The effect of calcium on the interaction of protein C with thrombin and the elastase fragment of thrombomodulin: Analysis by analytical ultracentrifugation

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Abstract
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Keywords
Chemistry, Biochemistry, Biophysics, General

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The effect of Ca$^{2+}$ on the interaction of protein C with thrombin and the elastase fragment of thrombomodulin: Analysis by analytical ultracentrifugation

Olsen, Philip Henry, Ph.D.

University of New Hampshire, 1991
THE EFFECT OF Ca²⁺ ON THE INTERACTION OF PROTEIN C WITH THROMBIN AND THE ELASTASE FRAGMENT OF THROMBOMODULIN. ANALYSIS BY ANALYTICAL ULTRACENTRIFUGATION.

BY

PHILIP H. OLSEN
B.A., University of New Hampshire, 1984

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PROTEASE INHIBITORS

DEGR  dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone
DFP   Diisopropylfluorophosphate
PPACK D-phenyl-L-prolyl-L-arginine chloromethyl ketone

PROTEIN C

BPC  Bovine protein C
BAPC Bovine activated protein C
BAPC, Bovine activated protein C inhibited with DEGR
HPC  Human protein C
HAPC Human activated protein C
gdBPC Gla-domainless bovine protein C
gdHPC Gla-domainless human protein C
Gla  The amino terminal 42 amino acids of protein C

THROMBIN

BT   Bovine thrombin
BTP  Bovine thrombin inhibited with PPACK
BTD  Bovine thrombin inhibited with DFP

THROMBOMODULIN

elTM elastase fragment of rabbit thrombomodulin
TM   Rabbit thrombomodulin
ABSTRACT

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Thrombin and elTM form a strong 1:1 complex that is relatively unperturbed by the addition of Ca\(^{2+}\). Thrombin and protein C form two complexes of 1:1 and 1:2 thrombin:protein C stoichiometry as suggested by the association model that fits the data best. The interactions between thrombin and protein C are strongest in 0 mM Ca\(^{2+}\), but become much weaker with the addition of Ca\(^{2+}\). Thrombin, protein C and elTM form a single 1:1:1 complex that behaves similarly to the thrombin/protein C mixture upon the addition of Ca\(^{2+}\). Studies with the 42 amino acid Gla (\(\gamma\)-carboxyglutamic acid) domain peptide and Gla-domainless protein C indicate that, at low Ca\(^{2+}\) concentrations, the interaction of protein C with thrombin and elTM may be mediated primarily through this Gla domain. The interaction of activated protein C with thrombin alone, and thrombin with elTM is also examined. Surprisingly, there is little difference in the interaction of activated protein C as compared to protein C. This is true regardless of whether the thrombin inhibitor is CK which may block some of the substrate recognition site, or DFP, which blocks only the catalytic site. As suggested by these results, activated protein C is a good inhibitor of protein C activation.
INTRODUCTION

Blood coagulation

The process of blood coagulation, a series of complex proteolytic reactions which are initiated explosively upon vascular damage in vivo, leads to an insoluble fibrin/platelet clot. A reading of the articles reviewing blood coagulation in the last fifteen years clearly demonstrates that the process of coagulation is far from simply a linear cascade, but is instead a process involving the formation of discrete enzyme complexes and multiple feedback pathways (Davie and Fujikawa, 1975; Nemerson and Furie, 1980; Jackson and Nemerson, 1980; Mann et al., 1988; Furie and Furie, 1988). These references are excellent and comprehensive reviews of blood coagulation. Nonetheless, let's consider briefly the triggering of the cascade, the components involved and the regulation of the coagulation process.

Traditionally, the blood coagulation pathway has been divided into the intrinsic and extrinsic pathways (see Fig. 1) which converge at the activation of factor X (Davie and Ratnoff, 1964; MacFarlane, 1964). The intrinsic pathway is initiated when plasma comes in contact with a negatively charged, nonmembraneous surface, whereas the extrinsic pathway is initiated when the endothelial cells lining the blood vessels are injured, resulting in the secretion of thromboplastin, also known as tissue factor. For several
Figure 1. The Blood Coagulation Cascade. See text for complete discussion. The dashed lines show points of interconnection between the pathways. PS = protein S, a protein cofactor involved in the inactivation of factors Va and VIIIa.
Figure 1.

INTRINSIC PATHWAY

XII → XIIa

XI → XIA

EXTRINSIC PATHWAY

THROMBOPLASTIN

IX → IXa

VIII → VIIIa

PROTHROMBIN

THROMBIN

FIBRINOGEN → FIBRIN

□ = COFACTORs

TH = THROMBOPOIEtIN

APC = ACTIVATED PROT. C

+,− = POS. OR NEG. FEEDBACK

XIII → XIIIa

CROSsLINKED FIBRIN
reasons it is currently thought that the division of blood coagulation into two pathways is artificial and not reflective of what is relevant in vivo: 1) there are a number of interconnections between the pathways (dashed lines in Fig. 1), and 2) people individually deficient in factor XII, prekallikrein (not shown in Fig. 1) or HMWK exhibit no bleeding disorders (Furie and Furie, 1988). Thus, it appears that the coagulation cascade may be a single pathway in vivo.

The components in the coagulation pathway include plasma proteins (most of the proteins shown in Fig. 1), several integral membrane proteins, and platelets. Though it is not shown in Fig. 1, most of the reactions also require phospholipid membranes and calcium ions. For example, optimal prothrombin to thrombin conversion requires phospholipid membranes, and when they are included in an in vitro reaction mixture consisting of factors Xa and Va and calcium, the rate of conversion increases nearly 1,000 times over that of the mixture without phospholipid membranes (Mann et al., 1986). This observation suggests a role for protein/phospholipid interactions in addition to the protein/protein interactions that are known to exist. Furthermore, these interactions are mediated through specific regions of the coagulation proteins.

The blood coagulation proteins have very different functional properties, but share a number of structural domains (Furie and Furie, 1988). Indeed, all of the enzymes of the pathway are serine proteases. A common feature of many of
the plasma proteins is an amino-terminal domain of approximately 45 amino acid residues that contains about 10 γ-carboxyglutamic acid (Gla) residues. These are glutamic acid residues which have been modified by a carboxylase, as yet unpurified, located in the liver, where most plasma proteins involved in coagulation are synthesized (Stenflo and Suttie, 1977; Furie and Furie, 1990; Vermeer, 1990). There is evidence that the Gla domain can bind negatively charged phospholipid membranes, presumably via a calcium ion bridge (Mann et al., 1982). However, a role in protein/protein interaction via the Gla domain should not be excluded (Sakai et al., 1990).

Another feature commonly found in many of the blood coagulation proteins is the epidermal growth factor-like (EGF) domain. There is often more than one EGF-like domain in series in the sequence (see, for example, Fig. 2). The EGF-like domain was so-named because of its primary sequence similarity to a 53 amino acid protein, the epidermal growth factor, first identified by Carpenter and Cohen (1979). This domain appears to be involved primarily in protein/protein interactions. This has been demonstrated, for example, by the binding of urokinase to its receptor (Appella et al., 1987), and by a study of the functionally important regions of protein S (Dahlbäck et al., 1990).

The enzyme components in the cascade are all synthesized as zymogens, so that the process of initiating coagulation is essentially one of converting these zymogens to active
enzymes. However, once this conversion process has been initiated, how is it terminated? In the absence of a rapid and effective inhibitory system, it is possible to envision a completely clotted plasma pool. The central reaction in blood coagulation appears to be the conversion of prothrombin to thrombin (see Fig. 1). Among a number of its procoagulant functions, thrombin catalyzes the conversion of soluble fibrinogen to insoluble fibrin and amplifies its own conversion reaction. It is inhibited by the plasma molecules heparin and antithrombin III (Rosenberg and Rosenberg, 1984). Recently, it has been shown that thrombin also initiates the protein C anticoagulation system (Clouse and Comp, 1986). This system shuts off the thrombin conversion reaction. Thus, as soon as the coagulation system is turned on, an anticoagulation system is initiated, resulting in the tight regulation of the clotting process.

The protein C anticoagulation system

The protein C anticoagulation system consists of three proteins: protein C, thrombin, and thrombomodulin (Esmon, 1987; Esmon, 1989). The protein C anticoagulation system was postulated to be operative in vivo in 1980 when thrombin was found to activate the plasma zymogen protein C at a physiologically relevant rate when the proteins were injected into a Langendorff heart preparation (Esmon and Owen, 1981). This experiment implied the existence of an endothelial cell
surface protein which was then subsequently isolated, purified, and named thrombomodulin (TM) (Esmon et al., 1982a).

Protein C is a serine protease zymogen synthesized as a single chain in the liver (Fig. 2), and has been isolated from bovine and human blood (Stenflo, 1976; Kisiel, 1979). Its molecular weight is on the order of 60,000 g/mol. The primary sequence of bovine protein C was determined by amino acid sequencing (Fernlund and Stenflo, 1982; Stenflo and Fernlund, 1982), and that of human protein C from the cDNA sequence (Foster and Davie, 1984). The mature form of protein C is a disulfide linked heterodimer of a light and a heavy chain. The light chain contains a Gla domain and 2 EGF-like domains. The heavy chain contains the catalytic site. Activation of bovine protein C is accomplished by the thrombin catalyzed removal of a 14 amino acid activation peptide from the N-terminal of the heavy chain (Kisiel et al., 1976). Circular dichroic, fluorescence and immunologic studies of protein C have shown that large conformational changes take place when the protein binds calcium (Johnson et al., 1983; Church et al., 1989). Calcium binds to several sites in the Gla domain with an affinity of about 200 μM, as well as to a single site in the EGF domain that has a Kₐ of about 50 μM. This latter site appears to contain an aspartic acid residue that has been posttranslationally modified to β-HO-aspartic acid (labelled Hya in figure 2) (Öhlin et al., 1988). Calcium is required for the activation of protein C by the thrombin-TM complex at
Figure 2. A model of the bovine protein C molecule. Hya labels the 2-hydroxyaspartic acid residue and the arrow indicates where thrombin cleaves the heavy chain to activate protein C. This figure was adapted from that found in the Ph.D. thesis by Öhlin (1989).
physiological rates (Esmon et al., 1983a), even though no calcium appears to bind the heavy chain where activation occurs. This phenomenon is probably explained by an allosteric interaction between the light chain and the heavy chain of protein C (Stearns et al., 1988; Orthner et al., 1989).

The action of protein C, once activated, is to inactivate factors VIIIa and Va by proteolytic degradation. These are cofactors in the conversion of factor X to Xa and prothrombin to thrombin, respectively (Walker et al., 1979; Marlar et al., 1982). The inactivation of factors Va and VIIIa involves protein S as a cofactor and is thought to occur on a membrane surface. Activated protein C may also be involved in fibrinolysis by neutralizing an inhibitor of plasminogen activator, thus increasing the level of active plasminogen, a fibrinolytic agent (Comp and Esmon, 1981; van Hinsbergh et al., 1985).

