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Degradation of human complement component C4b in the presence of the C4b-binding protein–protein S complex

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Vitamin K-dependent protein S and the higher-molecular-weight form of C4b-binding protein (C4bp-high) interact, forming a 1:1 complex with a K_D of approx. 1×10^{-7} M [Dahlbäck (1983) *Biochem. J.* **209**, 847–856]. In the present study the effect of protein S on the degradation of C4b by Factor I (C3b inactivator) and C4bp was investigated both in fluid phase and on cell surfaces, with the use of highly purified components. Fluid-phase degradation of C4b was monitored on sodium dodecyl sulphate/polyacrylamide-slab-gel electrophoresis, and the effect on surface-bound C4b was estimated by haemolytic assay. No effect of protein S could be demonstrated in any of the systems used. Thus, although bound to C4bp, protein S is neither involved in, nor does it affect, the interaction between C4bp and C4b. This indicates that the binding sites on the C4bp molecule for protein S and for C4b are independent and different.

Activation of the first complement component (C1) by antigen–antibody aggregates, or by antibodies bound to surface antigens, is the initiating event in the activation of the classical complement pathway (for a review see Porter & Reid, 1979). The serine proteinase $\overline{C1s}$, a subcomponent of activated C1 ($\overline{C1}$), cleaves a peptide bond near the *N*-terminal end in the α-chain of C4, yielding the C4a peptide (M_r approx. 8000) and C4b (M_r approx. 180000), which is composed of the modified α-chain (α′-chain), the β-chain and the γ-chain (Schreiber & Müller-Eberhard, 1974; Bolotin *et al.*, 1977). The α-chain in native C4 contains an internal thioester bond that is cleaved on activation by $\overline{C1s}$ (Campbell *et al.*, 1981; Harrison *et al.*, 1981). Nascent C4b binds covalently, via a reactive glutamyl group in its α′-chain, to acceptor sites on cell surfaces and on antigen–antibody aggregates (Law *et al.*, 1980; Campbell *et al.*, 1980; Janatova & Tack, 1981). $\overline{C1s}$ also cleaves a peptide bond in the single-chain C2 molecule (M_r approx. 108000), giving rise to an active serine proteinase composed of the two non-covalently linked polypeptides, C2a (M_r approx. 74000) and C2b (M_r approx. 34000) (Nagasawa & Stroud, 1977; Kerr, 1979). The active site is located in the C2a chain. After activation by $\overline{C1s}$, C4 and $\overline{C2}$ form the proteolytically active C3 convertase, $\overline{C42}$ (as reviewed by Reid & Porter, 1981). This occurs on antigen–antibody aggregates, on cell surfaces and in fluid phase. The $\overline{C42}$ is labile, owing to dissociation of C2a from the complex. The biological activity of the C3 convertase is also controlled through proteolysis of C4b by the enzyme Factor I (previously named C3b inactivator) (Cooper, 1975; Shiraishi & Stroud, 1975; Pangburn *et al.*, 1977). Factor I cleaves two peptide bonds in the α′-chain of C4b, giving rise to the C4d fragment (M_r approx. 44500), which is released from the rest of the molecule, and to two end products, α_3 (M_r approx. 25000) and α_4 (M_r approx. 12000), both linked to the β-chain by disulphide bridges (Fujita *et al.*, 1978; Nagasawa *et al.*, 1980; Press & Gagnon, 1981), C4b-binding protein (C4bp) has been shown to be required as a cofactor for the degradation of C4b in fluid phase (Scharfstein *et al.*, 1978; Fujita *et al.*, 1978; Nagasawa *et al.*, 1980), whereas no absolute requirement for C4bp was found for the degradation of surface-bound C4b (Gigli *et al.*, 1979; Iida & Nussenzweig, 1981).

It was reported recently that C4bp in plasma forms a complex with the vitamin K-dependent protein S (Dahlbäck & Stenflo, 1981). In the two preceding papers (Dahlbäck, 1983*a*,*b*) characteristics of the formation of the C4bp–protein S complex, both in plasma and in a system with

The nomenclature of complement components and of cell intermediates is that recommended by the World Health Organisation (1968, 1981). All other abbreviations used are the same as in the two preceding papers (Dahlbäck, 1983*a*,*b*).

