Protein C

Product Monograph 1995

CHROMOGENIX

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Protein C

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Preface

The blood coagulation system is carefully controlled in vivo by several anticoagulant mechanisms, which ensure that clot propagation does not lead to occlusion of the vasculature. The protein C pathway is one of these anticoagulant systems. During the last few years it has been found that inherited defects of the protein C system are underlying risk factors in a majority of cases with familial thrombophilia. The factor V gene mutation recently identified in conjunction with APC resistance is such a defect which, in combination with protein C deficiency, increases the thrombosis risk considerably.
The Chromogenix Monographs [Protein C and APC-resistance] give a didactic and illustrated picture of the protein C environment by presenting a general view of medical as well as technical matters. They serve as an excellent introduction and survey to everyone who wishes to learn quickly about this field of medicine.

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Determination of Protein C Activity with Snake Venom and S-2366™

Protein C is vitamin K-dependent inactive precursor of a serine protease (APC), which is the key component in a physiologically important natural anticoagulant system. Genetic defects associated with this system are the major underlying cause of familial thrombophilia. This monograph reviews the biochemistry and clinical implications of protein C and presents a functional protein C assay using a snake venom activator and the chromogenic substrate S-2366™.

Blood coagulation is an enzymatic event initiated in response to tissue damage. The binding of coagulation factor VII to exposed tissue-factor starts a cascade of reactions that ultimately leads to the formation of thrombin (Figure 1).[1]

Thrombin is the central enzyme in the coagulation system. It clots blood by converting soluble fibrinogen to clot-forming fibrin monomers and activates factor XIII leading to the strengthening of the clot by cross-linking. Thrombin also provides a link between the coagulation cascade and cell-mediated hemostasis by activating platelets. Furthermore, via a positive feed-back reaction thrombin accelerates its own generation by the activation of factors V and VIII. These proteins bind to negatively charged phospholipids on the surface of activated platelets and serve as cofactors for factors IXa and Xa, respectively. The total effect is a dramatic burst of thrombin and resulting fibrin deposition at the site of injury.

It is obvious that the autocatalytic nature of thrombin would, if unchecked, clot the blood content of a person within minutes. Anticoagulant control in humans is primarily achieved by two principally different mechanisms. A direct type of regulation is provided by a group of circulating enzyme inhibitors of which the two, antithrombin and the tissue factor pathway inhibitor, are of particular importance. The second type of anticoagulant mechanism is associated with intact blood vessels and is initiated by thrombin itself. When thrombin binds to the protein thrombomodulin on the surface of endothelial cells, a remarkable transformation takes place that removes most of its procoagulant functions. Instead, thrombin becomes a potent activator of protein C, the key component in the protein C anticoagulant system.[2,3] Together with protein S and factor V as potentiating cofactors, the activated protein C (APC) effectively degrades membrane-bound factors Va and VIIIa, thus limiting further coagulation.

The physiological importance of the protein C anticoagulant system is demonstrated clinically by the diseases associated with it.[2-5] Severe thrombosis known as purpura fulminans may occur in neonates with a rare homozygous protein C deficiency, whereas individuals with the more common heterozygous protein C deficiency may suffer an increased risk of venous thrombosis later in life. Inherited protein S deficiency has a similar risk and is found to be equally frequent, about 2-5%, in cohorts of thrombosis patients.[3] Of great interest recently is the described mutation in the factor V gene. The defect results in resistance to APC and is reported to be the major cause of familial thrombosis.[3]

The laboratory evaluation of this natural anticoagulant system is clearly of utmost importance. In this monograph, we describe various assay methods for protein C and present an assay kit; COAMATIC® protein C, highly suitable for the routine clinical laboratory.

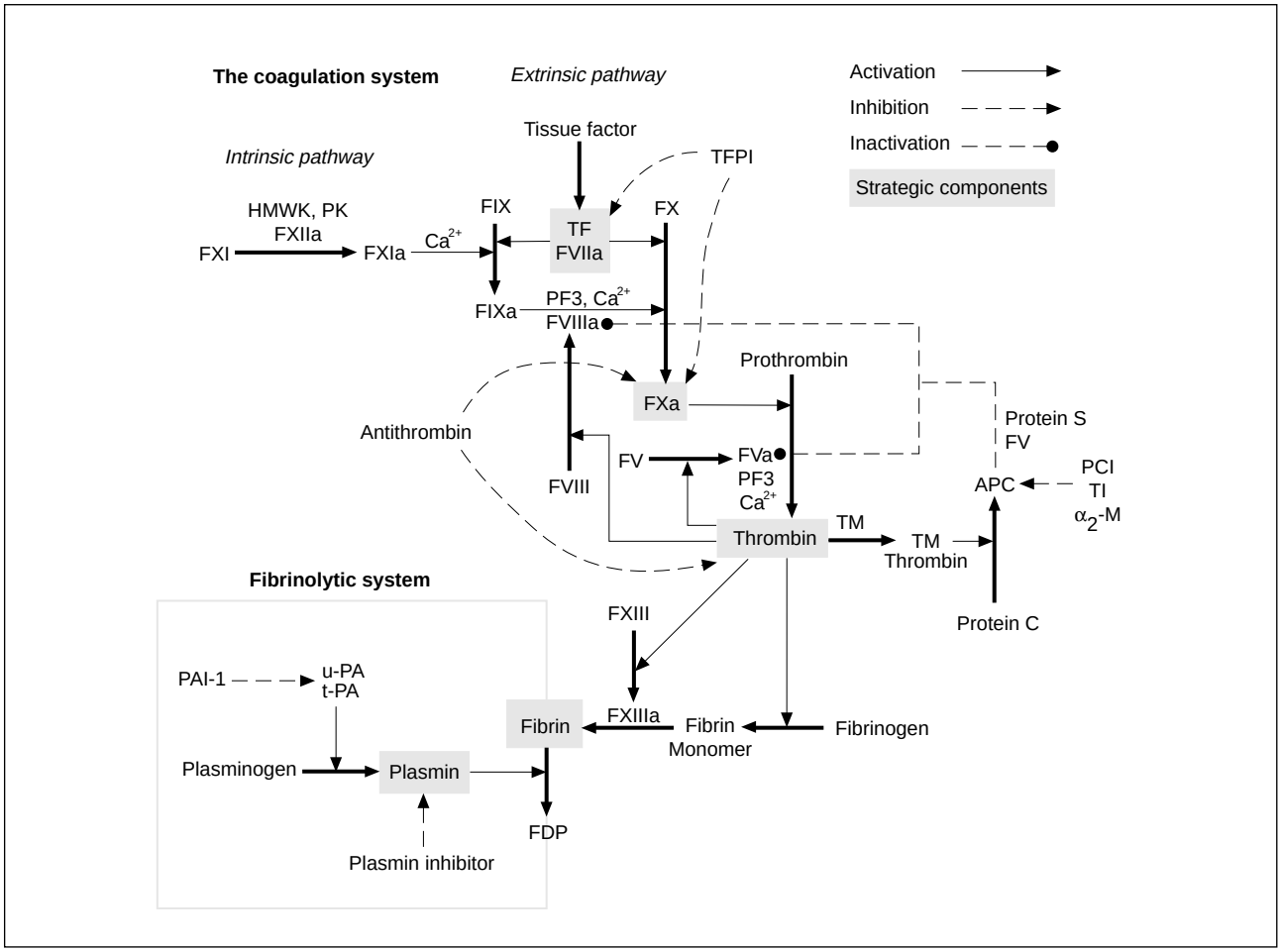


Figure 1. The coagulation system. *Coagulation factors are represented by their Roman numerals (a = activated). Abbreviations: HMWK= high molecular weight kininogen, PK= prekallikrein, K= kallikrein, TF= tissue factor, TFPI= tissue factor pathway inhibitor, PF3= phospholipid, TM= thrombomodulin, PC= protein C, APC= activated protein C, PCI= protein C inhibitor, TI= trypsin inhibitor, α_2M= α_2-macroglobulin, FDP= fibrin degradation products.*