Thrombin has been introduced in the previous section on blood coagulation. This protein is the active form of prothrombin which is synthesized in the liver. Thrombin has a molecular weight of 37,500 g/mol and can be approximated as a sphere with a diameter of 4.5 nm. There are two basic regions ('lysine patches') thought to be involved in substrate binding, and situated on opposite sides of the active site cleft (Bode et al., 1989). Thrombin does not appear to bind calcium and undergoes no conformational changes in its presence. However, when bound to TM, the active site appears
to undergo conformational changes (Musci et al., 1988). These conformational changes may account for the great improvement in thrombin's ability to activate protein C when bound to TM, as well as the other concomitant changes in its specificity—such as the decreased ability to activate factor V and platelets (Esmon et al., 1982b; Esmon et al., 1983b). Thrombomodulin also appears to compete with fibrinogen for the same binding site on thrombin, preventing the production of fibrin molecules (Hofsteenge and Stone, 1987; Jakubowski and Owen, 1989). Thus, there are two effects which result from the binding of thrombin to TM: 1) the increase in the activation rate of protein C, and 2) the elimination of the procoagulant functions of thrombin.

Thrombomodulin is an integral membrane protein with molecular weight on the order of 70,000 g/mol (Fig. 3). First identified in rabbit heart, it has been isolated from human, bovine, mouse, rat, and rabbit (Dittman and Majerus, 1990). It is a stable protein that can be subjected to boiling in SDS and when repurified, still remains active. However, when subjected to reducing agents its function is destroyed (Esmon et al., 1982). Intact TM can be incorporated into phospholipid vesicles or used in a 0.02% Lubrol buffer system (Esmon et al., 1983; Galvin et al., 1987). However, to further examine structure and function relationships, TM was subjected to limited proteolysis by a number of proteases (Kurosawa et al., 1987). TM was cleaved into two major fragments when digested
Figure 3. Schematic model of thrombomodulin. Filled circles located within the EGF-like domain represent points of putative N-linked glycosylation.
with either trypsin or elastase. In the case of trypsin, both fragments bound phospholipid vesicles, and the larger fragment (molecular weight 54,000 g/mol) was active as a cofactor. However, only the smaller of the two fragments from the elastase digest bound phospholipid vesicles, whereas the larger fragment (molecular weight of 50,000 g/mol as determined by polyacrylamide gel electrophoresis) was soluble in non-detergent containing buffer and functioned as a cofactor. This latter fragment has been used extensively in protein C activation studies. It is now commonly referred to as the elastase fragment of thrombomodulin, or elTM, and is shown in Figure 3.

ELTM contains six EGF-like domains which have been shown to contain the primary binding site for thrombin, as well as the site required for functional activity as a cofactor (Suzuki et al., 1989). Specifically, the fifth and sixth EGF domains (from the amino terminus) compete with protein C for thrombin binding, but this fragment contains no functional cofactor activity (Kurosawa et al., 1988). The smallest fragment which has functional activity is that which contains the fourth, fifth, and sixth EGF domains (Zushi et al., 1989; Stearns et al., 1989).

Recently the function of carbohydrate moieties found in many of the blood coagulation proteins has received attention. Protein C, thrombin and thrombomodulin all contain complex N- and O-linked carbohydrate structures. For example, protein C
contains nearly 25% carbohydrate by weight depending on species (Kisiel, 1979). Furthermore, TM contains two regions of N- and O-linked carbohydrate structures (see Fig. 3). The O-linked structures appear to be an additional, but secondary site of importance for the binding of thrombin (Preissner et al., 1990). The N-linked sites do not appear to be necessary for thrombin binding (Stearns, et al., 1989).

**Kinetics of protein C activation and the objectives of this research**

The enzyme kinetics of protein C activation is particularly complex with respect to its dependence on the calcium ion concentration. In this section, discussion is devoted to a model of protein C activation by the thrombin/thrombomodulin complex that is almost entirely based on kinetic evidence. Finally, a summary of research objectives is presented.

The current model describing protein C activation by the thrombin/TM complex is shown in Figure 4 (taken from Esmon, 1987). It is based primarily on kinetic studies of the activation of protein C by thrombin alone and the thrombin/TM or thrombin/elTM complex. While thrombin alone can recognize protein C, the conformational change in protein C brought about by calcium ions prevents this recognition. However, thrombin and thrombomodulin recognize each other regardless of the availability of calcium ions or a membrane surface. The
Figure 4. This model of protein C activation by the thrombomodulin/thrombin complex is more fully described in the text. Thrombin can bind protein C without being bound to thrombomodulin, as long as no calcium ions are bound to protein C. The binding of thrombin to thrombomodulin alters thrombin’s active site, allowing thrombin to bind protein C only in the presence of calcium ions. Protein C has shared binding sites on thrombin, thrombomodulin and the plasma membrane. Some of this binding may be mediated through protein C’s Gla domain (the rounded protruding region of protein C in the figure). BT = bovine thrombin. (From Esmon, 1987).
thrombin/TM interaction results in a conformational change in the thrombin active site, enabling it to recognize protein C in the presence of calcium ions (Esmon et al., 1983). The Gla domain of protein C appears to be necessary for its optimal activation. Membrane-incorporated TM/T complex has a ten fold smaller $K_m$ (around 0.7 $\mu$M) over that of the detergent solubilized TM/T complex for protein C. The $K_m$ of the membrane-incorporated TM/T complex for protein C is identical to the $K_m$ determined using cultured endothelial cells (which express thrombomodulin on their surface). However, the $K_m$ of the TM/T complex for Gla-domainless protein C ($\approx 7.0$ $\mu$M) remains unchanged regardless of whether TM is membrane incorporated or detergent solubilized (Galvin et al., 1987).

Recently, it has been shown that the T/elTM complex has a $K_m$ for protein C similar to that of the membrane incorporated TM/T complex. Furthermore, to achieve this similar $K_m$ for protein C by either of the complexes, the presence of the Gla domain is required. Thus, it has been suggested that the elTM fragment presents a site to protein C that is normally exposed when intact TM is incorporated into phospholipid vesicles (Kurosawa et al., 1987). It is this site which is thought to interact with the Gla domain of protein C. This is interesting in that most experimental evidence suggests a membrane binding role for the Gla domain. For example, the prothrombin Gla domain interacts with membrane surfaces (Mann et al., 1982). Therefore, it is thought that
the protein C Gla domain additionally interacts with phospholipid membranes.

Finally, a fluorescence study of the molecular architecture of the protein C, thrombin and TM complex supports the spatial arrangement of these molecules as shown in Figure 4 (Lu et al., 1989).

The goals of this research are to further examine the physical nature of each of the macromolecules in the protein C activation complex, as well as their interactions, particularly with regard to their binding energies and stoichiometries of association. In place of thrombomodulin, the e1TM fragment is used since it results in similar kinetic parameters for protein C activation as the intact phospholipid bound thrombomodulin and is soluble without the use of detergents. The macromolecular interactions will be studied as a function of the calcium ion concentration and as a function of the Gla domain of protein C. The data from these experiments will then be used to predict the kinetic behaviour of protein C activation and compared with the results from kinetics experiments.

The research to be presented in the following pages uses several techniques of analytical ultracentrifugation to directly study the nature of the protein C activation complex. Techniques of this kind have previously been used successfully in the study of heterogenous molecular complexes such as the structure of Factor Va (Laue et al., 1984) and the interaction
of prothrombin with factor Vₘ (Luckow, 1989; Luckow et al., 1989).

The techniques of analytical ultracentrifugation can measure association stoichiometries and binding strengths between molecules, distinguish between reversible and irreversible associating systems and determine general molecular shapes. Though this type of data cannot be easily obtained by other methods, the techniques of analytical ultracentrifugation are not often used because of the technical expertise required to run the Spinco Model E analytical ultracentrifuge and the difficulty of data analysis. A new centrifuge now in design and production should eliminate the need for technical expertise (Schachman, 1989). The studies done for this dissertation utilize a modified Model E analytical ultracentrifuge described in the Materials and Methods section and Appendix A.
Materials and Methods

Materials

The following proteins were provided by Dr. Charles Esmon and were purified using published methods: bovine thrombin (Owen et al., 1974), rabbit el-TM (Kurosawa et al., 1987), bovine protein C (Walker et al., 1979), human protein C (Vigano-D'Angelo et al., 1986), Gla-domainless protein C and the Gla peptide (Esmon et al., 1983), and activated protein C (Vigano-D'Angelo et al., 1986). Calcium chloride hydrate, 99.99%, was purchased from Aldrich Chemical Co. Dansyl-L-glutamylglycyl-l-arginine chloromethyl ketone and D-phenyl-L-prolyl-L-arginine chloromethyl ketone were purchased from Calbiochem. The chromogenic substrate, S-2238, was purchased from Helena laboratories. All other buffer components were of reagent grade.

Protein preparation

Proteins were prepared for analytical ultracentrifugation in one of two ways. All proteins were dialyzed in buffer A (100 mM NaCl, 50 mM Tris-HCl, pH 7.65, 1 mM Benzamidine) individually or as mixtures. Before dialysis, thrombin and protein C (and its derivatives) were inhibited with D-phenyl-L-prolyl-L-arginine chloromethyl ketone or dansyl-L-glutamylglycyl-L-arginine chloromethyl ketone, respectively (Kettner and Shaw, 1981). In some cases, thrombin was inhibited with diisopropylfluorophosphate (Esmon et al.,
1982a). Proteins were dialyzed in buffer A containing 2 mM EDTA, 0.3 mM CaCl₂, or 5 mM CaCl₂. However, and this step is where the difference in protein preparation is manifested, for many of the short column sedimentation equilibrium experiments, Buffer A was treated with Chelex 100 (BioRad) and proteins were dialyzed along with Chelex 100 in the dialysate. In all cases dialysis was done for a minimum of 60 hours at 4°C, with three buffer changes. The retentate:dialysate volume ratio was a minimum of 1:1000. Protein degradation was monitored using SDS-polyacrylamide gel electrophoresis, under reducing conditions, by analyzing samples taken before dialysis, after dialysis, and after ultracentrifugation (Laemmli, 1970). Protein concentrations were estimated using the approximate amino acid chain weights and the following extinction coefficients (1%, 1 cm) at 280 nm: bovine thrombin, 21.0 (Owen et al., 1974); bovine el-TM, 6.0 (Kurosawa et al., 1987); bovine protein C, 13.7 (Kisiel et al., 1976); human protein C, 14.5 (Kisiel, 1979); and, Gla-domainless protein C, 13.7 (Esmon et al., 1983).

Buffer density and partial specific volume

Density and partial specific volume are important parameters in the calculation of molecular weight from sedimentation data. Buffer A densities, containing 2 mM EDTA, 0.3 mM CaCl₂, or 5 mM CaCl₂, were determined at 23.3 °C, using the published method (Kratky et al., 1973), with a magnetic oscillator density meter (Mettler/Par model DMA 02 D). The
values are 1.0040 g/ml for buffer A containing either 2 mM EDTA or 0.3 mM CaCl₂, and 1.0044 g/ml for buffer A containing 5 mM CaCl₂. EDTA free and calcium free buffers were taken to have densities of 1.0040 g/ml.

