High Affinity Interaction between C4b-binding Protein and Vitamin K-dependent Protein S in the Presence of Calcium

SUGGESTION OF A THIRD COMPONENT IN BLOOD REGULATING THE INTERACTION*

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The anticoagulant vitamin K-dependent protein S interacts with the complement regulatory protein C4b-binding protein (C4BP), both in purified systems and in plasma. The concentrations of these proteins in plasma are approximately equimolar (0.3 μM) and 30–40% of protein S in plasma is found in the noncomplexed state. Only the uncomplexed form of protein S displays anticoagulant activity and studies have shown that patients with a selective deficiency of free protein S have a high incidence of thrombosis. In this study, we report that the protein S-C4BP interaction is at least 100-fold tighter in the presence of Ca²⁺ than in EDTA. The KD in the presence of Ca²⁺ was estimated with a gel filtration technique to be less than 5 × 10⁻¹⁰ M, whereas in the presence of EDTA, it was approximately 100-fold higher. Ca²⁺ titration experiments suggested that the Ca²⁺ sites which function in the protein S-C4BP interaction are of high affinity which, in turn, suggests that they may be independent of the γ-carboxyglutamyl acid region and may be present in the epidermal growth factor-like domains of protein S. The high affinity of the protein S-C4BP interaction in the presence of Ca²⁺ suggested that virtually all of the protein S in whole blood should be complexed with C4BP. However, even though the protein S-C4BP interaction in Ca²⁺-containing serum was shown to have the same high affinity as in purified systems, approximately 30–40% of the protein S in serum was free. These results appear best explained by the presence of a third component in whole blood which regulates the protein S-C4BP interaction, keeping approximately 30–40% of circulating protein S in its free, functionally anticoagulant form. It is speculated that persons with little free protein S may be deficient in this hypothetical third component.

Protein S is a 75-kDa single-chain vitamin K-dependent protein present in human plasma at an approximate concentration of 25 mg/liter (0.3 μM) (DiScipio et al., 1977; Dahlbäck, 1983a). It functions as a non-enzymatic cofactor to activated protein C in the degradation of the activated forms of coagulation factors Va and VIIIa (Walker, 1980; Gardiner et al., 1984; Walker, 1984; Walker et al., 1987). The functional importance of protein S as an anticoagulant is reflected in the high incidence of thromboembolic episodes in patients with heterozygous protein S deficiency (Comp et al., 1984; Comp and Esmon, 1984; Schwarzs et al., 1984; Comp, 1986; Engesser et al., 1987). Recently, the primary structures of bovine and human protein S were elucidated (Dahlbäck et al., 1986a; Lundwall et al., 1986; Hoskins et al., 1987; Ploos van Amstel et al., 1987). Protein S is composed of discrete domains and contains several functionally important post-translationally modified amino acids (Dahlbäck et al., 1986a; Stenflo et al., 1987).

In human plasma, protein S forms a noncovalent complex with C4b-binding protein (C4BP)¹ (Dahlbäck and Stenflo, 1991; Dahlbäck, 1993b). C4BP, which is an important regulator of the classical complement pathway, is a large protein with seven identical 70-kDa α-chains, each of which contains a binding site for C4b (Scharfstein et al., 1978; Nagasawa et al., 1980; Dahlbäck, 1984; Law and Reid, 1988). The molecule has an unusual octopus-like structure (Dahlbäck et al., 1983; Dahlbäck and Müller-Eberhard, 1984). Recently, we found a unique β-chain in C4BP, and several observations suggest that it contains the single protein S-binding site (Hillarp and Dahlbäck, 1988; Hillarp et al., 1989; Dahlbäck and Dahlbäck, 1999). The plasma concentration of C4BP is around 0.3 μM (Dahlbäck, 1983b; Barnum and Dahlbäck, 1990). Approximately 40–50% of plasma protein S is free, and only this form functions as a cofactor for activated protein (Comp et al., 1984; Dahlbäck, 1986; Bertina et al., 1985). Of particular importance is the observation that many of the individuals with functional protein S deficiency have an abnormal distribution between free and complexed protein S. They have normal or only slightly reduced levels of total protein S but only little or no free protein S (Comp et al., 1986). The biochemical basis for this disequilibrium is unknown.