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purified components, were described. Protein S and the higher-molecular-weight form of C4bp were found to form a 1:1 complex with a K_D of approx. 0.7×10^{-7}–0.9×10^{-7}M both in plasma and in a system with purified components.

In the present study we investigated whether protein S is involved in the formation of the C4b–C4bp complex and whether protein S affects the degradation of C4b, both in fluid phase and on cell surfaces.

Methods and Materials

Materials

Rabbit anti-(sheep haemolysin) was obtained from Statens Bakteriologiska Laboratorium (SBL), Stockholm, Sweden. All other materials were the same as used in the studies described in the preceding papers (Dahlbäck, 1983*a*,*b*).

Protein purifications

Protein S and C4bp were purified as described in the preceding papers (Dahlbäck, 1983*a*,*b*). Two different types of C4bp preparations were used: C4bp(s) purified from a barium citrate eluate, and C4bp(Ba-sup) purified from the supernatant after barium citrate adsorption. C4bp(s) contained a trace amount of protein S, whereas C4bp(Ba-sup) contained no detectable protein S (see the preceding paper, Dahlbäck, 1983*b*). Protein S (0.05–0.5mg/ml) was modified by thrombin (10–30 NIH units/ml, equivalent to 4–12μg/ml) as described in an accompanying paper (Dahlbäck, 1983*a*). Human protein C was purified and activated by thrombin (Kisiel, 1979). Human C1 was purified from 1 litre of freshly frozen plasma by using the method of Gigli *et al.* (1976). C4bp, the major contaminant in the C1 preparation, was removed by passing the material over a column (2cm × 5cm) with an anti-C4bp serum immunoglobulin fraction coupled to CNBr–Sepharose (Cuatrecasas, 1970). The column was run in 50mM-Tris/HCl buffer, pH 7.5, containing 0.5M-NaCl, after which the C1 preparation contained no major contaminants, as assessed by agarose-gel electrophoresis. The preparation was stored at −70°C. $\overline{C1s}$ was purified from it as described by Gigli *et al.* (1976). Factor I was purified as described by Crossley & Porter (1980). Human C2 was purified from barium citrate-adsorbed plasma by a method involving QAE-Sephadex adsorption, $(NH_4)_2SO_4$ precipitation, CM-Sephadex chromatography and gel filtration on Sephacryl S-200 (B. Dahlbäck, unpublished work). The C2 preparation was contaminated with a small amount of haemopexin. It was stored at −70°C. Human C4 was purified essentially as described by Lundwall *et al.* (1981). The purified protein was dialysed against 25mM-Tris/HCl buffer, pH 7.5, containing 75mM-NaCl and 50% (v/v) glycerol, and was then stored at −20°C. Under these conditions C4 was stable for more than 1 year as judged by a haemolytic assay, by 5–15%-gradient SDS/polyacrylamide-slab-gel electrophoresis and by agarose-gel electrophoresis. C4 was converted into C4b by $\overline{C1s}$ with an enzyme/substrate ratio of 1:50 (w/w). The mixture was incubated at 37°C for 2h, and the cleavage of the α-chain was checked on 5–15%-gradient SDS/polyacrylamide-slab-gel electrophoresis under reducing conditions. Purified protein S, C4bp(s), C4bp(Ba-sup) and C4 were iodinated with Na^{125} by the lactoperoxidase method (Thorell & Johansson, 1971). After the addition of 5mg of bovine serum albumin/ml and 50% (v/v) glycerol, the labelled proteins were stored at −20°C.

The 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); C4, 8.3 (Nagasawa & Stroud, 1977). For the other proteins $A^{1\%}_{1cm}$ at 280nm of 10 was used.