Protein partial specific volumes were calculated from amino acid and carbohydrate composition (Edsall, 1943; McMeekin and Marshall, 1952). The partial specific volumes of amino acids and carbohydrates (Gibbons, 1972) used are good to three significant figures.

**Sedimentation equilibrium**

Both high speed meniscus depletion and short column sedimentation equilibrium experiments were used to examine the proteins and mixtures of them.

High speed meniscus depletion experiments were done as previously described (Yphantis, 1964) with the "externally loaded" cell fitted with sapphire windows and containing three pairs of solution/solvent channels 3mm in length (Ansevin et al., 1970). The solutions contained a single protein or equimolar mixtures of two or more of them (the retentate of the final dialysis step), and the reference solvent was the dialysate from the final step of the protein preparation dialysis. All experiments were done at 23.3 °C and sedimentation equilibrium data were collected at the indicated protein concentrations and rotor speeds.

Short column (0.7 mm) sedimentation equilibrium experiments were carried out as previously described
The advantages of short columns over that of 3 mm columns are that equilibrium is reached in an hour or two as opposed to 24 hours, and much smaller quantities of protein are required - about five times less. A disadvantage is that short columns do not fractionate the protein solution with as much resolution.

In most cases, for short column sedimentation equilibrium experiments, calcium chloride was added to the retentate and dialysate directly, just prior to centrifugation. For experiments that used Gla peptide, the peptide was dialyzed separately from the other proteins and also added to both the solution and reference channels just prior to centrifugation. The volumes and concentrations of CaCl₂ or the Gla peptide were adjusted appropriately to insure that the only thermodynamic difference between the solution and solvent columns was the protein, or the proteins, of interest. All experiments were done at 23.3 °C and sedimentation data were collected at the indicated protein concentrations and rotor speeds.

A Beckman Model E analytical ultracentrifuge equipped with an electronic speed control, RTIC temperature controller, and a HeNe laser light source for Rayleigh interference optics was used (Williams, 1978). Data were collected using Kodak Technical Pan film and logged into a Vax microcomputer (Digital Equipment Corp.) via an automated plate reader for earlier experiments. Later experiments utilized the real-time
data acquisition and analysis system which gathers interference fringe data via a solid state TV camera (Laue, 1981; also, please see Appendix A).

Data were collected at estimated equilibrium times (Yphantis, 1964). Verification that equilibrium had been reached was done by comparing data with other data taken up to two hours after the estimated equilibrium time had been reached. Blank corrected data were obtained by subtracting blank (using distilled water in the solution/solvent channels) interferograms, obtained before and after each experiment, from the experimental data (Yphantis, 1964).

**Data analysis**

Blank correction and data editing were performed using the REEDIT program (kindly provided by D. Yphantis). Between 75 and 400 data points were measured from each interferogram with a maximum distance of 10 μm between each data point in the high gradient region. Data were truncated, to avoid Weiner skewing, by excluding fringe gradients above 15 mm/cm². These blank corrected, edited data were analyzed using a nonlinear least squares estimation computer program called NONLIN (Johnson et al., 1981). This program provides values and the 65% confidence intervals for the fitted parameters. Data from one or more channels, at different loading concentrations, radial positions, and rotor speeds can be fit simultaneously to a selected model. The simplest model (i.e., the one with the fewest fitting parameters) which adequately fits the data
is considered to be the "correct" model. This decision is a comparative one based on the variance of fit which should approximate the experimental noise level, and the degree of systematic variations in the residuals of the various models examined (Johnson et al., 1981).

Two models were found useful for fitting the data and are further described here.

The first model consists of a monomer-dimer (or higher oligomer) equilibrium with a hypothetical monomer of molecular weight that is the approximate weight average molecular weight of the mixture of the associating proteins. Thus,

\[
y(r) = \delta + e^{(\ln a + \xi \cdot 2B(r - \delta))} + e^{(N\ln a + \xi \cdot 2B(r - \delta) + \ln K)},
\]

where \(y(r)\) is the fringe displacement (in fringes) at a radius \(r\), \(\delta\) is the baseline offset, \(a\) is the monomer activity at the arbitrary reference radius \(r_0\), \(\sigma\) is the reduced molecular weight, \(\xi = (r^2 - r_0^2)/2\), \(B\) is the coefficient of nonideality, \(N\) is the stoichiometry of the association, and \(K\) is the association constant of the complex (Johnson et al., 1981).

The reduced molecular weight of a component is defined as \(\sigma = M(1 - \tilde{v}_q)\tilde{w}^2/RT\), where \(M\) is the molecular weight of the component, \(\tilde{v}\) is the partial specific volume of the component, \(\tilde{v}_q\) is the solvent density, \(\tilde{w}\) is the angular velocity of the rotor, \(R\) is the universal gas constant, and \(T\) is the solution
temperature in degrees kelvin (Yphantis and Waugh, 1956). This model (eq. 1) can be used to describe the sedimentation behaviour of individual proteins, but also is appropriate for describing heterogenous associations in cases where the difference in the reduced molecular weights of the two associating species is no more than 2.5 cm$^2$. However, it is best if the difference is less than 0.65 cm$^2$ (Luckow et al., 1989).

Whereas the model discussed above can be used to implicitly describe the fringe displacement data, the following model explicitly describes the total fringe displacement as the sum of the displacements due to the individual components and their reversible complex (-es). This model assumes that all components are ideal:

\[
Y(r) = \delta + \exp(\ln \alpha_A + \sigma_A \xi) + \exp(\ln \alpha_B + \sigma_B \xi) + \exp(\ln \alpha_A + \ln \alpha_B + (\sigma_A + \sigma_B) \xi + \ln K_{AB}),
\]

where \(Y(r)\) is the fringe displacement (in fringes) at a radius \(r\), \(\delta\) is the baseline offset, \(\alpha_A\) and \(\alpha_B\) are the monomer activities of the A and B species at the arbitrary reference radius \(r_0\), \(\sigma_A\) and \(\sigma_B\) are the reduced molecular weights (as defined above), \(\xi=(r^2 - r_0^2)/2\), and \(K_{AB}\) is the association constant of the AB complex (Johnson et al., 1981). This model is useful only when there is a difference in the reduced molecular weights between A and B of at least 0.65 cm$^2$. It is
the only model that will give an adequate fit when this difference is greater than 2.5 cm\(^2\) (Luckow et al., 1989). As will be seen in the Results section, it was necessary to modify this model to describe the experiments describing the interaction of thrombin and BPC or BAPC. This is because thrombin and protein C apparently form a 1:2 complex in addition to a 1:1 complex.

As mentioned earlier, both models return confidence intervals. These describe only the precision of the fit to a particular model and do not reflect the accuracy of the determination. Thus, the error bars to be found in the following Results section are only a measure of the precision of the determined value. Furthermore, the error bars (confidence intervals) are often not symmetric, which is to be expected when evaluating nonlinear functions (Johnson et al., 1981).

Conversion of units

All fits by NONLIN were done using units of fringe displacement. Conversion from the fringe displacement scale to other, and more accepted concentration scales was made by using the refractive increment of 0.185 ml/g at the He-Ne laser wavelength of 632.8 nm (Perlman and Longsworth, 1948). Molecular weights were calculated from the corresponding reduced molecular weight returned by NONLIN. The units of the association constant \(\ln K_{\text{meq}}\) returned by NONLIN, were converted from inverse fringes to inverse molarity units (represented by
lnK_m), by using the best-fit molecular weights for each case and the protein refractive increment (Laue et al., 1984) For monomer-dimer associating systems:

\[ K_m = K_{\text{fringe}}\left(\frac{M}{2}\right)(Y_T/C_T), \]

where M is the monomer molecular weight and YT/C_T is the specific fringe displacement (3.52 fringes·L/g at 632.8nm).

For an (A+B)-type association, the corresponding unit conversion equation for the association constant is:

\[ K_m = K_{\text{fringe}}\left(M_A M_B / M_{AB}\right)(Y_T/C_T), \]

where M_A, M_B and M_{AB} are the molecular weights of A, B, and the AB complex, respectively. The dissociation constant, K_d was calculated by taking the inverse of the association constant.

The dissociation free energies (\(\Delta G''\)) of the complexes were calculated using:

\[ \Delta G'' = -RT\ln(K_d) \]

where R is the universal gas constant in units of kcal/mole/K and T is the temperature in Kelvin. Finally, the nonideality coefficient, B, was converted to ml/g by using the best-fit value returned by NONLIN and multiplying it by the best-fit molecular weight, also returned by NONLIN.
Assay of protein C activation

This work was done with Dr. Naomi Esmon in the laboratory of Dr. Charles Esmon at the Oklahoma Medical Research Foundation, Oklahoma City, OK.

It became clear from the results of sedimentation equilibrium experiments that activated protein C might be a very good inhibitor of protein C activation. Protein C activation by thrombin alone and by thrombin and elTM was done under optimal activation conditions as follows. An assay for protein C activation has been described previously (Esmon et al., 1983b).

Protein C activation by thrombin alone was done in 1.0 mM EDTA, 0.1 M NaCl, 0.02 M Tris-HCl, 0.1% gelatin, pH 7.5, 37 °C for ten minutes. Total reaction volume was 50 μl. The reaction was stopped with 10 μl of antithrombin III (350 μg/ml final concentration). The thrombin concentration was 5 nM and the protein C and activated protein C concentrations are indicated in the figure legends. Activated protein C for use as an inhibitor was inactivated with DEGR and run over a Mono Q (0.5 x 5 cm) column (Pharmacia LKB) connected to a protein liquid chromatography system. Residual activity of BAPC, was quantitated and subtracted from the final results.

Protein C activation by thrombin and elTM was carried out similarly to activation by thrombin alone. The reaction time was decreased to five minutes and 0.3 mM calcium chloride was used instead of EDTA. The elTM concentration was 20 nM and the
protein C and BAPC, concentrations are indicated in the figure legends. To insure that the elTM was maximally bound by thrombin, a mixture of PPACK-inhibited thrombin (15 nM) and thrombin (0.08 nM) was used. The PPACK inhibited thrombin was prepared by incubating thrombin with PPACK for thirty minutes, and then the thrombin was run over a Mono Q column to remove excess PPACK. Residual thrombin activity was negligible (>99% inhibition). As described above, the reaction was stopped with 10 μl of antithrombin III.

The concentration of activated protein C was measured by the hydrolysis of H-D-Phe-pippecoyl-Arg-p-nitroanilide (S-2238) that was at an initial concentration of 0.2 mM. The reaction was monitored at 405 nm with a Molecular Devices V_mox spectrophotometer at room temperature (21-25 °C) in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5, 0.1% gelatin. The concentration of protein C was determined by using a standard curve that related the rate of p-nitroanilide production to the standard concentration of protein C (Esmon et al., 1983b).
RESULTS

Partial specific volume

The partial specific volume is a critical quantity in the calculation of molecular weights from the reduced molecular weights returned by the nonlinear least squares fitting of analytical ultracentrifuge data. A 1% error in the partial specific volume translates into a 3% error in the calculated molecular weight (Schachman, 1957). Partial specific volumes of proteins used in this research are shown in Table 1. The $\bar{v}$ values in this table are used in the calculation of the molecular weights of proteins for this thesis. This is because the mean difference between the calculated and measured values of the partial specific volume for a number of proteins is less using data from Cohn and Edsall as compared to other data sets, such as the data set from Zamyatin (Perkins, 1986).