Complex formation between C4BP and protein S has previously been characterized both in a purified system and in plasma using an agarose-gel electrophoresis technique (Dahlbäck, 1983b). In this method, complexed and free protein S were separated in an agarose gel with a buffer containing EDTA. The KD for the C4BP-protein S interaction in the purified system was determined to be approximately 0.0 × 10⁻⁷ M. An unusual property was the slow rate of association (Dahlbäck, 1983b). By analysis of the equilibrium in plasma, a KD of about 0.7 × 10⁻¹⁰ M was calculated. While most of the early characterizations of the protein S-C4BP interaction were made using buffers devoid of Ca²⁺, it was determined

¹ The abbreviations used are: C4BP, C4b-binding protein; BSA, bovine serum albumin; RIA, radioimmunoassay.

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that the distribution of protein S between its free and complexed forms did not change upon recalcification and coagulation of plasma.

During studies of the binding of protein S-C4BP complexes to phospholipid vesicles, Schwalbe et al. (1990) reported that the affinity of association of the protein S-C4BP complex was much higher in Ca\(^{2+}\)-containing buffers than in EDTA. This suggested that the affinity between protein S and C4BP was much higher in Ca\(^{2+}\) than in EDTA.

The present study was initiated to examine the effect of calcium on the protein S-C4BP interaction using a variety of methods and to determine the basis for the apparent incompatibility of the different reports outlined above. An important goal of this project was to determine if the high affinity also applied to proteins in more crude mixtures. All results suggested a substantial effect of calcium on this interaction with approximately a 100-fold higher affinity. Despite the fact that the protein S-C4BP interaction in serum was also shown to be of high affinity, the ratio of free/total protein S was the same as in EDTA-plasma. These findings are best reconciled by the presence of a third, as yet unidentified, regulatory component in serum which may also be the basis for deficiency in families that display reduced free protein S.

**EXPERIMENTAL PROCEDURES**

**Proteins**—Protein S was purified from human plasma as described (Dahlback, 1983a). Human C4b-binding protein devoid of protein S was purified as previously reported (Dahlback, 1983b; Dahlback and Müller-Eberhard, 1984). Protein S was cleaved with thrombin as described elsewhere (Dahlback, 1983a; Dahlback et al., 1986b).

**Binding of Protein S to Immobilized C4-binding Protein in Ca\(^{2+}\) and EDTA**—Microtiter plates (Dynatech Removastrips) were coated overnight, at 4–8 °C, with C4BP (10 μg/ml in 50 mM carbonate buffer, pH 9.6, 100 μl/well). The wells were washed three times with 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, and then incubated at room temperature for 2 h with 150 μl of bovine serum albumin (BSA), 10 mg/ml, in the same buffer containing either 2 mM Ca\(^{2+}\) or 10 mM EDTA. This was followed by three washes with 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5, containing 0.1% Tween and either 2 mM Ca\(^{2+}\) or 10 mM EDTA. A radiolabeled protein S tracer (40,000 cpm/well) was incubated with unlabeled protein S at various concentrations (0.5 mM to 0.5 μM) for 30 min at room temperature. The buffer was 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5. The mixture was centrifuged at 15,000 rpm in an Eppendorf centrifuge. Two analyses were made on each sample. One was made after the addition of 10 mM (final concentration) EDTA to the serum. These samples were incubated for 2 h at 37 °C before PEG precipitation. The other analysis was made in Ca\(^{2+}\)-containing buffer. Protein S was measured in the starting material and in the supernatants after PEG precipitation using the RIA.