Haemolytic assays

Buffers and reagents used in the haemolytic assays were prepared as described by Rapp & Borsos (1970). The following abbreviations are used: VBS/sucrose/G/M^{2+} medium, I 0.065, 5mM-sodium 5,5-diethylbarbiturate/HCl buffer, pH 7.35, containing 57mM-NaCl, 0.1% gelatin, 1mM-$MgCl_2$, 0.15mM-$CaCl_2$ and 0.17M-sucrose; VBS/G/EDTA medium, 5mM-sodium 5,5-diethylbarbiturate/HCl buffer, pH 7.35, containing 142mM-NaCl, 10mM-EDTA and 0.1% gelatin; C-EDTA medium, fresh guinea-pig serum diluted 1:50 in VBS/G/EDTA medium. EA cells were prepared from sheep erythrocytes by using the immunoglobulin M fraction of a rabbit anti-(sheep haemolysin) as described by Rapp & Borsos (1970). The cells were then converted into EA-C1 cells with highly purified human C1, essentially as described by Rapp & Borsos (1970); EA cells (1×10^9/ml) were incubated with C1 (10μg/ml) at 30°C for 20min in VBS/sucrose/G/M^{2+} medium, I 0.065, and then twice washed with ice-cold VBS/sucrose/G/M^{2+} medium, I 0.065. EA-C14 cells were then prepared from the intermediate EA-C1 cells (Rapp & Borsos, 1970); EA-C1 cells (1×10^9/ml) in VBS/sucrose/G/M^{2+} medium, I 0.065, were incubated with highly purified C4 (20μg/ml) at 30°C for 20min, twice washed with ice-cold VBS/sucrose/G/M^{2+} medium, I 0.065, and then resuspended in the same buffer, stored at 4°C and used within 3 days. Pooled guinea-pig serum diluted 1:50 in iso-osmotic VBS/G/EDTA medium (referred to as C-EDTA medium; see above) was used as a source of C3–C9. The biological activity of C4 and C2 was measured by haemolytic titration (Rapp & Borsos, 1970).

Electrophoretic techniques

Agarose-gel electrophoresis and SDS/polyacrylamide-slab-gel electrophoresis were performed as described in the preceding papers (Dahlbäck, 1983*a*,*b*).

Results

Effects of protein S on the C4bp–C4b interaction

To investigate whether protein S was important for the binding of C4b to C4bp, the two different preparations of C4bp, i.e. C4bp(s) and C4bp(Ba-sup), were mixed with C4b and then analysed by agarose-gel electrophoresis. Both C4bp(Ba-sup) and C4bp(s) were found to form a complex with C4b (results not shown), indicating that the C4bp–protein S interaction was not required for the C4b–C4bp binding. The effect of a higher concentration of protein S on the C4bp–C4b interaction was estimated by using the same technique. As Fig. 1 shows, protein S did not interfere with the C4bp–C4b interaction. A control experiment with a ^{125}I-labelled protein S tracer instead of the ^{125}I-labelled C4bp tracer demonstrated that protein S was part of the C4bp–C4b complex and that it was not displaced by increasing concentrations of C4b (not shown in Fig. 1). These results indicate that protein S and C4b have different binding sites on the C4bp molecule. As shown in Fig. 1, the migration rate of C4bp gradually increased with increasing concentrations of C4b, indicating an increased ratio of C4b to C4bp in the C4b–C4bp complex. At a C4b concentration of approx. 200 mg/l, the complex migrated as isolated C4b. On a molar basis the ratio between C4b and C4bp was 6:1 at this C4b concentration. Similar results were obtained when a thrombin-modified protein S (Dahlbäck, 1983*a*) was used.

Effects of protein S on the degradation of fluid-phase C4b

The following set of experiments were performed to investigate whether protein S had any function in the degradation of C4b in the fluid phase. In the first experiment the cofactor functions of C4bp(s) and C4bp(Ba-sup) were compared (Fig. 2). The C4 concentration was physiological, and a trace amount of ^{125}I-labelled C4 was included to facilitate detection of the degradation products. When intact C4 was applied to the gel, two additional bands (M_r values approx. 54000 and 40000) besides the α-, β- and γ-chains were observed. These bands, which were not seen after conversion of C4 into C4b by $\overline{C1s}$, represent a specific autolytic cleavage of the α-chain induced by denaturation of the C4 molecule by SDS, and indicate that the C4 preparation was native and contained an intact thioester bond in the α-chain (Janatova & Tack, 1981). After the addition of Factor I and C4bp(s) or C4bp(Ba-sup), the α′-chain of C4b was rapidly cleaved, yielding two fragments, one with apparent M_r 70000, the other with M_r 13000. In the absence of C4bp(s) or C4bp(Ba-sup) no cleavage of the α′-chain was detected, even after prolonged incubation of C4b with Factor I (not shown in Fig. 2). The 70000-M_r polypeptide, observed on the polyacrylamide gels, had a slightly faster migration rate than had the β-chain of C4b. On the original gel radioautogram the two bands were distinctly separated, although it has proved difficult to preserve this clarity in photographic reproduction. The 13000-M_r fragment appeared to be an end product, whereas, after prolonged incubation, the 70000-M_r component was cleaved again, giving rise to two polypeptide chains, one with apparent M_r 45000, the other with M_r 25000. The degradation patterns of C4b in the two parallel reaction mixtures in Fig. 2 appeared to be identical, with regard to both the peptide bonds