Analytical ultracentrifugation of individual proteins

Before examining the combinations of proteins in the analytical centrifuge, the sedimentation behaviour of individual proteins must be studied. For example, some proteins exhibit self-association. This would be an important consideration for the proper interpretation of sedimentation results of a mixture of several proteins, one of which might be self-associating.

Table 2 shows the molecular weights of individual
Table 1: Partial Specific Volumes of Selected Proteins.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide Chain M, (g/mol)</th>
<th>( \psi'_1 ) (ml/g)</th>
<th>( \psi'_2 ) (ml/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPC</td>
<td>46,800</td>
<td>0.712</td>
<td>0.699</td>
</tr>
<tr>
<td>gdBPC</td>
<td>42,000</td>
<td>0.712</td>
<td>0.700</td>
</tr>
<tr>
<td>BAPC</td>
<td>45,100</td>
<td>0.712</td>
<td>0.700</td>
</tr>
<tr>
<td>HPC</td>
<td>47,200</td>
<td>0.711</td>
<td>0.698</td>
</tr>
<tr>
<td>gdHPC</td>
<td>42,400</td>
<td>0.710</td>
<td>0.698</td>
</tr>
<tr>
<td>HAPC</td>
<td>45,800</td>
<td>0.711</td>
<td>0.699</td>
</tr>
<tr>
<td>elTM</td>
<td>27,400</td>
<td>0.692</td>
<td>0.681</td>
</tr>
<tr>
<td>BT</td>
<td>35,300</td>
<td>0.731</td>
<td>0.717</td>
</tr>
</tbody>
</table>

*Partial specific volumes calculated with data from Cohn & Edsall (1943).*

*Partial specific volumes calculated with data from Zamyatnin (1972).*
Table 2. Properties of the individual components. See text for discussion.

'a' Cell loading concentration.
'b' Protein dialyzed against buffer A containing the addition listed.
'c' Simultaneous fit to data acquired at the listed rotor speeds.
'd' Molecular weight determined and the 65% confidence interval (in parentheses).
'e' Product of the second virial coefficient and the fitted molecular weight and the 65% confidence interval (in parentheses).
'f' Square root of the variance of fit in units of fringe displacement.
Table 2: Properties of the Individual Components.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc. (μM)</th>
<th>Buffer Add. (mM)</th>
<th>Speed (10^3 rpm)</th>
<th>M₅ (g/mol)</th>
<th>BM (ml/g)</th>
<th>r-s</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPC</td>
<td>9.42</td>
<td>EDTA 24,28</td>
<td>(62,200-62,700)</td>
<td>62,400</td>
<td>0</td>
<td>.014</td>
</tr>
<tr>
<td></td>
<td>9.42</td>
<td>Ca²⁺ 20,24</td>
<td>(60,100-60,700)</td>
<td>60,400</td>
<td>0</td>
<td>.015</td>
</tr>
<tr>
<td></td>
<td>9.42</td>
<td>Ca²⁺ 20,24</td>
<td>(61,300-61,900)</td>
<td>61,600</td>
<td>0</td>
<td>.015</td>
</tr>
<tr>
<td>gdBPC</td>
<td>15.0</td>
<td>EDTA 20,24</td>
<td>(54,200-54,900)</td>
<td>54,600</td>
<td>8(6-10)</td>
<td>.019</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>Ca²⁺ 20,24</td>
<td>(55,100-55,800)</td>
<td>55,500</td>
<td>16(13-18)</td>
<td>.023</td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>Ca²⁺ 20,24</td>
<td>(54,800-55,300)</td>
<td>55,100</td>
<td>17(15-18)</td>
<td>.025</td>
</tr>
<tr>
<td>HPC</td>
<td>16.6</td>
<td>EDTA 20,24</td>
<td>(60,900-61,900)</td>
<td>61,400</td>
<td>0</td>
<td>.030</td>
</tr>
<tr>
<td></td>
<td>16.6</td>
<td>Ca²⁺ 20</td>
<td>(61,100-62,700)</td>
<td>61,900</td>
<td>0</td>
<td>.031</td>
</tr>
<tr>
<td></td>
<td>7.50</td>
<td>Ca²⁺ 20,24</td>
<td>(62,000-62,800)</td>
<td>62,400</td>
<td>0</td>
<td>.026</td>
</tr>
<tr>
<td>gdHPC</td>
<td>10.0</td>
<td>EDTA 20,24</td>
<td>(53,500-54,700)</td>
<td>54,100</td>
<td>13(8-18)</td>
<td>.024</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>Ca²⁺ 20,24</td>
<td>(53,700-55,000)</td>
<td>54,400</td>
<td>13(10-18)</td>
<td>.023</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>Ca²⁺ 20,24</td>
<td>(52,800-54,300)</td>
<td>53,600</td>
<td>17(12-22)</td>
<td>.027</td>
</tr>
<tr>
<td>e1TM</td>
<td>18.3</td>
<td>EDTA 20,24</td>
<td>(34,700-35,100)</td>
<td>34,900</td>
<td>0</td>
<td>.022</td>
</tr>
<tr>
<td></td>
<td>18.3</td>
<td>Ca²⁺ 20,24</td>
<td>(34,500-34,900)</td>
<td>34,700</td>
<td>0</td>
<td>.018</td>
</tr>
<tr>
<td></td>
<td>18.3</td>
<td>Ca²⁺ 20,24</td>
<td>(33,900-34,200)</td>
<td>34,100</td>
<td>0</td>
<td>.022</td>
</tr>
<tr>
<td>BTp</td>
<td>42.0</td>
<td>EDTA 20,24</td>
<td>(38,600-39,000)</td>
<td>38,800</td>
<td>0</td>
<td>.020</td>
</tr>
<tr>
<td></td>
<td>42.0</td>
<td>Ca²⁺ 20,24</td>
<td>(39,200-39,800)</td>
<td>39,500</td>
<td>0</td>
<td>.022</td>
</tr>
<tr>
<td></td>
<td>42.0</td>
<td>Ca²⁺ 20,24</td>
<td>(38,800-39,400)</td>
<td>39,100</td>
<td>0</td>
<td>.021</td>
</tr>
</tbody>
</table>
proteins as determined by the standard sedimentation equilibrium technique (Yphantis, 1964). Most of the proteins were modeled best as ideal monomers, irrespective of whether or not the dialysis buffer contained calcium ions. BPC and HPC behaved variably depending on the protein preparation. The data collected for these two proteins in the presence of calcium could sometimes be made to fit a monomer-dimer model (dissociation constant on the order of millimolar). However, the most consistent model is the ideal monomer model, and this was the one chosen to fit the data.

Not clearly evident from Table 2 is that the Gla domain of protein C appears to be responsible for the variability with regard to self-association, since any tendency toward self-association was eliminated when the Gla domainless counterparts of BPC and HPC were examined. Prothrombin, another protein containing a Gla domain, also exhibits this variability (data from T. Laue not shown). However, the Gla-domainless counterpart, thrombin, behaves as an ideal monomer.

The short column sedimentation equilibrium technique was used to examine the activated form of bovine protein C, BAPC (Yphantis, 1960). To activate protein C, a 14 amino acid activation peptide is cleaved from the amino terminus of the heavy chain. Before sedimentation, BAPC is separated from the ≈1600 g/mol activation peptide. However, the sedimentation behaviour of BAPC was similar to that of BPC (Figure 5). The molecular weight determined by short column sedimentation
Figure 5: The z-average molecular weights of BPC and BAPC as a function of the Ca\(^{2+}\) concentration. See text for full discussion. Short column sedimentation equilibrium, as described in Materials and Methods, was used to determine the sedimentation behaviour of BPC (20 μM) and BAPC (20 μM) as a function of the calcium chloride concentration. Each point represents a simultaneous fit to three speeds: 20, 24 and 28 thousand rpm.
Comparison of BPC and BAPC

\[ M_z \text{ vs. } \text{CaCl}_2 \text{ concentration} \]

- ▲ BAPC
- ● BPC

Figure 5.

\[ [\text{CaCl}_2]_{\text{initial}} \text{ mM} \]
equilibrium is a z-average of all the species present. Large aggregates are weighted more than lighter ones with this type of average. Thus, the observation noted above (that there may be some self-association, or aggregation of protein C) is supported here. There is an alternative explanation.

Sedimentation velocity studies conducted by the Laue laboratory (unpublished) have shown that activation of BPC results in the condensing of the protein structure. The activation results in a decrease in the partial specific volume of the protein, and the concomitant increase in the apparent molecular weight, as determined by sedimentation equilibrium. The increase in the apparent molecular weight occurs despite the lost mass which is due to the removal of the activation peptide.

Since BAPC sedimented similarly to BPC, all models used to fit experiments involving BAPC utilized the reduced molecular weight determined for BPC.

Thus, the sedimentation behaviour of all the proteins was essentially invariant with the calcium ion concentration and, as a result, an average molecular weight in conditions of calcium ions and no calcium ions was calculated and used to fit models involving the interactions of two or more proteins. Though the Gla domainless protein C molecules exhibited detectable nonideality, this fact was neglected for the remainder of this study. At the moment, the inclusion of nonideality in association models is too complicated.
Analytical ultracentrifugation of elTM with BTP

The strongest interaction observed was that between elTM and BTP. These results were determined using standard 3 mm columns and are shown at the top of Table 3. At the concentrations of protein examined, the strength of this interaction could not be determined in EDTA. This places an upper limit on the dissociation constant of $\approx 10^9$ M (Laue et al., 1984). The two proteins appeared to behave as a single component of 1:1 stoichiometry with an expected molecular weight of 73,400 g/mol. The presence of calcium ions weakened this interaction, though not significantly until a concentration of 5 mM was reached. The best model that was found to fit this data was an ideal monomer-dimer model.

Analytical ultracentrifugation of elTM with BPC, BAPC or gdBPC

These results form the bulk of the data shown in Table 3. As was the case for the elTM/thrombin interaction an ideal monomer-dimer model was used to fit the interaction of elTM with BPC and BAPC. This interaction was weak and relatively calcium ion insensitive. Surprisingly elTM and BAPC interacted more strongly than elTM and BPC. That is, the product of the activation, activated protein C, interacts more strongly with elTM than the substrate, protein C. The weight average molecular weight of elTM and gdBPC of 44,850 g/mol is in reasonable agreement with the value returned by NONLIN. Therefore, it was not possible to detect the presence of an interaction between elTM and gdBPC at the protein
Table 3: The interactions of BTP, BPC, BAPC and gdbPC with e1TM. See text for discussion.