**Other Methods**—Protein S concentrations were measured with a radiimmunoassay (RIA) as previously described (Malm et al., 1988). Protein S was labeled with \(^{125}\)I using Iodobeads (Pierce Chemical Co.) according to the manufacturers instructions and isolated by gel filtration at room temperature on a column (0.9 × 55 cm) packed with Sephacryl S-300. The buffer was 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5, containing 0.5% BSA (or with 0.1% ovalbumin for the Ca\(^{2+}\)-titration experiment), 0.03% NaN\(_3\), and either 2 mM Ca\(^{2+}\) or 10 mM EDTA. The labeled protein S that was isolated in Ca\(^{2+}\) was used for all experiments with Ca\(^{2+}\)-containing buffer whereas that isolated in EDTA was used for all the experiments with EDTA-containing buffers. The Ca\(^{2+}\) concentrations in buffers were measured by atomic absorption spectroscopy at the Clinical Chemistry Laboratory, Malmo General Hospital.

**RESULTS**

The Effect of Calcium on Protein S-C4BP Interaction—Binding of protein S to immobilized C4BP was used as the first of several methods to determine whether the affinity of the C4BP-protein S interaction was higher in the presence of Ca\(^{2+}\) than in EDTA (Fig. 1). When tracer levels of radiolabeled protein S were added to wells containing immobilized C4BP, about 3-fold more protein S bound in buffers containing Ca\(^{2+}\) than in buffers containing EDTA. This experiment, which was performed multiple times with similar results, provided qualitative evidence that Ca\(^{2+}\) enhanced the C4BP-protein S interaction. Higher affinity binding of protein S to C4BP in the presence of calcium was also evident when the data were plotted from 0 to 100% of maximum binding (Fig. 1, inset). Although suggesting that calcium enhanced the protein S-C4BP interaction, several properties indicated that this technique did not allow determination of equilibrium binding constants. For example, binding seemed to approach a maximum well below 100% of added radiolabel, and this is not expected for an equilibrium process.

Ca\(^{2+}\) titrations indicated that relatively low levels of calcium were needed for enhanced binding (Fig. 2). To minimize Ca\(^{2+}\) contamination, all buffers were treated with Chelex 100 and ovalbumin was used to quench the microtiter wells instead of BSA. Intact and thrombin-cleaved protein S were used to determine whether both forms demonstrated the Ca\(^{2+}\)-de-
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Fig. 1. Calcium stimulates the binding of protein S to C4b-binding protein. Radiolabeled protein S was mixed with increasing concentrations of unlabeled protein S either in the presence of Ca^{2+} (●) or EDTA (●), and the mixtures were then added to microtiter wells with immobilized C4BP. After overnight incubation in the cold room, the amount of protein S bound to the wells was determined. The binding data are shown both as % of added protein bound and as % of maximum binding (inset). These demonstrate both the difference in maximum binding of the tracer in the presence of Ca^{2+} and the lower binding affinity in EDTA.

Fig. 2. Calcium concentration-dependence of protein S binding to C4b-binding protein. A, C4BP was immobilized in microtiter wells under calcium-free conditions. Radiolabeled native (●) or thrombin-cleaved (▲) protein S, that had been dialyzed against Chelex 100-treated buffer to remove traces of Ca^{2+}, were added to the wells in buffer containing the indicated concentration of EDTA or Ca^{2+}. After overnight incubation at 37 °C, the wells were washed and the amount of bound protein S measured in a γ-counter. B, similar experiments as in A with the exception that all buffers contained 100 μM EDTA.

Dependence. Once again, binding in EDTA-containing buffer was lower than in Ca^{2+}-containing buffers. In fact, the effect was nearly maximal without added calcium. This suggested that low levels of contaminating Ca^{2+} were adequate or that another contaminating ion was removed by the EDTA. The experiment in part B of Fig. 2 suggested that Ca^{2+} was the required ion. Added Ca^{2+} equal to or greater than the 100 μM EDTA were adequate to enhance protein S-C4BP interaction.