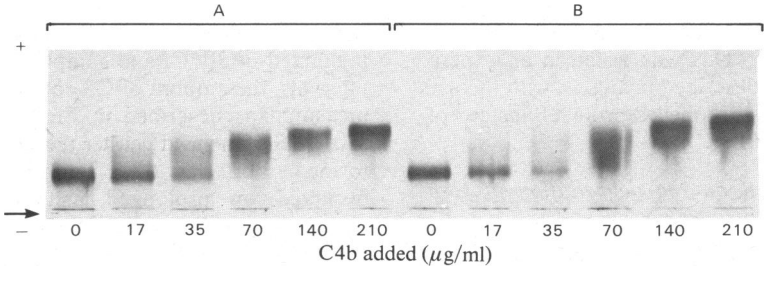

Fig. 1. *Effects of protein S on the binding between C4bp and C4b*

C4bp(s) (200 μg/ml, 0.35 μM), with a ^{125}I-labelled C4bp(s) tracer, and protein S (100 μg/ml, 1.18 μM) were incubated at 37°C for 1 h in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl and 10 mg of bovine serum albumin/ml. Samples of the incubation mixture were then mixed with equal volumes of C4b (final concentrations are given in the Figure) and incubated at 37°C for 2 h before being subjected to agarose-gel electrophoresis. The left half of the Figure (A) represents the control experiment without added protein S. The application slots are indicated by an arrow. The Figure represents the radioautography.