- Cell loading concentration of each protein.
- Proteins dialyzed against buffer A containing the addition listed.
- Simultaneous fit to data acquired at the listed rotor speeds.
- Molecular weight determined and the 65% confidence interval (in parentheses).
- Dissociation constant determined between the two proteins in the experiment and the 65% confidence interval (in parentheses).
- Square root of the variance of fit in units of fringe displacement.
- und = no dissociation constant could be detected.
- This experiment was done using the short column centerpiece.
- No addition was made to buffer A in this case, but the buffer and glassware was treated as described in methods to remove calcium ions.
- none = no association constant could be detected.
- This value was held constant during the fit.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc. (µM)</th>
<th>Buffer Addition (mM)</th>
<th>Speed (x10³ rpm)</th>
<th>M (g/mol)</th>
<th>K (µM)</th>
<th>rms (fr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BTp + elTM</td>
<td>21.5</td>
<td>2.0 EDTA</td>
<td>20,24</td>
<td>(72,500-74,000)</td>
<td>und</td>
<td>0.043</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 Ca²⁺</td>
<td>20,24</td>
<td>69,900</td>
<td>und</td>
<td>0.032</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0 Ca²⁺</td>
<td>20,24</td>
<td>36,700</td>
<td>0.042</td>
<td>0.032</td>
</tr>
<tr>
<td>BPC + elTM</td>
<td>4.7</td>
<td>2.0 EDTA</td>
<td>15,20</td>
<td>48,100</td>
<td>82.0</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 Ca²⁺</td>
<td>15,20</td>
<td>48,100</td>
<td>71.0</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0 Ca²⁺</td>
<td>15,20</td>
<td>48,100</td>
<td>69.0</td>
<td>0.017</td>
</tr>
<tr>
<td>BAPC + elTM</td>
<td>7.0</td>
<td>none</td>
<td>20,24,28</td>
<td>48,100</td>
<td>35.0</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 Ca²⁺</td>
<td>20,24,28</td>
<td>48,100</td>
<td>53.0</td>
<td>0.016</td>
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<td></td>
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<td>4.0 Ca²⁺</td>
<td>20,24,28</td>
<td>48,100</td>
<td>52.0</td>
<td>0.013</td>
</tr>
<tr>
<td>gdBPC + elTM</td>
<td>5.0</td>
<td>2.0 EDTA</td>
<td>15,20</td>
<td>49,400</td>
<td>none</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 Ca²⁺</td>
<td>15,20</td>
<td>49,200</td>
<td>none</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0 Ca²⁺</td>
<td>15,20</td>
<td>51,800</td>
<td>none</td>
<td>0.046</td>
</tr>
</tbody>
</table>
concentrations studied. This suggests that the bulk of the interaction of elTM and protein C may be mediated directly or indirectly through the Gla domain.

Another way of presenting some of the results in Table 3 that highlights the calcium dependence of complex formation is shown in Figure 6 for the mixture of elTM and BAPC. The dashed line in the figure (at $M_z = 48,100$ g/mol) represents the $z$-average molecular weight of elTM and BAPC, and indicates where the points should fall if there was no association of the proteins. So, using the reasonable assumption from earlier results that these proteins do not undergo significant self-association, this figure demonstrates that there is some association between elTM and BAPC. It appears that there may be a greater tendency for elTM and BAPC to associate in the absence of calcium ions, an observation reflected in Table 3. In addition, two separate experiments gave similar results. This was a typical example of the reproducibility found during the course of this research. Plots of $M_z$ vs. $[\text{CaCl}_2]_{\text{TOTAL}}$, like Figure 6, will be used to present all the initial experimental results that remain.

**Analytical ultracentrifugation of Thrombin with BPC and BAPC**

The interaction of thrombin inhibited with D-phenyl-L-prolyl-L-arginine chloromethyl ketone (BTp) with BPC and BAPC was examined (Figure 7). The interaction between BTp and BPC
Figure 6: The z-average molecular weight of the elTM-BAPC complex as a function of the concentration of Ca$^{2+}$. The results shown here are described in the text. The dashed line is the weight average molecular weight of elTM and BAPC when there is no interaction between them. Experiments A and B were done with short columns at equimolar protein concentrations of 7 and 10 $\mu$Molar respectively. Each data point represents a simultaneous fit to the data from 20, 24 and 28 thousand rpm.
elTM-BAPC Complex Formation

$M_z$ vs. $\text{CaCl}_2$ Concentration

△ Experiment A
● Experiment B

Figure 6.

$[\text{CaCl}_2]_{\text{TOTAL}}$ mM
Figure 7: BPC and BAPC binding to BTP - the z-average molecular weight of the complexes formed as a function of the Ca$^{2+}$ concentration. The results shown here are described in the text. These BTP/BAPC and BTP/BPC short column experiments were done at equimolar protein concentrations of 7.5 and 10 μM, respectively. Each point represents a simultaneous fit of the data to three speeds: 20, 24 and 28 thousand rpm.
BPC and BAPC Binding to BTp

$M_z$ vs. CaCl$_2$ Concentration

\[ \text{BTp + BAPC} \quad \text{□ BTp + BPC} \]

Figure 7.
or BAPC was virtually indistinguishable. Nor were calcium ions necessary for the interaction. In fact, the addition of calcium chloride immediately weakened the interaction, and in 4 mM calcium chloride the interaction is nearly undetectable as a noninteracting equimolar mixture of BTP and BPC would have a molecular weight of 50,300 (the dashed line in Figure 7).

A complex of 1:1 BTP:BPC (BAPC) stoichiometry has a molecular weight of 100,600 g/mol, represented by the solid line in Figure 7. This is much less than the z-average molecular weight determined in 0 mM calcium chloride. It is clear that structures with stoichiometries greater than 1:1 must be present. Therefore, no single $K_d$ is sufficient to describe the data.

In order to determine the stoichiometries of the species present in the BTP/BPC (BAPC) mixture, a more detailed analysis was made at each calcium chloride concentration. While there is absolutely no question that a BTPBPC complex is present, it is the higher oligomer(s) that is (are) difficult to identify. Some of the models tried are compared in Table 4. The best model is the last one in the table, which predicts a composition of the following four species: 1) BTP; 2) BPC; 3) BTPBPC; and, 4) BTPBPC$_2$. This model fit the data consistently better than the others based on the root mean square values.
Table 4: Comparison of the goodness of fit of several models used to interpret the BTp - BPC interaction.

<table>
<thead>
<tr>
<th>MODEL</th>
<th>rms (fringes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single component</td>
<td>0.030</td>
</tr>
<tr>
<td>BTp + BPC &lt;-&gt; BTpBPC (monomer - dimer)</td>
<td>0.080</td>
</tr>
<tr>
<td>BTp + BPC &lt;-&gt; BTpBPC (heteroassociation)</td>
<td>no convergence</td>
</tr>
<tr>
<td>2BTp - 2BPC &lt;-&gt; (BTpBPC); (heteroassociation)</td>
<td>no convergence</td>
</tr>
<tr>
<td>2BTp + 2BPC &lt;-&gt; (BTpBPC); (monomer - dimer - tetramer)</td>
<td>0.025</td>
</tr>
<tr>
<td>2BTp + BPC &lt;-&gt; BTpBPC (heteroassociation)</td>
<td>0.094</td>
</tr>
<tr>
<td>BTp + 2BPC &lt;-&gt; BTpBPC; (heteroassociation)</td>
<td>0.019</td>
</tr>
</tbody>
</table>

Experimental conditions: 10 μM each of BTp and BPC in 0 mM calcium chloride buffer, pH 7.6. F_μμ = 1.062
returned by NONLIN and the randomness of the residuals. Figures 8 and 9 show the dissociation energies, determined using this model, of BTPBPC (or BAPC) and BTPBPC$_2$ (or BAPC$_2$) as a function of the calcium chloride concentration, respectively. Interestingly, the same amount of energy is released on addition of the second protein C molecule to thrombin as is released when adding the first.

**The effect of decreased thrombin inhibitor size on substrate vs. product binding**

It was surprising to find that BPC and BAPC bound BTP with little difference. Since PPACK might be covering or blocking several important amino acids in the thrombin substrate recognition site necessary for BPC binding, diisopropylfluorophosphate (DFP) was used to inhibit thrombin (BTd). DFP binds to thrombin's active site serine as diisopropylphosphate and probably blocks the arginine pocket.

PPACK, which has the amino acid sequence, Phe-Pro-Arg, is a very specific inhibitor of thrombin. In particular, arginine sits in the arginine pocket of thrombin and proline and phenylalanine both interact with residues in the insertion loop segments of thrombin, which define and deepen the active site cleft (Bode et al., 1989). This interaction appears to prevent substrates from binding to the thrombin active site cleft properly.

The binding of BPC and BAPC to BTd, rather than BTP,
Figure 8: The free energy of dissociation between the BTp/BPC and the BTp/BAPC complexes. This figure is more fully described in the text. Using the following model, the energy of dissociation of BTp/BPC (or BAPC) to BTp and BPC (or BAPC) was determined from the data shown in Figure 3 and plotted against the calcium chloride concentration:

\[ \text{BTp} + 2\text{BPC} \rightleftharpoons \text{BTpBPC} + \text{BPC} \rightleftharpoons \text{BTpBPC}_2. \]
The BTp/BPC and BTp/BAPC Complexes

Energy of Dissociation vs. [CaCl₂]

Figure 8.

[CaCl₂] mM
Figure 9: The free energy of dissociation between the BTp/BPC$_2$ and the BTp/BAPC$_3$ complexes. Using the same model as described for figure 8, the energy of dissociation of BTpBPC$_2$ (or BAPC$_3$) to BTpBPC (or BAPC) and BPC (or BAPC) is plotted as a function of the calcium chloride concentration.
BTP/BPC$_2$ and BTP/BAPC$_2$ Complexes

Energy of Dissociation vs. [CaCl$_2$]

Figure 9.

\[ \Delta G^o \text{ kcal/mol} \]

\[ \text{[CaCl$_2$] mM} \]
is shown in Figure 10. As was found with BTp, there does not appear to be a great deal of discrimination between BPC and BAPC. However, BTd and BPC tend to result reproducibly in a slightly higher z-average molecular weight than the BTd and BAPC mixture. This may indicate that BTd and BPC interact more strongly than BTd and BAPC. The data sets used to produce Figure 10 were deconvoluted using the same model that describes the BTP/BPC (or BAPC) interaction, because this model fit best (Figures 11 and 12). Again, as was found with BTp, it appears that the same amount of energy is required to add the second protein C molecule as the first. However, at higher calcium ion concentrations, the second protein C molecule seems to be bound less tightly to BTd than when using PPACK inhibited thrombin.

