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PROTEIN C AND RELATED ANTICOAGULANTS

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On the Cover

Model of the combined human protein C EGF-1 and EGF-2 domains. The carbon backbone of EGF-1 is yellow and the side chains are magenta. The carbon backbone of EGF-2 is green and the side chains are dark blue. The β-OH aspartic acid is shown in red and the aspartic acids 46 and 48 are light blue. Graphic made using a digital MicroVax II with a star ST-50 array processor and an Evans and Sutherland PS390 picture system running *Discover* and *Insight* molecular imaging software.
Graphic: Dr. Partha Manavalan and Dr. Robert Wydro, Genzyme Corporation

Foreword

There is a need for additional anticoagulant agents for the treatment of clotting phenomena that take place in many disease states. Drugs, such as aspirin, heparin, and warfarin have played a very important role in providing essential health care for the world. However, these drugs have potentially dangerous bleeding complications which limit their usefulness.

Protein C, a vitamin K-dependent zymogen of a serine protease, is a potent anticoagulant and a critical protein in hemostasis. The protein was reported in the literature as early as 1960 and was first called autoprothrombin II-A. Later, it was shown that autoprothrombin II-A is an activated form of protein C. The activated molecule behaves as an anticoagulant via inhibition of the intrinsic coagulation pathway by proteolysis of factors VIIIa and Va. Preclinical studies of activated protein C have shown that it can control the formation and extension of blood clots by regulating the extent of fibrin and platelet deposition. Perhaps, the most important attribute of protein C in animal models is that it appears to be an effective anticoagulant, while having little effect on other hemostatic parameters (that is, bleeding). Clearly, the hope is that this property will be borne out in clinical trials.

The development of immuoaffinity chromatography has contributed to the recovery of gram quantities of trace proteins from blood plasma. This technology has made large quantities of biologically active protein C available for *in vitro* characterization. In addition, quantities of protein C are now available for preclinical and clinical evaluation. An important futuristic goal of protein C research is to engineer systems which produce large quantities of protein C which can be purified economically. This will only be achieved through cross-disciplinary efforts of life scientist and biological engineers. It will be possible to manufacture kilogram quantities of these important proteins when bioprocess engineers utilize the marvelous discoveries of basic science and achieve scale-up of the essential processes for

commercialization. This will require that great strides be made in both upstream and downstream processing technology. The possibility that transgenic large animals might be used as bioreactors is promising and could help to solve the upstream synthesis problems.

Replacement of the present therapy for protein C deficiency with a safe biologic at a cost that is not prohibitive to the general population should be the ultimate objective of such a collaborative effort. Only time will tell if this goal will ever be reached or if an alternate route such as gene replacement therapy will solve the problems associated with hereditary diseases.

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Table of Contents

Development of New Anticoagulants

Anticoagulant Therapies and Clinical Studies

The Potential of Protein C

Regulation and Production of Anticoagulants

1

Regulation of Coagulation:
The Nature of the Problem

Charles T. Esmon
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Protein C is a vitamin K-dependent zymogen of a natural anticoagulant. It is activated by the thrombin-thrombomodulin complex, and the enzyme, activated protein C, works in concert with protein S on cell surfaces to inactivate factors Va and VIIIa. Inflammation down regulates the system by decreasing free protein S and increasing the amount of C4bBP-protein S complex. Thrombomodulin expression on the endothelial cell surface is also decreased. For activated protein C to function as an anticoagulant, the Gla domain is required. Functional levels of protein C are reduced in patients on warfarin. This may play a role in warfarin induced skin necrosis. While the protein C pathway can be inhibited by inflammatory mediators, activated protein C and other components of the pathway are capable of protecting from shock induced by *E.coli*, while other more potent anticoagulants are not. These properties suggest that activated protein C and other components of the pathway may have unique advantages as antithrombotic agents.

Blood clotting involves a series of reactions, which, if unchecked, would amplify, ultimately leading to complete conversion

of fibrinogen to fibrin. *In vivo*, such failure to limit clotting would lead to massive thrombosis. Recent studies have elucidated three main pathways involved in regulating coagulation. One pathway involves antithrombin III inhibition of coagulation proteases.This reaction appears to be accelerated by vascular heparin-like molecules.[1] The second pathway involves an inhibitor, referred to as either LACI (lipoprotein associated coagulation inhibitor) or EPI (extrinsic pathway inhibitor) that blocks the activity of the factor VIIa-tissue factor complex.[2] The third pathway involves activated protein C, which functions to neutralize factors Va and VIIIa.[3] Thus, these major inhibitory pathways work in concert to inhibit both the proteases and regulatory proteins(cofactors) of the coagulation system. Physiological significance for two of these three inhibitory mechanisms is indicated by the observation that patients with antithrombin III and protein C deficiency both exhibit thrombotic complications.[1,4]