Estimation of the Magnitude of the Calcium Effect—The slow rates of dissociation of the protein S-C4BP complex in both EDTA and Ca^{2+} (Dahlbäck, 1983b; Schwalbe et al., 1990) are on the time frame of gel filtration chromatography so that the extent of complexed and free protein S may be estimated by this approach. We have also previously used this technique to demonstrate the presence of C4BP-protein S complex in human plasma (Dahlbäck and Stenflo, 1981). Equimolar mixtures of protein S and C4BP, at the indicated concentrations, in either EDTA or Ca^{2+}, were subjected to gel filtration chromatography (Fig. 3). Control experiments, in which protein S or C4BP were gel-filtered alone (results not shown) in the presence of EDTA or calcium, gave elution profiles similar to those previously reported (Dahlbäck and Stenflo, 1981). In EDTA and at 10^{-7} M C4BP and protein S, approximately 50% of the protein S eluted as a high molecular weight complex with C4BP (Fig. 3F). As expected from an equilibrium process, at 10^{-6} M the majority of the protein S was recovered in the complexed form (Fig. 3D), whereas at 10^{-5} M the majority of protein S was uncomplexed (Fig. 3F). If dissociation of the protein S-C4BP complex during chromatography was negligible, these results would be consistent with a KD of approximately 5 × 10^{-8} M, a value close to that previously reported (Dahlbäck, 1983b). If significant dissociation of the complex took place during chromatography, the amount of free protein S would be overestimated, and the binding constants would be correspondingly lower. The important comparison was that in the presence of Ca^{2+}, the situation was quite different. At 10^{-7} M of protein S and C4BP, almost all protein S was recovered in the complexed form (Fig. 3A). Even at protein concentrations of 10^{-6} M, most of protein S was complexed (Fig. 3B). Approximately 50% of protein S eluted as free protein at 10^{-5} M protein S and C4BP (Fig. 3C). If dissociation during chromatography was negligible, this result would be consistent with a Kd for the protein S-C4BP interaction in the presence of Ca^{2+} of approximately 5 × 10^{-10} M. Again, dissociation of the complex during chromatography would result in overestimation of the concentration of free protein S and the dissociation constant would be lower. In any event, any value less than 5 × 10^{-10} M is far below the concentration of protein S in the serum and provides the same conclusions presented below. Overall, the protein S-C4BP binding affinity is about 100-fold higher in Ca^{2+} than in EDTA.

The Amount of Complexed Protein S in Serum Is Essentially Independent of Calcium—The experiments using purified proteins suggested a very high affinity for the interaction between protein S and C4BP in the presence of Ca^{2+}. It has previously been determined that 40-50% of protein S in plasma is free, and the remainder is bound to C4BP (Dahlbäck and Stenflo, 1981; Dahlbäck, 1983b). This was found to be the case for both citrated and EDTA plasma. Both of these anticoagulants
provide essentially Ca\(^{2+}\)-free milieu for the protein S-C4BP interaction. This raised the question whether or not these earlier results, obtained in plasma, reflected the in vivo situation where calcium levels are high. Based on the high affinity for the protein S-C4BP interaction obtained in the purified systems and assuming a simple binary interaction, virtually all of the protein S in whole blood should be complexed.

To approach the in vivo situation, we determined the distribution of protein S in serum, which contains physiological concentrations of Ca\(^{2+}\). To avoid technical problems, two experimental approaches were used to separate free and complexed protein S. One technique used PEG-precipitation (Malm et al., 1988) to separate the complexed protein S. The total to free ratio of protein S was then determined with the RIA for protein S (Table I). Alternatively, complexed and free protein S in serum were separated by gel-filtration chromatography (Fig. 4). Both techniques gave similar results. The distribution of protein S between free and complexed form was essentially the same in serum as in plasma (about 50% free protein S), and the addition of EDTA to the serum did not change the distribution. Thus, the results obtained in crude mixtures did not correlate with the expectations derived from studies of the purified systems.