**Analytical ultracentrifugation of thrombin with elTM and BPC and BAPC**

Since thrombin was not able to discriminate between BPC and BAPC to any large extent, it might be possible that the cofactor, elTM, plays an important role in this respect. In Figure 13 the interaction of BTP, elTM and BPC (or BAPC) is examined, and though there is a difference between the binding of BPC and BAPC, it is not large. As was for the case of the thrombin and protein C studies, calcium chloride is not required for these proteins to interact. In fact, as before, the addition of calcium chloride causes their dissociation.
Figure 10: BPC and BAPC binding to BTd - the z-average molecular weight of the complexes formed as a function of the Ca\(^{2+}\) concentration. The results shown here are described in the text. These BTd/BAPC and BTd/BPC short column experiments were done at equimolar protein concentrations of 12.0 and 12.0 \(\mu\text{M}\), respectively. Each point represents the simultaneous fit of data to three speeds: 20, 24 and 28 thousand rpm.
BPC and BAPC Binding to BTd

$M_z$ vs. CaCl$_2$ Concentration

- $\bullet$ BTd + BAPC
- $\square$ BTd + BPC

![Graph showing the relationship between $M_z$ and CaCl$_2$ concentration](image-url)
Figure 11: The free energy of dissociation between the BTd/BPC and the BTd/BAPC complexes. This figure is more fully described in the text. Using the following model, the energy of dissociation of BTd/BPC (or BAPC) to BTd and BPC (or BAPC) was determined from the data shown in Figure 10 and plotted against the calcium chloride concentration:

$$\text{BTd} + 2\text{BPC} \rightleftharpoons \text{BTdBPC} + \text{BPC} \rightleftharpoons \text{BTdBPC}_2.$$
The BTd/BPC and BTd/BAPC Complexes
Energy of Dissociation vs. [CaCl₂]

\[ \Delta G^\circ \text{ kcal/mol} \]

\[ [\text{CaCl}_2] \text{ mM} \]

Figure 11.
Figure 12: The free energy of dissociation between the BTd/BPC, and BTd/BAPC, complexes. Using the same model as described for figure 11, the energy of dissociation of BTdBPC, (or BAPC,;) to BTdBPC (or BAPC) and BPC (or BAPC) is plotted as a function of the calcium chloride concentration.
BTd/BPC₂ and BTd/BAPC₂ Complexes
Energy of Dissociation vs. [CaCl₂]

Figure 12.
Figure 13: The binding of BPC and BAPC to the eITM/BTp complex - the z-average molecular weight of the complexes formed as a function of the Ca\(^{2+}\) concentration. The three proteins were mixed in equimolar concentrations (8 µM for the BPC experiment and 7 µM for the BAPC experiment) and subjected to short column sedimentation equilibrium. Each data point represents a simultaneous fit of data collected at three speeds: 15, 20 and 24 thousand rpm.
BPC and BAPC Binding to eITM-BTp

$M_z$ vs. $\text{CaCl}_2$ Concentration

$\Delta$ BAPC  $\square$ BPC

Figure 13.

$[\text{CaCl}_2]_{\text{total}}$, mM
A similar study was made using DFP inhibited thrombin, with much the same result (Figure 14). However, there does seem to be a greater difference in the affinity of BPC vs. BAPC for elTM/BTd than for elTM/BTp, particularly at the lower calcium chloride concentrations. This was previously noted for the thrombin and protein C studies (compare Figures 7 and 10).

Using NONLIN, an attempt was made to quantitate the stoichiometry of the elTM/BTp/BPC complex and the strength of the BPC interaction with the elTM/BTp complex (Table 5). This was done for the 0 mM calcium chloride case only, because the elTM/BTp complex begins to weaken upon addition of calcium ions, and the analysis of a three protein mixture is not possible without more data. Thus, the elTM/BTp complex was treated as a single protein component and the BPC (or BAPC) as the other protein component. The data was well fit in all cases with a monomer/dimer model using a monomer molecular weight of 67,600 g/mol for experiments involving BPC and BAPC. These molecular weights represent half the total molecular weight of a 1:1:1 stoichiometry of elTM:BTp:BPC (or BAPC or gdBPC). There was no evidence for higher oligomers. A dissociation constant of 1 μM corresponds to a dissociation free energy between BPC and the elTM/BTp complex of about 8 kcal/mol.

**Analytical ultracentrifugation of elTM with thrombin and gdBPC**

Temporarily setting aside the question of why BPC and
Figure 14: The binding of BPC and BAPC to the elTM/BTd complex - the z-average molecular weight of the complexes formed as a function of the Ca\textsuperscript{2+} concentration. The three proteins were mixed in equimolar concentrations (8 μM for the BPC experiment and 8 μM for the BAPC experiment) and subjected to short column sedimentation equilibrium. Each data point represents a simultaneous fit of data collected at three speeds: 15, 20 and 24 thousand rpm.
BPC and BAPC Binding to eI TM-BTd

$M_z$ vs. $\text{CaCl}_2$ Concentration

- △ BAPC
- □ BPC
Table 5: Estimates of the $K_d$ between elTM-BTp(d) BPC, BAPC and gdBPC.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc. (µM)</th>
<th>Buffer Addition (mM)</th>
<th>Speed (x10^3 rpm)</th>
<th>$M_r$ (g/mol)</th>
<th>$K_d$ (µM)</th>
<th>$\text{rms}^f$ (fr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPC + elTM-BTp</td>
<td>8.0</td>
<td>none</td>
<td>15,20,24</td>
<td>67,600</td>
<td>1.70</td>
<td>0.030</td>
</tr>
<tr>
<td>BAPC + elTM-BTp</td>
<td>7.0</td>
<td>none</td>
<td>15,20,24</td>
<td>67,600</td>
<td>0.890</td>
<td>0.024</td>
</tr>
<tr>
<td>BPC + elTM-BTd</td>
<td>8.0</td>
<td>none</td>
<td>15,20,24</td>
<td>67,600</td>
<td>1.00</td>
<td>0.031</td>
</tr>
<tr>
<td>BAPC + elTM-BTd</td>
<td>8.0</td>
<td>none</td>
<td>15,20,24</td>
<td>67,600</td>
<td>4.60</td>
<td>0.015</td>
</tr>
<tr>
<td>gdBPC + elTM-BTp</td>
<td>7.0</td>
<td>none</td>
<td>15,20,24</td>
<td>64,400</td>
<td>181</td>
<td>0.012</td>
</tr>
</tbody>
</table>

*Cell loading concentration of each protein. *No addition was made to buffer A in this case, but the buffer and glassware was treated as described in methods to remove calcium ions. *Simultaneous fit to data acquired at the listed rotor speeds. *This molecular weight was held constant during the fit. *Dissociation constant determined and the 65% confidence interval (in parentheses). *Square root of the variance of fit in units of fringes.
BAPC appear to recognize thrombin and the thrombin/elTM complex with very similar affinity, an attempt was made to examine whether the Gla domain was involved in the recognition of protein C by the thrombin/elTM complex. Limited digestion of BPC results in the removal of the Gla domain (the amino terminal 42 amino acids) from the NH₂ end of the light chain. This form of BPC, gdBPC, was centrifuged with BTP and elTM at a series of calcium chloride concentrations. gdBPC had very limited interaction with BTP/elTM (Figure 15). The dissociation constant between gdBPC and elTM/BTP is shown at the bottom of Table 5. The removal of the Gla domain resulted in nearly a 100-fold increase in the dissociation constant over that of BPC with the elTM/BTP complex.

The effect of the Gla peptide on the interaction of thrombin and elTM with protein C

Purified Gla peptide from the chymotrypsin digestion of BPC was used to assess whether it might bind directly to thrombin and elTM. The peptide has a molecular weight, as determined from the amino acid sequence, of 4,860 g/mol. Table 6 shows the results of short column sedimentation equilibrium of the Gla peptide in 0 mM calcium chloride. The peptide is in a monomer-dimer equilibrium. In the following experiments where the peptide was used, it was considered to be fully dimerized.

When the Gla peptide was included with equimolar mixtures of BTP and BPC or BTP and BAPC, the z-average molecular weight
Figure 15: The binding of BPC and gdBPC to the eITM/BTp complex - the z-average molecular weight of the complexes formed as a function of the Ca\(^{2+}\) concentration. The three proteins were mixed in equimolar concentrations (8 μM for the BPC experiment and 7 μM for the gdBPC experiment) and subjected to short column sedimentation equilibrium. Each data point represents a simultaneous fit of data collected at three speeds: 15, 20 and 24 thousand rpm.
gdBPC and BPC Binding to eITM-BT

\( M_z \) vs. CaCl\(_2\) Concentration

- ■ gdBPC
- ▲ BPC

![Graph showing \( M_z \) vs. CaCl\(_2\) concentration](image)

*Figure 15.*
Table 6: Properties of the BPC Gla peptide as determined from sedimentation equilibrium in Ca\(^{2+}\) depleted buffer. Modelled as an ideal monomer and as a monomer-dimer equilibrium.

<table>
<thead>
<tr>
<th>Conc. (μM)</th>
<th>Speed (x10^3 rpm)</th>
<th>M_\text{r} (g/mol)</th>
<th>K_d (μM)</th>
<th>rms (fr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.0</td>
<td>40.48</td>
<td>9,000</td>
<td>und</td>
<td>0.0085</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(8,200-9,900)</td>
<td></td>
</tr>
<tr>
<td>4,860*</td>
<td>4.53</td>
<td>0.0084</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(2.90-6.82)</td>
<td></td>
</tr>
</tbody>
</table>

*Cell loading concentration of peptide. *Simultaneous fit to data acquired at the listed rotor speeds. Molecular weight determined and the 65% confidence interval (in parentheses). Monomer-dimer dissociation constant determined and the 65% confidence intervals (in parentheses). Square root of the variance of fit in units of fringes. und = undetermined. *This value was held constant during the fit.
decreased (Figure 16). This is consistent with the idea that BPC and BAPC are being competed off BTp by the peptide. Unfortunately, it is not possible to distinguish whether one, or both of the protein C molecules are competing with the Gla peptide for a binding site. It is unlikely that the Gla domain is solely responsible for the interaction of BPC (or BAPC) with BTp, since excess peptide failed to completely eliminate the BTp/BPC (or BAPC) interaction. If the interaction had been eliminated, one would have expected a z-average molecular weight of 50,300 g/mol. Furthermore, there may be Gla domain specificity since the z-average molecular weight of the BTp/BPC mixture decreased from 115,000 g/mol with no peptide, to only 105,000 g/mol when 45 \( \mu \)M of Factor X Gla peptide was used.

The situation was markedly different when the BPC Gla peptide was included with equimolar mixtures of elTM and BTp or elTM and BAPC (Figure 17). The z-average molecular weight of the elTM/BTp mixture increased by about 10,000 g/mol with the addition of 8 \( \mu \)M peptide and did not change with the addition of more peptide. This molecular weight gain is what would be anticipated if the elTM/thrombin complex is binding one Gla peptide dimer. Thus, the result suggests that the elTM/BTp complex can bind only one BPC molecule. The interaction between elTM and BAPC, as indicated by the z-average molecular weight, showed very little change upon addition of the Gla peptide. The fact that there was little
Figure 16: The effect of the Gla peptide on the z-average molecular weight of mixtures of BTP/BPC and BTP/BAPC. Equimolar mixtures of BTP and BPC (5 μM) and, BTP and BAPC (5 μM) were titrated with the indicated concentrations of Gla peptide in 0 mM calcium chloride. Short column sedimentation equilibrium was used to determine the z-average molecular weight. Each data point represents a simultaneous fit to three speeds: 20, 24, and 28 thousand rpm.