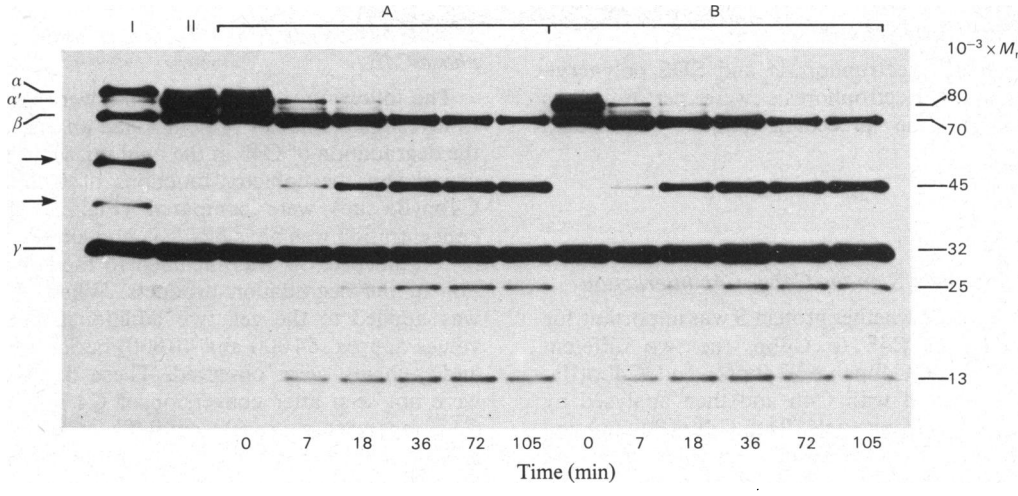

Fig. 2. *Degradation of C4b in the fluid phase: comparison between the effects of C4bp(s) and C4bp(Ba-sup)*
C4 (0.32 mg/ml), with a ^{125}I-labelled C4 tracer, was converted into C4b by incubation with $\overline{C1s}$ (6 μg/ml) at 37°C for 2 h in 50 mM-Tris/HCl buffer, pH 7·5. Tracks I and II respectively are samples taken before and after the incubation. C4b (0.2 mg/ml) was then incubated with Factor I (10 μg/ml) and with either (A) C4bp(Ba-sup) (50 μg/ml) or (B) C4bp(s) (50 μg/ml). Samples were withdrawn at intervals and analysed by 5–15%-gradient SDS/polyacrylamide-slab-gel electrophoresis. The radioautography is shown in the Figure. The three chains of C4 are denoted α, β and γ. The modified α-chain, resulting from proleolysis by $\overline{C1s}$, is marked α'.

cleaved and the rate of the two cleavages, indicating that protein S was not required for the proteolytic breakdown of C4b. The rate of degradation of C4b by Factor I was also followed in two parallel incubation mixtures, one containing C4bp(s) and the other C4bp(s) plus extra protein S (50–100 μg/ml). C4b degradation appeared to be identical, whether protein S was included or not, and the degradation patterns were identical with those shown in Fig. 2. Similar results were obtained when a thrombin-modified protein S preparation was used. The experiments described were performed in the absence of bivalent cations. However, control experiments in the presence of $CaCl_2$ (5 mM) and $MgCl_2$ (5 mM) gave essentially identical results. Furthermore, the protein S molecule was not cleaved during the C4b degradation. This was tested in an experiment similar to that described in Fig. 2, with the use of a trace amount of ^{125}I-labelled protein S instead of the ^{125}I-labelled C4 tracer.

Effects of proteins S on the degradation of C4b bound to erythrocyte membranes

Efforts were also made to investigate whether protein S influenced the degradation of C4b covalently bound to the surface of sheep erythrocytes (EA-C14 cells). The biological activity of membrane-bound C4b was measured by a conventional haemolytic assay technique. The possible effect of protein S on the time of maximal reactivity ($t_{max.}$) of EA-C14 cells was studied both in the presence and in the absence of Factor I (Fig. 3). As evident in the Figure, the activity of surface-bound C4b was inhibited by preincubation with Factor I. The inhibitory effect of Factor I was uninfluenced by the presence of protein S. Furthermore, preincubation of EA-C14 cells with protein S alone did not affect the haemolytic activity. In the experiment described, protein S was washed away before the addition of C2, and a possible effect of protein S on the stability of the cell-bound C3 convertase (EA-C142) was therefore not investigated. Instead, this was done in another experiment (not shown in Fig. 3), in which protein S (final concn. 10 μg/ml) was added to EA-C14 cells (final concn. 0.5×10^8/ml) at the same time as C2. The C2 was diluted to give about 1–2 haemolytic sites per erythrocyte. The cells were incubated at 30°C, and samples were withdrawn at intervals, the amount of C3 convertase formed being measured as described in Fig. 3. The presence of protein S influenced neither the amount of EA-C142 formed, nor the $t_{max.}$, nor the activity decay observed on prolonged incubation. The curves obtained were very similar to that in Fig. 3 representing EA-C14 cells not preincubated with Factor I.

The effect of increasing concentrations of C4bp(s) or C4bp(Ba-sup) on the haemolytic activity of EA-C14 cells in the presence or in the absence of Factor I is shown in Fig. 4. Increasing concentrations of C4bp(s) or C4bp(Ba-sup) resulted in a gradual loss of haemolytic activity. This is in

