp–protein S complex on the degradation of C4b.

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