Figure 17: The effect of the Gla peptide on the z-average molecular weight of mixtures of eLTM/BTP and eLTM/BAPC. Equimolar mixtures of eLTM and BTP (5 μM) and, eLTM and BAPC (5 μM) were titrated with the indicated concentrations of Gla peptide in 0 mM calcium chloride. Short column sedimentation equilibrium was used to determine the z-average molecular weight. Each data point represents a simultaneous fit to three speeds: 20, 24, and 28 thousand rpm.
Gla Peptide Effect on BTP Complexes
$M_z$ vs. BPC Gla Peptide Conc.

- ▲ - BTP + BPC
- ▼ - BTP + BAPC

Figure 16.

[BPC Gla Peptide] microMolar

Gla Peptide Effect on eITM Complexes
$M_z$ vs. BPC Gla Peptide Conc.

- ▲ - eITM + BTP
- ▼ - eITM + BAPC

Figure 17.

[BPC Gla Peptide] microMolar
competition reflects the weakness of the elTM/BAPC interaction. Recalling the result that no interaction could be detected between elTM and gdBPC (Table 3), the result here suggests that the Gla domain is not directly responsible for the interaction between elTM and BPC. Rather, the removal of the Gla domain from BPC may induce a conformational change in BPC which prevents it from interacting with elTM.

The inhibition of BPC activation by BAPC

Results discussed earlier indicated that BAPC might be a very good inhibitor of BPC activation by both thrombin alone and by the elTM/thrombin complex.

This is indeed the case, since it was found that BAPC, inhibited with DEGR, inhibits thrombin activation of BPC with an apparent $K_i$ of around 0.5 μM in 0 mM calcium chloride (the condition for optimal activation of BPC by thrombin). Figure 18 shows a typical Dixon plot for this inhibition. This apparent $K_i$ is approximately identical to the $K_m$ of thrombin for BPC. It is also nearly the same as the $K_i$ of PPACK or DFP inhibited thrombin for BPC (and for BAPC) as determined by sedimentation equilibrium in 0 mM calcium chloride (Figures 8 and 11).

BAPC, inhibited with DEGR, also inhibited the activation of BPC by the elTM/thrombin complex in 0.3 mM calcium chloride (the optimal condition for BPC activation by the elTM/thrombin complex). Figure 19 is a representative Dixon plot for this inhibition. There was a wide range of apparent $K_i$ values of
Figure 18: A Dixon plot for the inhibition by BAPC, (BAPC inhibited with DEGR) of BPC activation by thrombin alone in 1mM EDTA. The initial concentrations of BPC used were 0.4, 0.8 and 1.2 μMolar. Each point represents the average of duplicate determinations. Standard deviations were smaller than the size of the points.
BAPC\textsubscript{i} Inhibition of BPC Activation by Thrombin

- 0.4 $\mu$M BPC
- 0.8 $\mu$M BPC
- 1.2 $\mu$M BPC

Figure 18.
Figure 19: A Dixon plot for the inhibition of BPC by BAPC, of BPC activation by the eITM/thrombin complex in 0.3 mM calcium chloride. The initial concentrations of BPC used were 0.1, 0.2, 0.4 and 0.8 μMolar. Each point represents the average of duplicate determinations. Standard deviations were smaller than the size of the points.
BAPC<sub>i</sub> Inhibition of BPC Activation by the Thrombin/eITM Complex

- 0.1 μM
- 0.2 μM
- 0.4 μM
- 0.8 μM

Figure 19.
from about 3.0-5.0 μM. Also, the points on the graph in Figure 19 for 0 μM BAPC, were omitted as they resulted in far too low an initial rate. Control experiments later showed that the BPC added to the reaction mixtures was sticking to the plastic tubes. BPC adhesion to the tubes was not noted for the activation of BPC by thrombin alone, because no calcium chloride was involved in the reaction. The $K_i$ (0.5-1.0 μM) of BAPC, for the elTM/thrombin complex is in good agreement with the $K_i$ of BAPC, for the elTM/BTd complex in 0 mM calcium chloride as determined by sedimentation equilibrium.

**Human versus bovine protein C**

The study of differences in the interactions of the bovine and the human protein C species with elTM and thrombin is incomplete.

Figure 20 shows the effect of Ca$^{2+}$ on the interaction of BTp, elTM, and human protein C. The calcium ion dependence appears similar to that seen when using bovine protein C (compare with Figure 13). However, it also appears that the interaction of HPC with thrombin and elTM is not as strong as compared to the interaction of bovine protein C with thrombin and elTM.

The following data helps to explain the latter observation in that human protein C does not appear to interact as strongly as bovine protein C with either thrombin or elTM (Table 7). The interaction of thrombin and protein C was modeled as a monomer-dimer-tetramer. That is, as a system
Figure 20: The binding of HPC to the elTM/BTp complex - the z-average molecular weight of the complexes formed as a function of the Ca\textsuperscript{2+} concentration. The three proteins, human protein C, BTP and elTM were mixed in equimolar concentrations (7.1 \mu M each) and subjected to short column sedimentation equilibrium. Each data point represents a simultaneous fit of data collected at three speeds: 15, 20 and 24 thousand rpm.
Figure 20.

HPC Binding to eITM-BTp

$M_z$ vs. CaCl$_2$ Concentration

\[ M_z \begin{align*} \text{[CaCl}_2\text{]}_{\text{total}} & \quad \text{mM} \\ \end{align*} \]
Table 7: The interaction of human protein C with BTP and elTM

<table>
<thead>
<tr>
<th>Protein</th>
<th>Conc. ( \mu M )</th>
<th>Buffer Addition ( \text{mM} )</th>
<th>Speed ( x10^3 \text{ rpm} )</th>
<th>( M_r ) ( \text{g/mol} )</th>
<th>( K_d ) ( \mu M )</th>
<th>( \text{rms} ) ( \text{fr} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPC + BTP</td>
<td>7.5</td>
<td>2.0 EDTA</td>
<td>15,20</td>
<td>49,300 ( ' )</td>
<td>11.5 ( (10.2 - 12.9) )</td>
<td>0.019</td>
</tr>
<tr>
<td>0.3 ( Ca^{2+} )</td>
<td>15,20</td>
<td>49,300 ( ' )</td>
<td>79.4 ( (63.7 - 98.9) )</td>
<td>0.027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 ( Ca^{2+} )</td>
<td>15,20</td>
<td>49,300 ( ' )</td>
<td>510 ( (425 - 611) )</td>
<td>0.050</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPC + elTM</td>
<td>4.5</td>
<td>2.0 EDTA</td>
<td>15,20</td>
<td>47,900</td>
<td>250 ( (47,000-49,000) )</td>
<td>( (190 - 320) )</td>
</tr>
<tr>
<td>0.3 ( Ca^{2+} )</td>
<td>15,20</td>
<td>49,200</td>
<td>250 ( (48,300-50,100) )</td>
<td>( (200 - 310) )</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>5.0 ( Ca^{2+} )</td>
<td>15,20</td>
<td>51,500</td>
<td>310 ( (50,600-52,500) )</td>
<td>( (260-430) )</td>
<td>0.021</td>
<td></td>
</tr>
</tbody>
</table>

\( ' \) Cell loading concentration of each protein. \( ' \) Proteins dialyzed against buffer A containing the addition listed. \( ' \) Simultaneous fit to data acquired at the listed rotor speeds. \( ' \) Molecular weight determined and the 65\% confidence interval (in parentheses). \( ' \) Dissociation constant between the two proteins in the experiment and the 65\% confidence interval (in parentheses). \( ' \) Square root of the variance of fit in units of fringe displacement. This value was held constant during the fit.
consisting of free thrombin and protein C, plus 1:1 and 2:2 thrombin:protein C complexes. From the more extensive studies done with bovine protein C and thrombin, this is probably not the correct model. However, as will be noted in the discussion section, the selection of the stoichiometry for the second complex does not appear to change the value of the 1:1 complex dissociation constant. So, for the interaction of human protein C with thrombin in Table 7, only the first dissociation constant is presented.

The human protein C interaction with e1TM was of 1:1 stoichiometry and relatively Ca²⁺-independent like the interaction of bovine protein C with e1TM. However, the dissociation constant for the human protein C interaction with e1TM was nearly four fold weaker.
DISCUSSION

The activation of protein C by thrombin and elTM is complex (Kurosawa et al., 1987). The studies done here suggest that the activation of protein C by thrombin alone is particularly complicated due to the presence of a 1:2 thrombin:protein C interaction in addition to the 1:1 interaction. Ca$^{2+}$ further complicates the interactions of the three proteins in the protein C activation complex. It appears that the interaction of primary importance is that between protein C and thrombin, and that the Gla domain of protein C plays the significant role in the interactions of protein C with thrombin and with elTM. The biggest surprise is that activated protein C is a potent inhibitor of protein C activation by thrombin alone and by thrombin and elTM. In addition, there seems to be a species dependence on the interaction of human versus bovine protein C with thrombin and elTM.

The effect of Ca$^{2+}$ on the interactions of the three proteins in the protein C activation complex

There are four basic combinations that thrombin, elTM and protein C can form. They are the thrombin and elTM, the thrombin and protein C, the elTM and protein C, and the thrombin, elTM and protein C combinations. Each of these mixtures was examined as a function of the calcium ion concentration.
Thrombin and elTM - Kinetic studies have revealed that the interaction of thrombin and elTM is strong; a result which was verified here. This 1:1 elTM:thrombin complex is strong enough in the absence of Ca$^{2+}$ that it was considered as a single protein component when modeling the behaviour of the thrombin:elTM:protein C mixture. The addition of Ca$^{2+}$ weakened the interaction to detectable levels at 5 mM.

However, though this weakening may not be sufficient to explain the decrease in the rate of activation of protein C by thrombin and elTM with increasing concentration of Ca$^{2+}$, this result has an impact on the conclusions that can be drawn from protein C activation studies done in the presence of millimolar amounts of Ca$^{2+}$. An assumption made in many of the kinetic studies examining this calcium ion dependence of activation is that there is no change in the affinity of thrombin for elTM (for example, Kurosawa et al., 1987), or for even smaller fragments of elTM which still retain cofactor activity (Hayashi et al., 1990). The $K_d$ of thrombin for elTM measured by sedimentation equilibrium was found to be $\approx 4 \mu$M in 5 mM Ca$^{2+}$. None of the kinetic studies cited above contain enough thrombin and elTM (or other catalytic fragment in place of elTM) to absorb this change in affinity. In fact, nearly all the elTM and thrombin is dissociated at the higher calcium concentrations studied.
Protein C and elTM - The protein C:elTM interaction has 1:1 stoichiometry, a $K_d$ of around 60 $\mu$M in EDTA or Ca$^{2+}$ depleted buffer, and is relatively unaffected by the addition of Ca$^{2+}$. The protein C:elTM interaction is not a likely candidate for the decrease in the protein C activation rate since this interaction is relatively Ca$^{2+}$ independent.

Protein C and thrombin - The protein C:thrombin interaction is an interaction that is perturbed significantly upon the addition of Ca$^{2+}$. The addition of small amounts of Ca$^{2+}$ (<0.3 $\mu$M) has an immediate effect on the weakening of the interaction between protein C and thrombin.