Factor	Name	Size [kDa]	Concentration [µg/ml]	Factor	Name	Size [kDa]	Concentration [µg/ml]
I	Fibrinogen	340	3000	IX	Christmas factor	55	5
II	Prothrombin	69	100	X	Stuart-Prower factor	59	8
III	Tissue factor	47	-	XI	Thromboplastin antecedent	160	5
IV	Calcium	-	-	XII	Hageman factor	80	30
V	Proaccelerin	330	10	XIII	Fibrin-stabilizing	320	10
VI	-	-	-	-	Tissue factor	37	-
VII	Proconvertin	48	0.5	-	Antithrombin	58	150
VIII	Antihemophilic factor	330	0.1	-	Heparin cofactor II	66	91

Table 1. *Coagulation factors and regulatory proteins.*

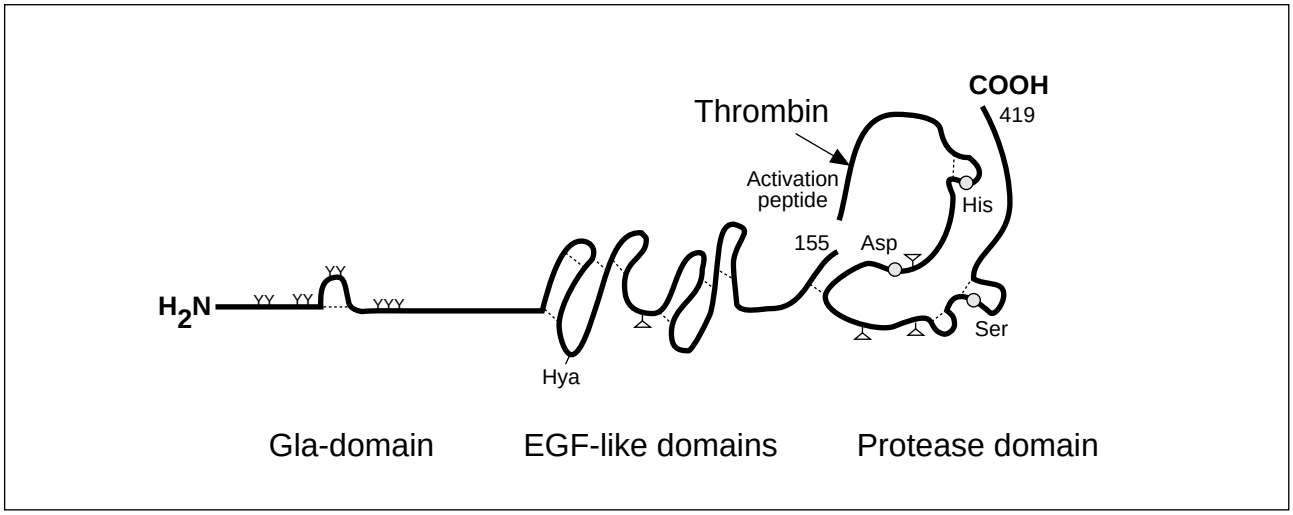


Figure 2. *Domain structure of human protein C. Symbols: Y = Gla-residues, Hya = erythro-β-hydroxyaspartic acid, O = catalytic residues, Δ = N-linked glycosylation sites*

Protein C biochemistry

Structure and functional aspects

Protein C is synthesized in the liver as a 461 amino acids long single chain precursor. Prior to secretion, it is processed into a disulfide-linked two chain polypeptide containing, one light (155 amino acids) and one heavy chain (262 amino acids). The molecular weight of the mature protein is approximately 62,000 daltons and its carbohydrate content about 23%. It circulates in human plasma at a concentration of 3-5 μg/ml and its half-life is 6-8 hours. Protein C is an inactive pro-enzyme or zymogen of a serine protease (APC).

From information based upon the protein C gene structure and amino acid sequence homologies with other proteins, it is clear that protein C contains several regions with discrete structural or functional properties (Figure 2).[2,3,6]

The amino terminal light chain consists of a *Gla-domain*, with nine glutamic acid residues that are γ-carboxylated in a vitamin K-dependent reaction in the liver. This region interacts with negatively charged phospholipid in the presence of calcium ions (Ca^{2+}) and is a prerequisite for the anticoagulant function of APC. The Gla-domain is followed by *two EGF-like domains*, which appear to interact with protein S. Together with the Gla-domain these domains are also important for the binding of protein C to the thrombin-thrombomodulin complex.

The heavy chain contains the *serine protease domain* with the 'catalytic triad' composed of histidine, aspartic acid and serine (located at position 211, 257 and 360, respectively). These amino acid residues are situated relatively far apart from each other in the primary structure, but are in close proximity in the folded protein. APC cleaves substrates with arginine in the P1 position, whereas the amino acid composition in the P2 and P3 positions are more varied (Figure 3). It is not known which structural features give APC its narrow specificity for factors Va and VIIIa although, it probably involves a secondary binding site located outside the catalytic center. Recent studies using synthetic peptides indicate that the amino acid residues 311-325 and 390-404 in APC are important for its anticoagulant interaction with substrates.[3]

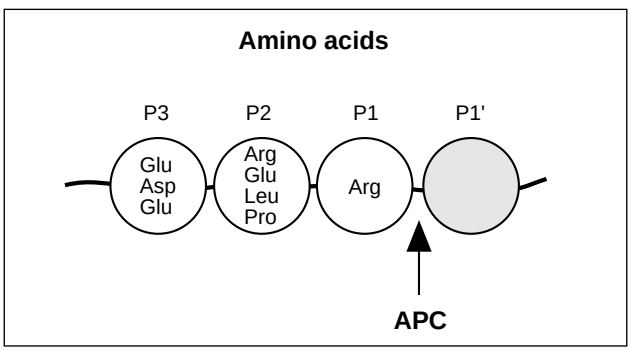


Figure 3. *The cleavage-site composition of APC's natural substrates, factors Va and VIIIa.[3]*

Protein C activation

Protein C must be transformed to an active serine protease to be physiologically functional. The activation of human protein C occurs through the enzymatic removal of a small activation peptide from the aminoterminus of the heavy chain.[7] In vivo, the activation is mediated by the thrombin-thrombomodulin complex.[8,9] In vitro, the activation can be achieved using thrombin alone, trypsin,[10] or proteases from various snake venoms.[11,12]

Thrombin-Thrombomodulin

Activation of protein C by thrombin alone is slow and has no physiological function. However, when thrombin binds to the integral membrane protein thrombomodulin (TM), present on the vascular endothelium, the result is a 20,000-fold increase in the rate by which thrombin activates protein C. TM also removes the procoagulant properties of thrombin and accelerates the thrombin-antithrombin reaction by a glycosaminoglycan moiety, identified as chondroitin sulfate.[13] Ca^{2+} is an important regulator that enhances protein C activation by the thrombin-TM complex, but inhibits the activation by thrombin.[14]

The mature TM molecule consists of 557 amino acids residues arranged in a lectin-like domain, six EGF-like domains, a Ser/Thr-rich domain, a transmembrane domain, and a short cytoplasmic tail (Figure 4). TM has a widespread distribution in the mammalian organism and with few exceptions, is expressed by endothelial cells of arteries, veins, capillaries, and lymphatic vessels. The highest concentration of TM is found in the capillaries, where the ratio of endothelial surface to the volume of circulating blood is high. Thrombin entering the microcirculation will therefore be extracted rapidly by TM and protein C will be activated. This is physiologically important as even small amounts of thrombin would generate enough fibrin to stop the blood flow in the microvasculature.