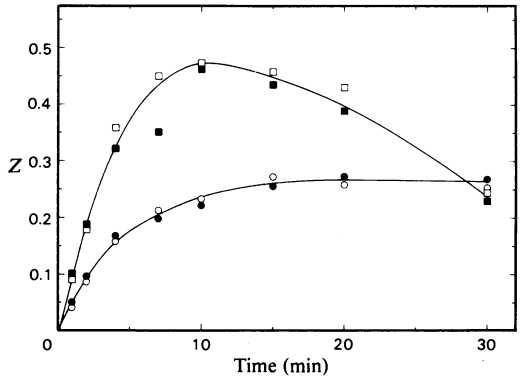

Fig. 3. *Effects of protein S and Factor I on the formation of C3 convertase on EA-C14 cells*
Samples with EA-C14 cells (1×10^8/ml) in VBS/sucrose/G/M^{2+} medium, *I* 0.065, were incubated with protein S (10 μg/ml) and/or Factor I (10 μg/ml) at 37°C for 1 h. The cells were then washed three times with ice-cold VBS/sucrose/G/M^{2+} medium and resuspended to the original concentration in VBS/sucrose/G/M^{2+} medium at 30°C. An equal volume of C2 (approx. 0.1 μg/ml) was added and the cells were incubated at 30°C. Samples were withdrawn at intervals, diluted 1:9 in ice-cold C-EDTA medium and kept on an ice bath. After the last sample point, the samples, incubated at 37°C for 1 h, were centrifuged, and the degree of haemolysis was estimated by measuring absorbance at 412 nm. *Z* represents the number of $\overline{C142}$ sites formed and is the negative natural logarithm of 1 − (% lysis). □, Control; ■, protein S; ○, Factor I; ●, protein S plus Factor I.

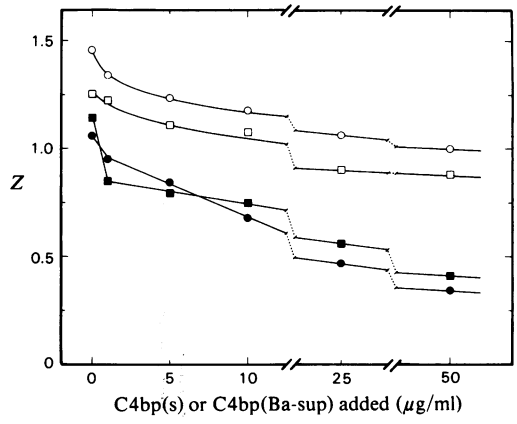

Fig. 4. *Comparison of the effects of the two different C4bp preparations on the formation of C3 convertase*
EA-C14 cells (1×10^8/ml) in VBS/sucrose/G/M^{2+} medium were incubated at 37°C for 1 h with increasing concentrations of C4bp(s) or C4bp(Ba-sup) in the presence or in the absence of Factor I (5.4 μg/ml). The cells were then washed three times and resuspended to the original concentration. The number of $\overline{C142}$ sites (*Z*) was then determined as described in the legend to Fig. 3. ○, C4bp(s); □, C4bp(Ba-sup); ●, C4bp(s) plus Factor I; ■, C4bp(Ba-sup) plus Factor I.

agreement with published results (Gigli *et al.*, 1979), and is presumably due to the binding of C4bp to cell-bound C4b with subsequent inhibition of either C2 activation or the binding of C2 to the surface-bound C4b. The two different C4bp preparations gave essentially identical results. Inhibition of haemolytic activity was greatly enhanced by the two combinations of Factor I and C4bp. No major differences in the effects of the two types of C4bp were observed, indicating that protein S was not required in the system.

The effect of increasing concentrations of protein S in the haemolytic assay system was also investigated (Fig. 5). Even relatively high concentrations of protein S did not affect the haemolytic activity of the EA-C14 cells. Incubation of the cells with Factor I or C4bp resulted in a slight inhibition of haemolytic activity, whereas incubation with Factor I and C4bp combined had a more pronounced inhibitory effect on haemolytic activity. The presence of increasing concentrations of protein S did not influence the results significantly. Similar results were obtained

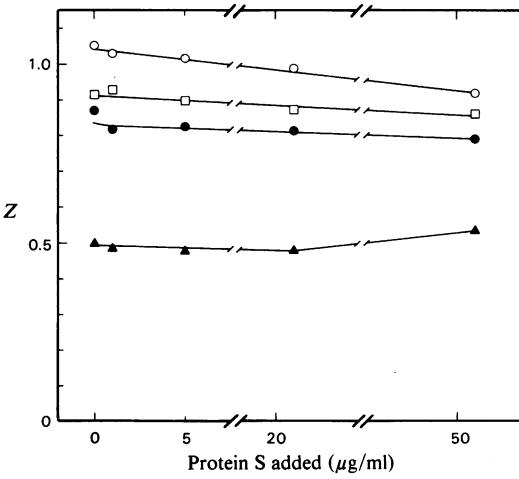

Fig. 5. *Effects of increasing protein S concentrations on haemolytic activity of EA-C14 cells*
EA-C14 cells (1×10^8/ml) in VBS/sucrose/G/M^{2+} medium, *I* 0.065, with Factor I (5.4 μg/ml) and/or with C4bp(s) (50 μg/ml) were incubated with indicated concentrations of protein S at 37°C for 1 h. The number of $\overline{C142}$ sites (*Z*) were then determined as described in the legend to Fig. 3. ○, Control without Factor I and C4bp(s); □, C4bp(s); ●, Factor I; ▲, Factor I plus C4bp(s).