There are several possible explanations for the lack of correlation in pure and crude systems. Two less intriguing explanations are that the stimulatory effect of Ca\(^{2+}\) on the protein S-C4BP interaction is only seen with purified components because they have been somehow modified during purification. Alternatively, protein S and/or C4BP may become modified during coagulation and they are therefore different in serum versus plasma or the purified state. Both these explanations are unlikely, as demonstrated below. A third alternative, with important biological and medical implications, is that the protein S-C4BP interaction in serum is of high affinity but that the distribution of protein S between the free and complexed states is actually regulated by another component that is present in serum.

**High Affinity Protein S-C4BP Interaction in Ca\(^{2+}\)-containing Serum**—To determine whether the protein S-C4BP interaction in serum was of high affinity and that calcium exerted the same effect as demonstrated with purified proteins, the distribution of protein S in 100-fold diluted serum was determined. In the diluted sample, the protein S and C4BP concentrations should be approximately 2-3 nM each. When used in the purified system (Fig. 3) these concentrations gave approximately 50% complexed protein S in the presence of Ca\(^{2+}\) and little complexed protein in the presence of EDTA. The diluted serum was incubated for 2 h at 37 °C to allow the protein S-C4BP interaction to reach equilibrium, and the samples were then subjected to gel filtration chromatography and the fractions analyzed with a RIA for protein S (Fig. 5). Undiluted serum, gel filtered in the presence of Ca\(^{2+}\) as control (Fig. 5A), showed that approximately 50% of the protein S was complexed. This was consistent with the results shown in Fig. 4.

The serum sample that was diluted in buffer containing Ca\(^{2+}\) gave essentially identical elution profiles whether it was directly applied to the column after dilution (not shown) or after 2 h of incubation at 37 °C (Fig. 5D). The elution profile of the sample diluted with EDTA-containing buffer showed considerable difference. When applied to the column immediately after dilution with EDTA-containing buffer, approxi-

**TABLE I**

| Protein S total (%) | Protein S free (%) |
|---------------------|-------------------|
| Serum Ca (n = 4)    | 111 (100-119)     | 42 (35-57)     |
| Serum EDTA (n = 4)  | 123 (110-133)     | 32 (30-36)     |
| Plasma EDTA (n = 4) | 107 (100-117)     | 30 (27-34)     |

**Fig. 4.** Ratio of free to bound protein S in plasma and serum estimated by gel filtration chromatography. Plasma (0.5 ml) or serum (0.5 ml ± added EDTA, 10 mM final concentration) were chromatographed on a column packed with Sephacryl S-300 as described in the legend to Fig. 3. The buffer either contained 10 mM EDTA (A and B) or 2 mM Ca\(^{2+}\) (C). The protein S concentration in the fractions was measured with a RIA.

**Fig. 5.** Gel filtration of serum diluted in either EDTA or Ca\(^{2+}\). Serum was diluted 100-fold in 10 mM Tris-HCl, 0.15 M NaCl, pH 7.3, 0.5% BSA, 0.05% NaN\(_3\) containing either 2 mM Ca\(^{2+}\) (B) or 10 mM EDTA (C). After 2 h at 37 °C, a 0.5-ml aliquot of each sample was subjected to gel filtration on Sephacryl S-300 as described in the legend to Fig. 3. Undiluted serum (A) was run with buffer containing 2 mM Ca\(^{2+}\) as a control. The protein S concentration in the fractions was determined with a RIA.
The initial study designed to determine whether protein S could subsequently bind C4BP to the membrane revealed that labeled protein S can only bind to C4BP which becomes available due to dissociation from unlabeled protein S molecules. This suggested a KD in plasma of approximately 0.7 $\mu M$ which agreed well with the clinical observation that patients with protein S deficiency have a high incidence of thrombosis (Comp et al., 1984; Comp and Eason, 1984; Schwarz et al., 1984; Comp, 1986; Engesser et al., 1987). Of particular importance in this regard is that several of the patients with functional protein S deficiency show a selective deficiency of free protein S, whereas their total protein S levels are approximately normal (Comp et al., 1986). Therefore, a critical observation in this study was that, in serum, even though the protein S-C4BP interaction was found to be extremely tight, only 60% of protein S is recovered in the complexed form.