Snake venoms

A number of snake venoms have been reported to contain protein C activating activity particulary from

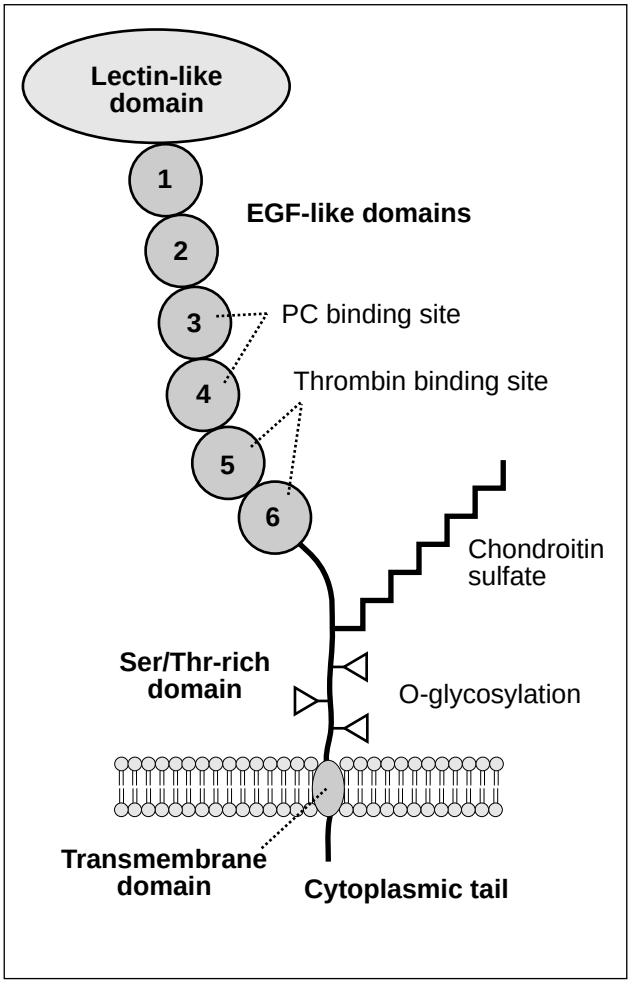


Figure 4. *Domain structure of thrombomodulin*

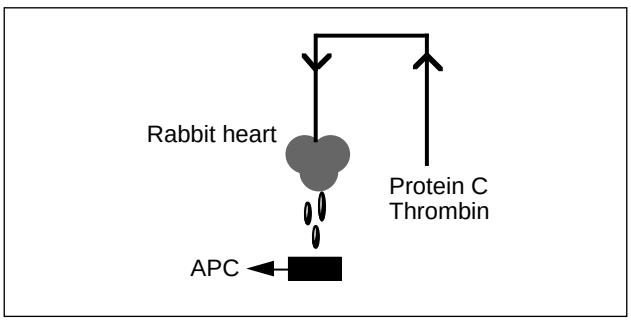


Figure 5. *In the experiments that lead to the discovery of thrombomodulin (Esmon and Owen 1981), the capillary bed of a rabbit heart (a so-called Langendorff heart preparation) was perfused with protein C and thrombin. Protein C was rapidly activated by thrombin bound to a high affinity receptor on the endothelium. This receptor was named thrombomodulin since it dramatically modulated the substrate specificity of thrombin.*

the Agkistrodon genus. Three serine proteases have been isolated and characterized from venoms of this family including the species of A. bilineatus,[15] A. halys halys,[16] and A. contortrix contortrix.[17,18] The enzymes are fast-acting specific protein C activators, with a single-chain structure in the molecular weight range of 35,000-38,000 daltons.

Function of APC

The anticoagulant function of APC is demonstrated by prolongation of both the prothrombin time and the activated partial thromboplastin time (APTT). APC cleaves and inactivates two membrane-bound procoagulant proteins, factor Va and factor VIIIa, resulting in decreased thrombin generation. Circulating non-activated forms of these proteins (factors V and VIII) are poor substrates for APC. In addition to its anticoagulant function, it has been suggested that APC possesses profibrinolytic activity.[19,20]

APC cofactors

The molecular mechanisms involved in the protein C anticoagulant system is highly complex and so far poorly understood. However, essential for the maximal catalytic efficiency of APC is probably the presence of helper proteins or cofactors, which promote the binding of APC to phospholipid on cellular surfaces and give support to the APC degradation of the membrane-bound factors Va and VIIIa.[3] Two cofactors are now associated with APC, one is the recently discovered cofactor-function of factor V,[21] the other is the established cofactor, protein S.[22]

Protein S

Protein S is a vitamin K-dependent plasma protein, which apart from being an APC cofactor binds a regulatory acute-phase protein of the complement system, C4b-binding protein (C4bBP), and mediates its attachment to phospholipid.[22] Approximately 60% of protein S in plasma is bound to C4bBP. Only the free form of protein S has a APC cofactor function.

Protein S enhances binding of APC to the phospholipid of platelets, platelet microparticles and endothelial cells.[3] In vitro experiments have shown that protein S abrogates factor Xa-mediated protection of factor Va and factor IXa-mediated protection of factor VIII, making the substrates available for APC.[3]

Factor V

A recent observation by Dahlbäck et al. suggests a possible anticoagulant role for factor V.[21] It was found that in the presence of both factor V and protein S, APC degrades its substrates efficiently, whereas APC alone or together with factor V was inefficient. The combination with APC and protein S was less potent than when factor V was also present. This suggets that protein S and factor V function as synergistic cofactors to APC in a multimolecular complex on the membrane surface.

APC inhibitors

APC is inhibited relatively slowly by at least three protease inhibitors in plasma, including protein C inhibitor (also known as PAI-3),[23] trypsin inhibitor,[24] and α_2-macroglobulin.[25] Heparin stimulates only the activity of the protein C inhibitor. The half-life of APC in the circulation is about 10-20 minutes.

Protein C data

Name:	Protein C
Synonyms:	Autoprothrombin II, PC
History:	Described by Seegers et al in 1960, isolated/identified by Stenflo in1976
Concentration:	3-5 µg/ml
Molecular weight:	62,000 daltons
Primary structure:	461 amino acids
Carbohydrate:	23%
Half-life:	6–8 hours
Gene:	11 kb, chromosome 2, position q14-q21, composed of 9 exons and 8 introns
Type:	Vitamin K-dependent zymogen of a serine protease
Function:	APC degrades factors Va and VIIIa
Importance:	Hereditary deficiency of PC is a risk factor for venous thromboembolism