when a thrombin-modified protein S was used (not shown in Fig. 5).

Walker (1980) has reported that protein S enhances the rate of inactivation of the coagulation Factor Va by the vitamin K-dependent serine proteinase, protein C_a. Therefore protein C_a was included in the haemolytic assay system to investigate whether, alone or combined with protein S and C4bp, it affected the haemolytic activity of EA-C14 cells. EA-C14 cells (1×10^8/ml) in VSB/sucrose/G/M^{2+} medium, I 0.065, were incubated for 1 h at 37°C with one of the following combinations: Factor I (5.4 μg/ml) and/or C4bp(s) (100 μg/ml) plus (1) protein S (23 μg/ml), (2) thrombin-modified protein S (23 μg/ml), (3) protein C_a (7 μg/ml), (4) protein C_a (7 μg/ml) and protein S (23 μg/ml), or (5) protein C_a (7 μg/ml) and thrombin-modified protein S (23 μg/ml). Haemolytic activity was measured as described above. Protein C_a, alone or combined with protein S (either before or after modification by thrombin), did not influence the inhibitory effects of Factor I and/or C4bp (results not shown).

Discussion

As discussed in the preceding paper (Dahlbäck, 1983*b*), protein S has presumably been present in trace amounts in the C4bp preparations used in earlier studies of C4b degradation (Fujita *et al.*, 1978; Nagasawa *et al.*, 1980; Press & Gagnon, 1981). Therefore it was important to determine whether trace amounts of protein S are responsible for any of the observed effects and whether, at more physiological concentrations, protein S influences C4b degradation in the fluid phase and on cell surfaces. The availability of C4bp preparations containing no immunochemically detectable protein S made the present experiments possible. In all experiments, protein S was found to have no influence on the degradation of C4b. Though several different preparations of protein S and C4bp were used, results remained consistent.

In the preceding paper (Dahlbäck, 1983*b*) it was demonstrated that protein S forms a complex with the higher-molecular-weight form of C4bp (C4bp-high) only, whereas the lower-molecular-weight form (C4bp-low) is unable to bind protein S. Fujita & Nussenzweig (1979) demonstrated that both C4bp-high and C4bp-low function as cofactors to Factor I for C4b degradation in the fluid phase. The present data bear this out, besides indicating that protein S was not involved in the C4bp–C4b interaction. The experiments on C4b degradation in the fluid phase and on cell surfaces, and the results reported in the two preceding papers (Dahlbäck, 1983*a*,*b*), indicate that protein S and C4b bind independently and to different sites on the C4bp molecule. Here it is of importance that C4b appears to bind equally well to C4bp-high and to C4bp-low, whereas protein S binds to C4bp-high only. Available data indicate that C4bp-high and C4bp-low contain an equal number of essentially idential subunits (about eight), presumably with one C4b-binding site per subunit, and that C4bp-high has an extra, modified or differing, subunit that interacts with protein S. Scharfstein *et al.* (1978), using an electroimmunoassay system, reported that approximately five C4b molecules bind per C4bp molecule; assuming one C4b binding site per subunit, however, their estimate would appear to be too low. Further study is required to clarify this point and to determine the binding characteristics of C4bp–C4b interaction.

In terms of the test system used in the present study, protein S appears to have no distinct function in the degradation of C4b. This, however, does not preclude a function for protein S in the complement system *in vivo*. The vitamin K-dependent protein S, through its affinity for phospholipids, may be important for localization of C4bp; this, for example, would be a means of controlling activation of the classical pathway of the complement system in the vicinity of damaged or lysed cells, exposing negatively charged phospholipids.

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