Unfortunately, the picture is complicated by the presence of an additional complex which has been identified as a thrombin:protein C complex with 1:2 stoichiometry. As indicated in the Results section, a model incorporating 1:1 and 1:2 thrombin:protein C complexes fits the data the best. The next best model was one which included a 1:1 thrombin:protein C complex along with a dimer (2:2) of this complex. What is most interesting about the comparison of these two models is that both of them returned approximately similar association energies for the 1:1 thrombin:protein C complex. There are several reasons why it is difficult to get a firm idea of what the higher order complex may be: 1) there is not as much of it relative to the other complex; and, 2) its presence is detected primarily in regions of the centrifuge cell having the higher concentration gradient, a
Thrombin, elTM and protein C - The interaction of the thrombin:elTM:protein C complex has 1:1:1 stoichiometry and the effect of Ca^{2+} on this complex is nearly identical to the effect of Ca^{2+} on the thrombin:protein C complex. There is no evidence for complexes of any other stoichiometry. The similarity of these results suggests that Ca^{2+} is somehow similarly affecting the affinity of protein C for thrombin and the thrombin:elTM complex. This 1:1:1 stoichiometry also suggests that elTM may be competing with protein C for a site on thrombin.

It was possible to determine a K_d for the affinity of protein C for the thrombin:elTM complex in EDTA or Ca^{2+}-depleted buffer, because the thrombin:elTM complex can be modeled as a single protein under these conditions. The K_d was around 1 μM which is equivalent to an association free energy of ≈ -8.0 kcal/mol. The association free energy for thrombin and protein C is ≈ -7.5 kcal/mol, and for elTM and protein C is ≈ -5.5 kcal/mol. Thus, the binding of protein C to thrombin and elTM is apparently noncooperative.

The role of the Gla domain in the interaction of protein C with thrombin and elTM

As mentioned in the Introduction, protein C contains two known intramolecular regions that bind Ca^{2+}: a β-hydroxy aspartic acid residue in the epidermal growth factor-like domain, and an amino terminal stretch of residues that
contains from 10 to 12 γ-carboxyglutamic acid residues - the Gla domain. The affinities for Ca$^{2+}$ are $\approx 50$ µM and $\approx 200$ µM, respectively. Kinetic studies have shown that the Gla domain is essential for the accelerated rate of protein C activation over that of Gla-domainless protein C activation.

Sedimentation equilibrium experiments of Gla-domainless protein C with the eI71:thrombin complex are in agreement with these results and show that the affinity of Gla-domainless protein C for the complex is over 100-fold weaker than the affinity of intact protein C for the complex. In addition, the removal of the Gla domain from protein C eliminates further weakening of the interaction by Ca$^{2+}$.

When the protein C Gla peptide was included in a mixture of protein C and thrombin, the z-average molecular weight of the mixture dropped, probably because protein C was being competed off thrombin by the Gla peptide. In a mixture of eI71 and thrombin, the z-average molecular weight increased, presumably because the Gla domain was binding to the thrombin:eI71 complex. Both of these experiments suggest that the Gla domain is involved directly in the interaction of protein C with thrombin and with the thrombin:eI71 complex. The Gla domain may also be involved indirectly in these interactions.

All of this suggests an extremely important point. It has been hypothesized by a number of authors that the interaction of protein C with eI71 is via a Ca$^{2+}$ bridge (Kurosawa et al.,
This is based on the fact that there is no detection of protein C activation by the thrombin:eLTM complex in EDTA, and that this rate increases with the addition of Ca\(^{2+}\) up to \(\approx 0.3\) mM, thereafter decreasing with the addition of Ca\(^{2+}\), presumably due to the dissolution of the Ca\(^{2+}\) bridge. In light of the results from sedimentation equilibrium, the model must be modified, because there are clearly strong interactions in the absence of Ca\(^{2+}\). Thus, since the recognition of the proteins by one another is at maximum strength in the absence of Ca\(^{2+}\), it may be only that a Ca\(^{2+}\)-induced conformational change is required for the maximal activation rate.

For the interaction of eLTM with thrombin and protein C, the role of eLTM may include, in addition to the elimination of one of two possible sites on thrombin for protein C, a means for forcing protein C to adopt a structure on thrombin that is optimal for activation. It is not difficult to imagine a scheme which might result in the acceleration of protein C activation by the thrombin:eLTM complex over the rate of activation of protein C by thrombin alone.

While it remains unclear what is going on at low Ca\(^{2+}\) concentrations, at the higher concentrations it is likely that the Gla domain is no longer able to interact with thrombin. The direct involvement of the Gla domain with thrombin may be via either one or both of the basic residue patches located near the active site cleft of thrombin (Bode et al., 1989),
and the weakening of the interaction due to Ca$^{2+}$ may be a result of the elimination of this electrostatic interaction. **Activated protein C is a potent inhibitor of protein C activation**

The most surprising discovery during the course of this research is that activated protein C is a potent inhibitor of protein C activation. This was first suggested by experiments which examined the interaction of protein C and activated protein C with PPACK inhibited thrombin. No great difference in the interactions was noted. This lead to the idea that PPACK may be interacting with a number of residues in the thrombin active site that are necessary for the discrimination between protein C and activated protein C by the thrombin active site cleft. In particular, the L-arginine residue of PPACK blocks the arginine pocket of thrombin, and the L-proline is held in a hydrophobic cage formed by the D-phenylalanine of PPACK and a number of amino acid residues of thrombin that define the active site cleft (Bode et al., 1989). Thus, three subsites of the active site cleft of thrombin are interacting with PPACK, and this means these three subsites cannot interact with protein C or activated protein C. Therefore, another much smaller inhibitor was used, diisopropylfluorophosphate.

Similar results to those obtained with PPACK-inhibited thrombin were observed. Diisopropylphosphate is bound to the active site serine of thrombin and probably also blocks the
arginine pocket, or subsite 1, of thrombin. Unlike PPACK, this molecule probably does not interact significantly with amino acids in the other subsites of thrombin that define the active site cleft.

When the protein C and activated protein C interactions were examined with thrombin and eITM, there was slightly more discrimination between protein C and activated protein C. This was reflected in the kinetic studies, since activated protein C is a better inhibitor of protein C activation by thrombin alone than by the thrombin:eITM complex. It is not clear how eITM is interacting with thrombin and protein C to bring about this discrimination.

It has been shown that the sequence for optimal thrombin cleavage on the carboxyl terminal of arginine is P4-P3-Pro-Arg-P1'-P2', where P4 and P3 are hydrophobic residues and P1' and P2' are nonacidic residues (Chang, 1985). In protein C all these requirements are met except that P3 is an aspartic acid residue. Protein C is not a good substrate for thrombin without the cofactor thrombomodulin or eITM. The role of eITM may be to eliminate the need for a good interaction of the P3 residue with subsite 3 of thrombin.

The physiological implications of this inhibition of protein C activation are unclear at present. These results may not be relevant to the activation of protein C by thrombin and thrombomodulin in vivo.
Effect of human versus bovine protein C on the interactions with thrombin and elTM

The study of the interaction of human protein C with thrombin and elTM are by no means complete. However, it does appear that the interaction of human protein C with both thrombin and elTM is not as strong as when bovine protein C is used. Both human and bovine protein C contain the same functional domains though their sequences are not identical. Since the structure of protein C probably plays an important role in the interactions with thrombin and elTM, even a small difference in sequence identity could give rise to the observed interaction differences between human and bovine protein C.

Kinetic studies have not been done with human protein C, thrombin and elTM. Sedimentation equilibrium results predict that human protein C would not be as good a substrate as bovine protein C for activation by thrombin and elTM.

Summary

The study of protein interactions is fundamental to the understanding of biochemical properties. Recently, a review article discussed the nature of protein-protein interactions. From an examination of x-ray crystallographic structures of protease-inhibitor complexes it was found that there are around thirty close-contact residues and about ten hydrogen bonds involved in the overall interaction (Janin and Chothia, 1990). Thus, protein-protein interactions are complicated, and the interactions in the protein C activation enzyme complex
are no exception.

Unfortunately, not all proteins or complexes of proteins can be studied by x-ray crystallography. In this study of the protein C enzyme complex, the utility of analytical ultracentrifugation as a technique for the survey and study of protein-protein interactions has been demonstrated. The direct involvement of the Gla domain in the interaction with both thrombin and with elTM is required for optimal protein C activation. The effect of Ca\(^{2+}\) on the rate of protein C activation was somewhat clarified. Finally, the possibility of potent product inhibition was predicted by analytical ultracentrifugation and demonstrated with kinetics.
The work undertaken to study the enzyme complex with the analytical ultracentrifuge described in this dissertation could not have been completed without some modifications made to the Beckman Model E analytical centrifuge.

The fundamental measurement made with the use of the centrifuge is that of concentration as a function of radial position. There are three optical methods that have been designed to do this, and they are the schlieren, absorbance and interference optical systems. While each of these systems has its own advantages, absorbance and interference optics are currently the most utilized.

Absorbance optics permits the use of very low concentrations of proteins, and the measurement of absolute concentration at any position. It is also possible to use it to observe specific proteins in a mixture, provided that the extinction coefficient is known and doesn't change with association. This is not the typical situation. In addition, absorbance measurements are easily automated.

However, measurements made with interference optics are more precise than those made using absorbance optics. Furthermore, the refractive index of proteins is relatively independent of size and association. Used together, the absorbance and interference measuring systems would be powerful. Unfortunately, at this time, it is not possible to
examine a mixture of proteins with both methods during a single centrifuge run.

Despite the advantages of the interference optical system (IOS), it has not been used extensively for the study of macromolecular associations because of the difficulty of gathering and analyzing data. Typically, photographs of interferograms are taken and the necessary measurements are made from these photos manually or with an automated measuring device. It takes several hours to read a single channel of data. The automated reader requires only twenty to thirty minutes. However, even this amount of time is prohibitively long, since most studies of interest demand a large data set. It is also an advantage to be able to examine data as it is collected without having to prepare photographs. For example, it is then possible to verify quickly during an experiment that sedimentation equilibrium has been reached. This is done by subtracting data taken at later times from data taken at earlier times. Thus, a real time, automated IOS was developed based on previous work (for example, Laue, 1981).

A block diagram of the modified real time IOS for the Model E (A) is shown in Figure 20. This system can be completely operated from a computer terminal. A computer (B) receives a rotor timing pulse from (C), and controls laser (D) synchronization with the rotor via a laser pulse controller (E). A solid state TV camera (F) sits on a translation stage
Figure 21: The block diagram of the Model E analytical ultracentrifuge and the real-time interference optical system. See text for full discussion. (A) Model E, (B) computer, (C) rotor timing circuit, (D) laser, (E) laser pulse controller, (F) TV camera, (G) translation stage, and (H) video monitor.
The Modified Model E Analytical Ultracentrifuge

Figure 21.
that is moved by a micrometer driver (G). The interferogram is displayed on a video monitor (H) from which the computer reads frames of data. At the time this research was done, the laser pulse controller was not yet installed.

The development of a real-time IOS allowed us to exploit the advantages of interference optics, without the difficulties mentioned above. Using this system, a single channel of data can be read in 10-15 seconds.
LIST OF REFERENCES