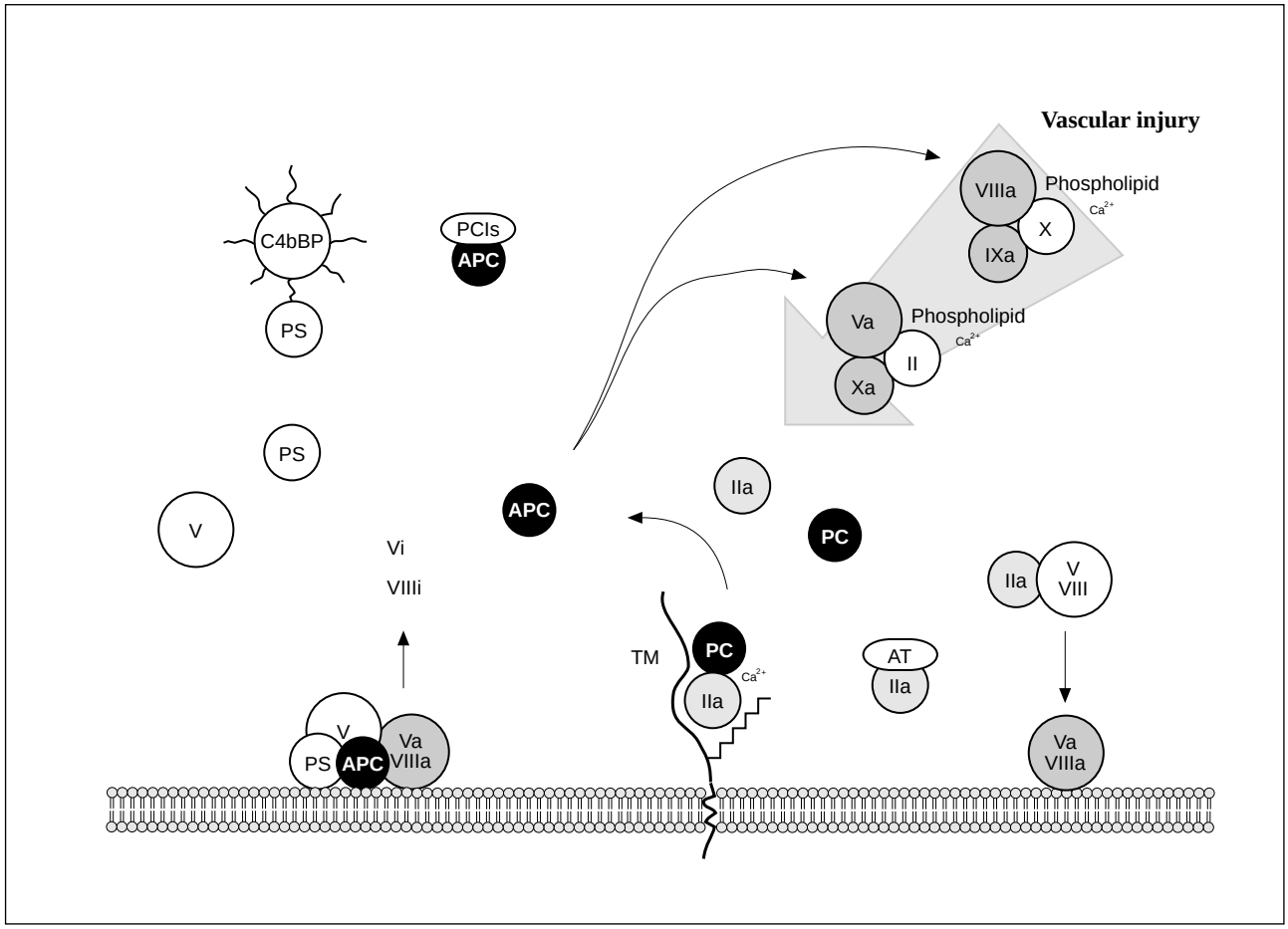


Figure 6. Schematic illustration of the protein C anticoagulant pathway. *Thrombin (IIa) escaping the site of vascular injury binds to a high affinity receptor called thrombomodulin (TM), located on the surface of intact endothelial cells. Thrombin bound to TM looses most of its procoagulant properties instead, it becomes a potent activator of protein C in the presence of calcium ions. Activated protein C (APC) specifically degrades the membrane-bound factors Va and VIIIa, whereas non-activated factors V and VIII are poor substrates for APC. The activity of APC is potentiated by protein S and factor V. These two plasma proteins function in synergy as membrane-bound cofactors to APC and probably also, localize and focus the APC activity to the surface of endothelium and platelets. About 60% of protein S in plasma circulates in a complex with the octupus-shaped C4b-binding protein (C4bBP). Only free protein S functions as a cofactor for APC. APC is slowly neutralized by protein C inhibitors (PCIs). Thrombin bound to TM will be inhibited either by antithrombin (AT) or by endocytosis of the thrombin/TM complex.*

Component	Size [kDa]	Concentration [µg/ml]	Function
Protein C	62	3-5	Zymogen activated by thrombin/TM
Activated protein C	-	-	Cleaves/inactivates factors Va, VIIIa
Protein S	71	20-25	Cofactor for APC
Factor V	350	10	Cofactor for APC
Thrombomodulin	60	0,02*	Cofactor for protein C activation
Protein C inhibitor	57	5	Specific inhibitor of APC
C4b-binding protein	570	150	Binds to protein S

Table 2. *Major components involved in the human protein C anticoagulant system. * Soluble thrombomodulin*

Protein C deficiency

Introduction

Venous thrombosis is a serious medical problem, which anually affects 1 in 1000 people.[26] The pathogenesis of the disease is very likely multifactoral, involving both circumstantial and genetic risk factors. The importance of identifying the underlying genetic cause has been obvious, as up to 40% of patients with thrombosis have positive family histories.[27] The major hereditary disorders of the coagulation system known to predispose for venous thrombosis, include APC resistance and deficiencies of protein C, protein S, and antithrombin. Together these defects may account for up to 60% of cases with familial thrombophilia or about 30% of unselected thrombosis patients.[3,27-32]

Hereditary protein C deficiency

The relevance of evaluating protein C levels in a patient's plasma became clear in 1981, when the first case of hereditary protein C deficiency associated with thrombotic disease was reported by Griffin et al.[33] Several studies have confirmed this initial report and it has been shown that individuals with an isolated protein C deficiency may run a 6 to 9-fold increased risk for venous thrombosis.[34,35]

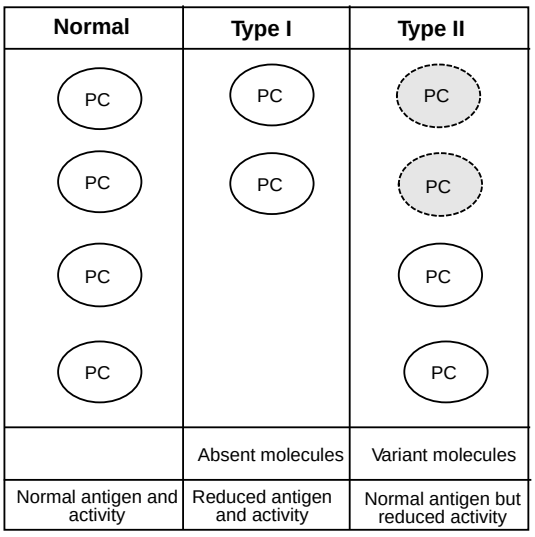


Figure 7. *Classification of protein C deficiency and the criteria for laboratory assessment.*

Defect	Prevalence
APC Resistance	20-30%
Protein C deficiency	2-5%
Protein S deficiency	2-5%
Antithrombin deficiency	1-5%

Table 3. *Prevalence of inherited blood protein defects in thrombosis patients.*

Protein C levels

Hereditary protein C deficiency is inherited as an autosomal dominant trait. Heterozygotes for protein C deficiency have protein C activity or antigen levels of 30 to 70% of normal, whereas homozygotes (or compound heterozygotes) with a severe defect have levels below 1%.[4] Homozygotes with a mild defect have also been reported with protein C levels of 10-24%.[4] The normal range of protein C in the adult is 70% to 130% of a normal plasma pool (defined as 100%).[36]

Prevalence

The prevalence of protein C deficiency is 2-5% in patients with thromboembolic disease.[27,29-31,35] Selected patient-groups with thrombosis occurring at a young age tend to have a higher prevalence (up to 10-15%).[37] Extrapolation into the general population would give an estimate prevalence for *symptomatic* protein C deficiency of one in 20,000 people. However, the identification of *symptom-free* individuals with protein C deficiency in large groups of healthy blood donors put the prevalence at between one in 200 and one in 500.[38,39] The majority of these individuals did not present family histories of thrombosis. It remains to be established whether clinical phenotypes reflect different defects in the protein C gene or if additional risk factors, genetic or circumstantial, are required for expression of the symptomatic phenotype.[39]

Classification

Two types of protein C deficiency states are recognized (Figure 7). In type I deficiency the plasma concentration of protein C is reduced both in func-

tional and immunological assays, reflecting a genetic defect causing a reduced biosynthesis of protein C. Type II deficiency is characterized by normal protein C antigen levels, but with decreased functional activity. This type of defect reflects the synthesis of abnormal molecules with reduced function. Type I deficiency is the most common type of disorder. The mutations in the protein C gene have been characterized in a recently published database.[40]

Clinical features

The most common clinical manifestation of symptomatic heterozygous protein C deficiency is deep venous thrombosis of the lower extremities.[3] The first thrombotic event usually occurs after puberty. Of the affected patients, 50-80% experience the event before the age of 30-45.[3,34] Important circumstantial risk factors known to be associated with the apperance of thrombosis include surgery, immobilization, pregnancy and oral contraceptives (Table 6).[3] However, approximately 50% of all the first thrombotic events and 65% of recurrences occur spontaneously without apparent cause.[3]

In contrast to the heterozygous state, which by itself is a relatively weak risk factor for thrombosis, patients with homozygous protein C deficiency usually suffer from severe and fatal thrombosis in the early stage of life. The clinical picture, known as purpura fulminans, is associated with skin necrosis (due to microvascular thrombosis), thrombosis in the brain, and DIC. The incidence of homozygous or compound heterozygous protein C deficiency is extremly rare (1 in 500,000 births).