The Protein C Pathway

The protein C pathway is outlined schematically in Figure 1. Human protein C activation involves the release of a 12 residue peptide from the amino terminal region of the protein C heavy chain.[5] Although thrombin can catalyze this cleavage, physiologically the pathway is triggered when thrombin binds to thrombomodulin. This reversible, high-affinity complex activates protein C at least 1000 times more effectively than free thrombin.[3] Complex formation between thrombin and thrombomodulin not only accelerates protein C activation but inhibits platelet activation and fibrinogen clotting as well as all other coagulation reactions examined to date.[3] The complex retains the ability to react with antithrombin III, and this reaction is accelerated by chondroitin sulfate moieties located on thrombomodulin.[6] Thus, thrombomodulin serves both to initiate the anticoagulant pathway and as a direct inhibitor of thrombin activity. Given that thrombomodulin may exceed 100nM in the microcirculation,[3] it

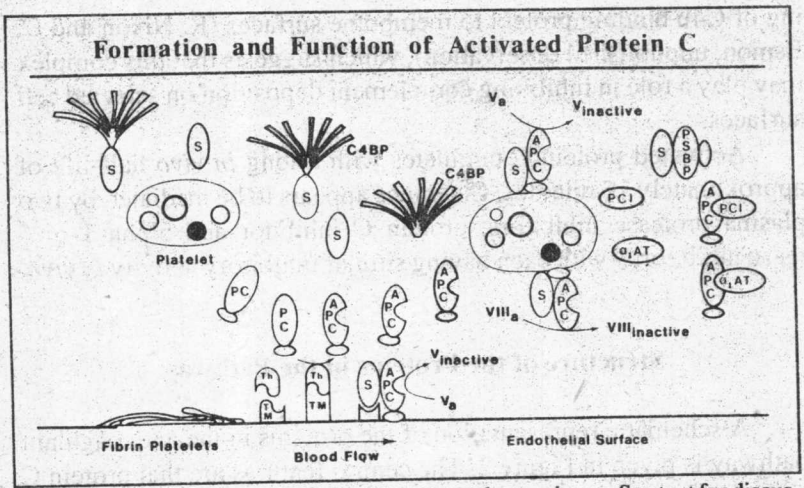

Figure 1. Interactions within protein C anticoagulant pathway. See text for discussion. Abbreviations: PC and APC, protein C and activated protein C; S, protein S; *Th*, thrombin; α_1AT, α_1-antitrypsin; PCI, protein C inhibitor; *PSBP*, protein S binding protein; *C4BP*, C4b binding protein. (Reprinted from Reference 3, with permission of the American Society of Biochemistry and Molecular Biology, and adapted from C.T. Esmon, *Science* 235, 1348, 1987, ©1987 by the American Association for the Advancement of Science.)

is likely that this protein may contribute significantly to thrombin inhibition.

Once protein C is activated, the enzyme interacts with cell surfaces to form a complex with protein S.[7] This complex is responsible for factor Va and VIIIa inactivation. Unlike most of the vitamin K-dependent clotting factors, protein S is not a zymogen of a serine protease but rather serves more like a regulatory protein. It should be noted, however, that protein S acceleration of factor Va inactivation is not nearly as dramatic as the factor Va acceleration of factor Xa activation of prothrombin. Protein S circulates both free and in complex with C4b binding protein, a regulatory protein of the complement system. This complex is not functional as a cofactor for activated protein C in factor Va inactivation.[8,9] Its role in regulating complement remains unknown, but protein S can facilitate bind-

ing of C4b binding protein to membrane surfaces (R. Nixon and C. Esmon, unpublished observation), which suggests that this complex may play a role in inhibiting complement deposition on selected cell surfaces.

Activated protein C circulates with a long *in vivo* half-life of approximately 15 minutes. Clearance appears to be mediated by two plasma protease inhibitors, protein C inhibitor and alpha 1 protease inhibitor,[10] with each having similar inhibitory activity *in vivo*.

Structure of the Proteins in the Pathway

A schematic representation of the proteins in the anticoagulant pathway is given in Figure 2. The central features are that protein C and protein S are both vitamin K-dependent factors (reviewed in Reference 3), contain 4-carboxy glutamic acid residues, and hence their binding to membrane surfaces is calcium dependent. This feature creates the paradoxical situation of having both an anticoagulant and procoagulant pathway inhibited simultaneously by oral anticoagulants. It is likely that it is this phenomena that contributes to warfarin-induced skin necrosis (see the chapter by Schwartz). These proteins also have epidermal growth factor (EGF) like repeats that contain sites for β-hydroxylation of aspartic acid and asparagine residues.[11,12] Although the function of these residues is unclear, these domains bind calcium ions and, at least in the case of protein C, this seems to be involved in the activation and function of the protein.[3] In protein C, the remaining domain contains the protease region. In protein S, this region is structurally related to the steroid binding proteins. The potential function suggested by this homology remains unknown.

Thrombomodulin differs from protein C and S in that it is an integral membrane protein. Three extracellular domains are apparent.[3] The amino terminal region, which has weak homology with the lectins,[13] six EGF-like domains, and an O-linked sugar region. Recent studies have shown that the growth factor domains are responsible for thrombin binding.[3,14]