These combined results are inconsistent with a simple binary interaction between protein S and C4BP in the blood. An intriguing possibility is that another, as yet unidentified, component is present in serum and that this is responsible for maintaining a substantial amount of free protein S. A deficiency of this hypothetical third component would be associated with low levels of free protein S in circulating blood since the protein S-C4BP interaction would be reduced to a simple binary interaction of very high affinity. The presence of a third component and related genetic deficiencies would provide a simple model for development and inheritance of a deficiency in free protein S. That is, rather than genetic variations in protein S or C4BP that must create higher affinity interactions between these proteins, the similar manifestations of free protein S deficiency arising in different families could all be the result of a simple deficiency of another component, possibly a protein. Whether this is the biochemical explanation for the selective deficiency of free protein S observed in patients remains to be determined and must await isolation of the hypothetical component(s). However, this study has provided a rationale and basis for detecting such a component.

Walker (1986) reported that bovine plasma contains a protein S-binding protein which is unrelated to C4BP. This protein has not been found in human plasma, and it is not possible to judge from available data whether the protein reported by Walker is related to any process observed in this study. In this context, it is important to note that bovine plasma is also different from its human counterpart in that no C4BP-protein S complex can be identified in bovine plasma (Dahlbäck, 1986b). We have tested bovine serum without being able to find a complexed form of protein S. This does not preclude the presence of a C4BP-protein S complex in the cow, but it indicates that comparisons between the two species must be made with care.

The Ca$^{2+}$ titration experiment (Fig. 2) suggested that the Ca$^{2+}$-binding site(s) which are important for the protein S-C4BP interaction were of high affinity. From the data presented, it was not possible to determine whether the Ca$^{2+}$-binding sites were located in protein S or on C4BP. However,

2 B. Dahlbäck, B. Frohm, and G. Nelsestuen, unpublished results.
previous studies have shown calcium-binding sites that are considerably tighter than the γ-carboxyl glutamic acid residue-dependent sites (Sugo et al., 1986). This affinity would be consistent with the calcium concentrations needed to elicit the high affinity protein S-C4BP interaction observed in this study. Furthermore, recent evidence suggests the presence of four extremely high affinity Ca\(^{2+}\)-binding sites in the epidermal growth factor-like domains of protein S (Dahlbäck et al., 1990). These sites require extensive dialysis against EDTA to remove residual calcium. It is possible that the resulting calcium-free protein S will not interact with C4BP at all. Of course, it is not yet known whether the epidermal growth factor-like domains are directly involved in the protein S-C4BP binding. Walker (1989) has recently suggested that the carboxyl-terminal region of protein S (G\(^{695-1614}\)) was involved in the interaction between protein S and C4BP. Whether the high affinity calcium-binding sites in protein S might affect the conformation in this region of protein S remains to be determined.

Overall, this study has suggested that calcium participates in many ways in supporting the various functions of the vitamin K-dependent plasma proteins. Future studies will be needed to answer many of the questions raised by the present investigation. These include the exciting possibility of a genetic deficiency related to an unidentified component of the protein S-C4BP interaction, identification of this hypothetical component, the role of the high affinity protein S-C4BP interaction in coagulation and/or complement, and the identity of the calcium-binding sites that function in the protein S-C4BP interaction.

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