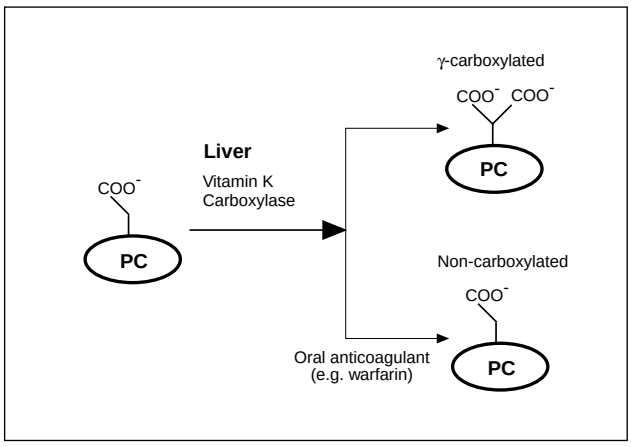


***Figure 6**. Oral anticoagulants interfere with the formation of the g-carboxyglutamic acid moiety (indicated above as COO-) of the protein C molecule during biosynthesis in the liver.*

Clinical manifestations
- Autosomal dominant
- Venous thrombosis of the lower limbs
- Occurence of thrombosis in the microcirculation
- 50% of first events begin before the age of 30-45
- Oral anticoagulant-induced skin necrosis

Laboratory diagnosis
- Low protein C activity levels (< 70% of normal)
- Low antigen levels (type II)
- Normal antigen levels (type I)

Treatment options
- Warfarin or heparin for long-term treatment
- Heparin for acute thrombotic events
- Protein C concentrates for homozygotes

***Table 4**. Features of hereditary protein C deficiency*

Drug-induced skin necrosis

Protein C deficiency is associated with an increased risk of developing skin necrosis in the initial phase of oral anticoagulant therapy with vitamin K-antagonists such as warfarin sodium and bishydroxycoumarin.[4,42] The drugs interfere in the vitamin K-dependent biosynthesis of several proteins in the coagulation system (e.g protein C, protein S, prothrombin, factor VII, factor IX, factor X). Skin necrosis is thought to develop as a result of the relatively short biological half-life ($t_{1/2}$ = 6–8 hours) of protein C compared to those of other vitamin K-dependent coagulation factors ($t_{1/2}$ ›40 hours).[43] During the initial phase of oral anticoagulant therapy this may lead to a sharp decline in protein C activity levels, causing a transient but severe hypercoagulable state.

Acquired protein C deficiency

The Protein C level is influenced by various diseases and drugs (Table 5). Acquired protein C deficiency is often associated with disseminated intravascular

Decreased levels
DIC
Deep vein thrombosis
Pulmonary embolus
Severe liver disease
Post-operative patients
Infection
Malignancy
L-asparaginase therapy
Adult respiratory distress syndrome
Hemolytic uremic syndrome
Thrombotic thrombocytopenic purpura
Oral anticoagulants
Vitamin K-deficiency
Neonatal period
Elevated PC levels
Diabetes
Nephrotic syndrome
Late pregnancy
Oral contraceptives
Anabolic steroids

Table 5. *Conditions and drugs associated with an elevated or decreased protein C level.*

Risk factors for DVT
Surgery
Increasing age
Malignancy
Immobilization
Heart failure or myocardial infarction
Previous DVT
Obesity
Pregnancy
Oral contraceptives
Stroke with limb paresis or paralysis

Table 6. *Common risk factors associated with deep venous thrombosis (DVT).*

coagulation (DIC), deep vein thrombosis, severe liver disease, sepsis, vitamin K deficiency, oral anticoagulant therapy and elective surgery.[44-47] The protein C activity level may in some cases indicate the severity of a disease and can be used as a prognostic parameter.[47]

Elevated protein C levels

Elevated protein C levels have been reported in diabetic and nephrotic patients, during late pregnancy, and with oral contraceptives and anabolic steroids.[4,36,48,49] Elevated levels have no known clinical significance. However, clinical conditions and drugs influencing the protein C level should always be considered for the correct laboratory diagnosis of hereditary protein C deficiency.

Treatment options

Treatments for protein C deficiency include heparin, warfarin, and protein C concentrates.[4,44] Acute thrombosis in protein C-deficient individuals should be treated with heparin. Warfarin is used for longer treatment periods to prevent thrombotic recurrences. When initiating the warfarin therapy it must be started at low doses in conjunction with heparin to prevent skin and fat necrosis.[4]

Prophylactic anticoagulation is mainly recommended for symptomatic patients in high risk situations (e.g. surgery, pregnancy). Symptom-free relatives of symptomatic patients with protein C deficiency may benefit from prophylactic anticoagulation in similar risk situations, since they run an increased risk of thrombosis compared to nondeficient individuals.[41]

Replacement of protein C can be carried out with fresh frozen plasma or pure protein C concentrates produced either from plasma or by recombinant techniques. There is currently major interest in the use of APC preparations in the management of acute DIC. Studies of APC in animal models show that it is a powerful antithrombotic agent, without the risk of bleeding as a side effect.[50,51]

Protein C assays

Introduction

The laboratory evaluation of protein C is the only definitive way of diagnosing hereditary protein C deficiency in thrombophilic patients. Various types of assays have been developed and some are available in commercial kit form.

Protein C is measured using either a functional assay, that tries to evaluate the biological activity of protein C, or an immunological assay, which determines the total amount of protein C related material in plasma. Each assay has a number of pros and cons. However, for the routine screening of hereditary protein C deficiency, a functional activity assay is generally recommended. This approach will detect low activity levels associated with both reduced (type I) as well as dysfunctional protein C (type II).[52-54]

Activity assays

Numerous activity assays have been described that use different types of activators and detection methodologies.[55-63] The majority of the proposed methods can be divided into three major steps: (1) isolation of protein C from plasma, (2) protein C activation, and (3) measurement of APC using either synthetic substrates or clotting-based assays (Table 8).

- Expression of normal antigen concentration (70-130%)
- Presence of Gla residues, calcium-binding
- Activation by the thrombin-thrombomodulin complex
- Interaction with phospholipids and protein S/factor V
- Recognition and degradation of factors Va and VIIIa
- Inhibition by protein C inhibitors

Table 7. *Criteria for optimal function of protein C*

Isolation step

In the first generation of functional protein C assays the activation of protein C was achieved either by thrombin alone, or by the thrombin-thrombomodulin complex. These reagents required an adsorption step prior to the protein C activation in order to isolate protein C from its plasma inhibitors and other interfering substances. The surface binding of protein C was obtained using either immunoadsorption techniques or insoluble salts (e.g barium citrate or aluminium hydroxide). The latter procedure exploited the ability of protein C and other vitamin K-dependent proteins to bind to insoluble salts via the Gla-domain.

Once protein C was isolated and activated, the thrombin excess has to be quenched or removed by specific thrombin inhibitors (e.g. I-2581, antithrombin) before the protein C activity could be quantified accurately. In general, these multi-step

Assay	Step 1 Separation of PC from interfering substances	Step 2 PC Activator	Step 3 APC activity measurement
Bertina et al.[55]	Al(OH)	Thrombin	S-2366™
Sala et al.[56]	Barium citrate	Thrombin/TM	S-2266™
Francis & Patch.[57]	Barium citrate	Thrombin	APTT
Francis.[58]	Barium citrate	Thrombin/TM	APTT
Vigano et al.[59]	Monoclonal antibody	Thrombin/TM	S-2238™ or FXa clot.
Martinolo & Stocker.[60]	-	Protac®	APTT
Gugliemone & Vides.[61]	-	Protac®	PT
McCall et al.[62]	-	Protac®	S-2366™
Odegaard et al.[63]	-	Protac®	S-2366™

Table 8. *Functional assays for protein C involve three major procedures: isolation of protein C, activation of protein C and measurement of protein C activity. At least one step from each group is necessary to develop a functional assay. The use of Protac® negates the need for an isolation step. APTT = activated partial thromboplastin time, PT = prothrombin time.*

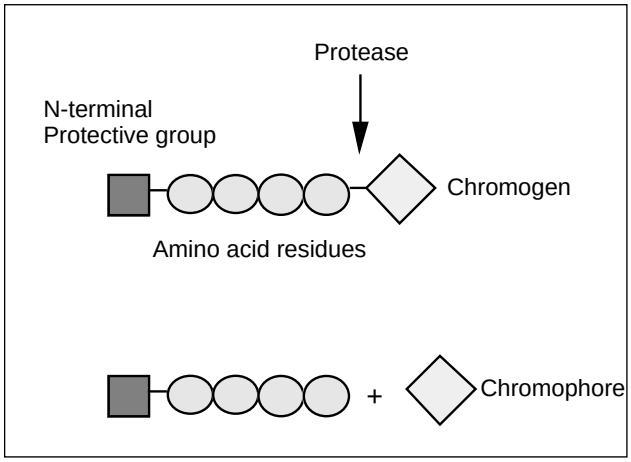


Figure 9. *Principle structure and function of synthetic peptide substrates.*

Chromogenic Substrates
S-2366™ pyroGlu-Pro-Arg-pNA
S-2266™ H-D-Val-Leu-Arg-pNA
S-2288™ H-D-Ile-Pro-Arg-pNA
S-2238™ H-D-Phe-Pip-Arg-pNA

Table 9. *Chromogenic substrates used in amidolytic protein C assays (see figure 3 for comparison with the natural substrates factor Va and VIIIa).*

assays have been shown to be specific for protein C but are time-consuming and unsuitable for clinical use.[64]

Snake venom activator

The determination of protein C was greatly facilitated by the use of a specific protein C activator with the trade name Protac® (American Diagnostica, Greenwich C.T., USA). The activator is a serine protease isolated and purified from the venom of the southern copperhead snake, Agkistrodon c. contortrix.[11,17,18] It rapidly activates both human and bovine protein C, probably via the same mechanism as thrombin, without interfering with other coagulation factors. The activation reaction is especially effective in the absence of calcium ions and conditions of low ionic strength. Furthermore, the venom activator does not hydrolyze the chromogenic substrates for protein C to any significant extent. Since the activation is rapid, it minimizes the efficiency of the protein C inhibitors and thus eliminates the need for isolating protein C in an adsorption step.

Measurement of APC

APC can be measured using either chromogenic substrates or clotting-based techniques.

Chromogenic substrates are small synthetic peptides that mimic the cleavage site of a natural substrate.[55] The peptides are generally composed of a sequence of 2-4 amino acids with the chromogen, 4-nitroaniline (pNA) attached to the end (Figure 9). When the chromogenic substrate is incubated with a proteolytic enzyme, such as APC, it is cleaved and pNA (yellow colour) is released. The release is measured at a wavelength of 405 nm, either during the reaction in a photometer cuvette (kinetic method), or discontinuously by stopping the reaction with acetic or citric acid (end-point method). The photometric signal is proportional to the enzyme activity in a properly-designed assay.

Substrates to be used in a chromogenic assay for protein C must be specific for the enzyme, and activators and contaminating factors should not cleave the substrate. One of the most suitable chromogenic substrate available for the assay of protein C activated by Protac®, appears to be S-2366™. The substrate has been shown to have little sensitivity to the isolated venom activator, although it is significantly hydrolyzed by normal plasma mixed with the activator.[18,65]

Clotting assays for the determination of protein C use the ability of activated protein C to prolong the clotting time. A widely-used method is the activated partial thromboplastin time, APTT (Figure 10). The use of a protein C-deficient plasma in these assays negates the possibility that deficiencies of other plasma proteins (e.g. protein S) are the cause of APTT prolongation.

APTT assays for protein C are generally less

precise and show a greater variability than chromogenic protein C assays. This is probably due to the influence of high levels of factor VIII (an acute phase protein), and/or the variable phospholipid reagent quality and composition. The interference may result in erroneously low protein C levels.[61]

Since the APTT assays are based on APC's ability to inactivate factors Va and VIIa in the natural environment, they offer the possibility of evaluating all the functions of the protein C molecule. This is important for a small minority of patients that have defects in the protein molecule not associated with amidolytic functions (Table 7).[66-68]

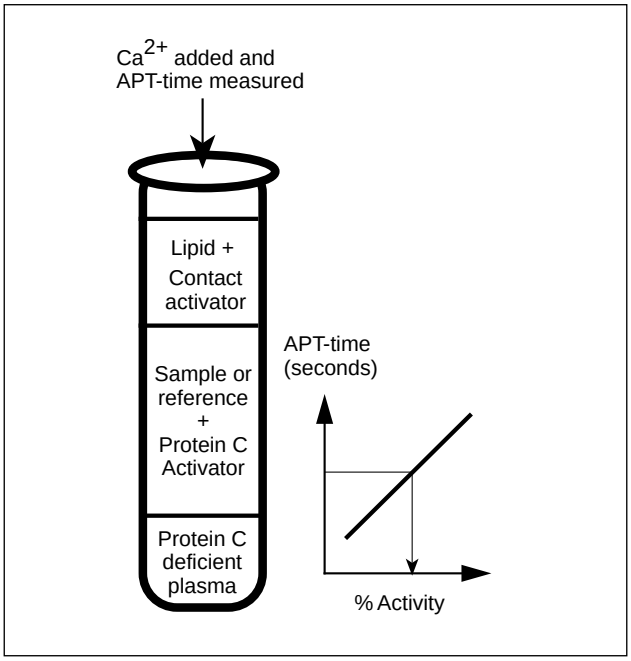


Figure 10. APTT assay
Generally, a citrated and diluted plasma sample is incubated with Protac® and added to a protein C-deficient plasma containing a phospholipid reagent and a contact activator (e.g. kaolin, ellagic acid). Coagulation is started by adding a $CaCl_2$-solution and the clotting time, proportional to the protein C activity, is recorded.

Immunological assays

Immunological assays for protein C are usually based on the use of monoclonal antibodies against protein C and include electroimmunoassays (EIA),[69] radioimmunoassays (RIA),[70] and enzyme-linked immunosorbent assays, ELISA (Figure 11).[71,72] The advantages of these assays are their specificity, reproducibility and accuracy. However, since immunological assays measure all types of protein C molecules in plasma without evaluating their function, they will not detect dysfunctional molecules (type II deficiency).

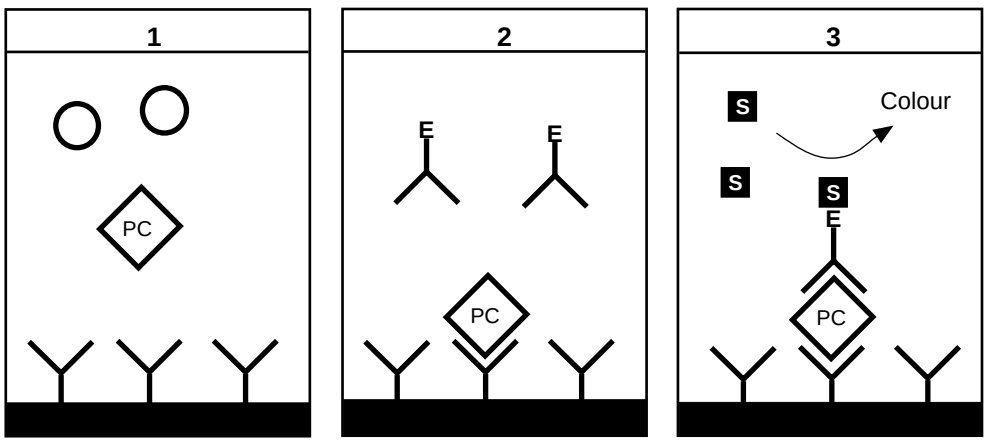


Figure 11. Enzyme-linked immunosorbent assay
*Protein C assay of the ELISA type are often commercially available. In general, a support is coated with protein C-specific antibody fragments. [**1**] The protein C antigen in the plasma sample is allowed to react with the antibody, followed by a washing step. [**2**] A conjugate (enzyme-labeled anti-protein C antibodies) is then added, forming a sandwich-like complex in the presence of protein C antigen. This is followed by a second washing. [**3**] Enzyme activity, proportional to protein C concentration, is determined by the cleavage of a substrate yielding a coloured or fluorescent product.*

Laboratory aspects

Oral anticoagulant therapy

The diagnosis of hereditary protein C deficiency is particularly problematic in patients on oral anticoagulant (OAC) therapy.[64] As explained previously, OAC therapy interferes with the formation of the γ-carboxyglutamic acid moiety of the protein C molecules during biosynthesis in the liver, which results in a loss of anticoagulant activity. Non-carboxylated forms of protein C molecules that are inactive in vivo can however, still be activated by snake venoms or thrombin-thrombomodulin and retain amidolytic activity in vitro.[65] Assays using chromogenic substrates will therefore over-estimate the true level of protein C activity in plasma from patients receiving OACs.

Theoretically, clotting-based assays would be preferable to use in patients on OAC treatment since they evaluate all of the functional domains of the APC molecule. However, clotting techniques have the principal disadvantage that protein C activity levels in all patients are very low, which makes it difficult to identify individuals with an inherited deficency. Certain statistical procedures may minimize this problem. Ratios of protein C activity to thrombin activity have been used to improve the discrimination between deficient and non-deficient patients on OAC therapy.[54]

Antigen assays are also difficult to interpretate in patients on OAC therapy, in that the loss of anticoagulant activity is more marked than the corresponding decrease in antigenic level. This antigen to activity ratio and its discrepancy during OAC therapy should always be considered when the appropriate immunological assay is to be chosen. Ratios of protein C antigen to factor X antigen and/or protein C antigen to factor II antigen are often used to determine a quantitative type I deficency in patients on OAC therapy (Figure 12).[52]

Contact factor proteases

In plasmas from patients on streptokinase therapy and in plasmas where contact activation is suspected, such as with DIC patients or individuals on oral contraceptives where cold activation may occur, there is a contribution to the chromogenic substrate cleavage that is not associated with the activity of protein C. This is because all known chromogenic protein C substrates are susceptible to some degree to activities generated in the contact activation system. To avoid the subsequent risk of overestimating the protein C levels in such plasmas it is recommended that the substrate blank activity, in the absence of Protac®, is measured and used for correction of the results.[73]

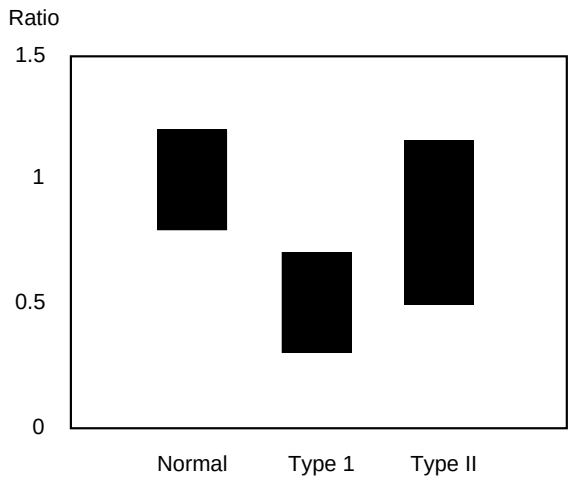


Figure 12. *Ratios of PC antigen to factor X antigen are often used to discriminate between non-PC deficient (normal) and type I-PC deficient patients receiving OACs. Type II-PC deficients are not possible to identify with this method.*

Diagnostic kits from Chromogenix

We invented the technology

The development of the first chromogenic peptide substrate S-2160 in the early Seventies, initiated the introduction of photometry in hematology. Today, Chromogenix has a wide range of chromogenic peptide substrates as well as complete assay kits with applications extending from routine analysis to front-line research in both coagulation and fibrinolysis (Table 10).[75] The tests can be performed manually or on automated analytical systems with high specificity, sensitivity and accuracy. Important, non-chromogenic based products include APTT-test for APC resistance and several ELISA kits (Table 11).

Protein C kit

COAMATIC® Protein C is a chromogenic substrate assay for the measurement of protein C activity in human plasma. It is easy to perform and is suitable for application in the majority of automated instruments. The kit contains a specific and rapid protein C activator (Protac®) from the venom of Akgistrodon c. contortrix, and the highly-sensitive and specific chromogenic substrate S-2366.

HEMOSTASIS
Prekallikrein
Factor VII
Factor VIII
Factor X
Soluble fibrin
Antithrombin
Heparin/LMW heparin
Protein C
α_2-macroglobulin
α_1-antitrypsin
Plasminogen
t-PA
PAI-1
Plasmin inhibitor
ENDOTOXIN
Endotoxin

Table 10. *Substances that can be determined with diagnostic kits based on synthetic peptide substrates (Examples from Chromogenix product range 1995).*

HEMOSTASIS
APC resistance
Anti-Cardiolipin IgG, IgM
D-dimer
Lipoprotein(a)
t-PA
PAI-1
INFLAMMATION/ SEPSIS
EndoCAb
IL-6
TFNa
Endotoxin

Table 11. *Substances that can be determined with kits from Chromogenix, based on ELISA or clotting techniques .*

Clinical indications for COAMATIC® Protein C

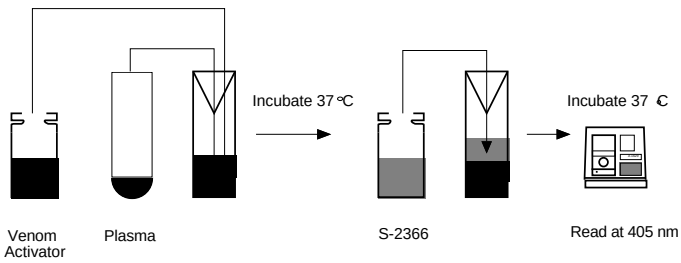
- **Detection and characterization of hereditary protein C deficiency**
- **Evaluating conditions with acquired protein C deficiency**
- **Monitoring of replacement therapy with protein C concentrates**

Assay procedure

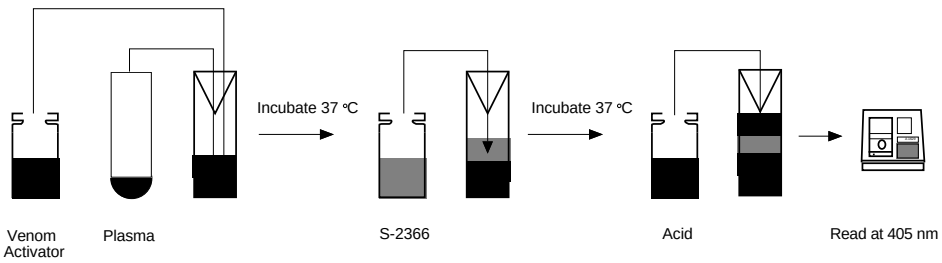
Product	Substrate	Activator	Plasma	Method
COAMATIC® Protein C	S-2366	Protac®	Undiluted	Kinetic/End-point

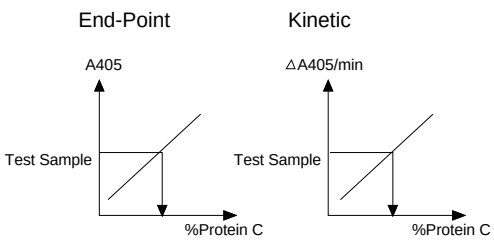
COAMATIC® Protein C

Kinetic Method



End-point Method





COAMATIC® Protein C

COAMATIC® Protein C is a chromogenic test kit for the specific determination of protein C activity in human plasma.

Measurement principle

Protein C is activated by a thrombin-like enzyme, Protac®, from the venom of the southern copperhead (Agkistrodon contorix contorix) and the amount of activated protein C is determined by the rate of hydrolysis of the chromogenic substrate S-2366. The pNA release measured at 405 nm is proportional to the protein C level up to 120% of normal plasma.

Article number: 82 20 98

The kit contains

S-2366	2 vials
Protein C activator	2 vials

Storage and stability in solution

S-2366	2–8 ˚C, 3 months
Protein C activator	2–8 ˚C, 1 month

Measuring range

0-120%

Detection limit

5%

Repeatability

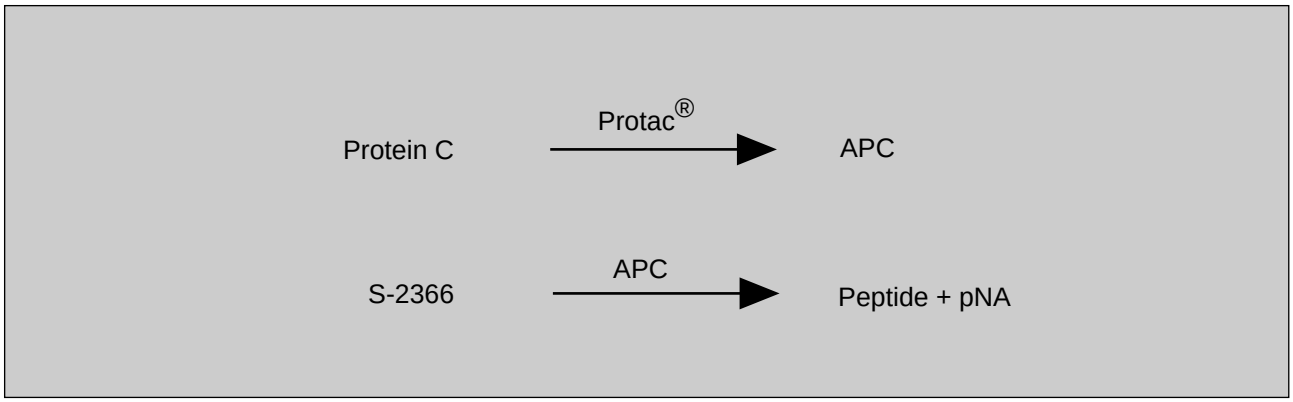
PC	CV within series	CV between series
50%	1.6%	2.8%
100%	0.8%	1.7%

Specificity and interfering factors

There is no interference of heparin levels ≤ 3 IU/ml. Sample blank activities should be determined and subtracted in plasmas from patients on streptokinase therapy as well as in plasmas where contact factor activation is suspected, e.g. in plasmas from DIC patients or individuals on oral contraceptives susceptible to cold activation.[73] Aprotinin therapy may cause an underestimation of protein C levels.[74]

Determinations per kit

Manual 72, automated up to 180, microplate 288



The measurement principle of COAMATIC® Protein C

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Definitions

Allele. One of an array of possible mutational forms of a gene at a specific locus

Amino acids. Basic building blocks of all proteins

Antibody. A molecule produced by animals in response to antigen.

Antigen. A molecule that induces the formation of an antibody.

APC resistance. An hereditary defect caused by a point mutation in the gene coding for factor V and characterized by a poor anticoagulant response to activated protein C.

Autosome. A chromosome other than a sex chromosome.

Chromosome. The darkly staining bodies within the cells made up of a large number of genes and a centromere region.

Embolism. Obstruction or occlusion of a vessel by a transported clot.

Endothelium. Cells lining blood vessels and lymphatics which control the passage of materials into and out of the bloodstream.

Enzymes. A protein with catalytic power.

Exon. Gene segment encoding protein.

Fibrin. An elastic filamentous protein derived from fibrinogen by the action of thrombin, which releases fibrinopeptides A and B from fibrinogen.

Fibrinogen. Factor I; a globulin of the blood plasma that is converted into the coagulated protein, fibrin, by the action of thrombin in the presence of calcium ions.

Fibrinolysis. The hydrolysis of fibrin by plasmin.

Gene. The unit of inheritance, located at a specific region on the chromosome.

Glycoprotein. One of a group of protein-carbohydrate compunds

Hemostasis. Process that arrests the escape of blood from injured vessels.

Homozygous. Condition of having identical alleles at one or more loci under consideration

Heparin cofactor II. Serpin with heparin cofactor abilities. Specific inhibitor of thrombin.

Hepatocytes. Cells in the liver that are arranged in folded sheets. They produce many of the blood proteins.

Heterozygous. Having a dissimilar alleles at one or more loci.

Intron. Gene segment between exons not encoding protein.

Locus. The position on a chromosome at which a particular gene is found.

Platelets. A small disk-shaped blood cell, containing granules in the central part and peripherally, clear protoplasma, but no nucleus. Numbering 200,000 to 300,000/µl.

Platelet factor 4. A heparin antagonist released from activated platelets. It interacts equally with high and low affinity heparin and like most heparin-binding proteins the interactions decrease with molecular weight.

Protamine. Protein derived from fish and used as a clinical antagonist for heparin. After injection it neutralizes all the anticoagulant avtivity of UF heparin and about 75% of the anti-Xa activity.

Proteases, proteinases. Enzymes hydrolyzing native protein, or polypeptides, making internal cleavages; they include pepsin, chymosin, trypsin, papain etc.

Proteins. A class of macromolecules that are built from a repertoire of twenty amino acids.

Proteoglycan. A macromolecular glycoconjugate composed of sulfated glycosaminoglycans covalently linked to a protein core.

Proteolysis. Enzymatic cleavage of protein.

Prothrombin. Factor II, zymogen of thrombin; a glycoprotein formed and stored in the parenchymal cells of the liver. Present in blood at approximately 100 µg/ml.

Receptor. A cell surface molecule which binds specifically to particular proteins or peptides in the fluid phase.

Sepsis. A clinical syndrome of serious bacterial infection.

Serine protease. Proteolytic enzyme with a serine residue at its enzymatically active site.

Serpin. Serine protease inhibitor.

Serum. The watery portion of blood remaining after fibrinogen has been removed from the plasma

Thrombocyte. Blood platelet

Thrombocytopenia. A conditon in which there is an abnormally small number of platelets in the circulating blood (usually less than 150,000/µl).

Thromboembolism. Refers to either thrombosis or embolism or a combination of both.

Thrombolytics. Biological and synthetic substances capable of activating the fibrinolytic system in plasma.

Thrombin. Active protease deriving from prothrombin (factor II). Induces conversion of fibrinogen into clot-forming fibrin monomers resulting in the coagulation of blood.

Thrombophilia. A disorder in which there is a tendency to develop thrombosis.

Thrombosis. The formation of a thrombus (blood clot).

Thrombotic. Relating to, caused by, or characterized by thrombosis.

Zymogens. The enzymatically inactive precursors of proteolytic enzymes.

Notes

Protein C

Chromogenix Product Monographs

Antithrombin
COAMATIC® Antithrombin
COAMATIC® AT 400
COAMATIC® LR Antithrombin
COATEST® Antithrombin
COACUTE® Antithrombin R

APC resistance
COATEST® APC Resistance
COATEST® APC Resistance – C
COATEST® APC Resistance – S
COATEST® APC Resistance – SC
COATEST® APC Resistance V
COATEST® APC Resistance V-S
COASET® FV-506

D-dimer
SimpliRED® D-dimer
DIMERTEST® GOLD EIA

Factor VIII
COAMATIC® Factor VIII
COATEST® Factor VIII
COATEST® VIII:C/4

Heparin
COATEST® Heparin
COATEST® LMWHeparin/Heparin
COACUTE® Heparin

Plasminogen
COAMATIC® Plasminogen
COATEST® Plasminogen

Protein C
COAMATIC® Protein C

t-PA
COASET® t-PA
COALIZA® t-PA

COAMATIC®
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