Protein C
Biochemical and Medical Aspects

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PREFACE

Thrombosis has been known since about 2 000 B.C. and pulmonary embolism was first described in 1676 by WISEMAN. In the 1840's Rudolf VIRCHOW recognized the connection between thrombosis and pulmonary embolism.

According to VIRCHOW's triad changes of the vessel wall, blood flow and blood properties are the most important pathogenic factors for the development of thrombi with the risk of consecutive embolism.

For a long time it was assumed that hypercoagulability is mainly caused by an increased intravascular thrombin generation. During the last decade a key role in the development of hypercoagulability was attributed to proteinase inhibitors. The importance of antithrombin III became well established when it was observed at the end of the 1960's that an inherited or acquired deficiency is combined with a high risk of thromboembolism.

A few years ago, it became evident that thrombin generation can be inhibited not only by proteinase inhibitors but also by the action of a specific proteinase - Protein C. The inhibition of coagulation by Protein C may be of equal importance to that of antithrombin III.

The protein, which has already been known since 1960 as autoprothrombin II a, had a rennaissance when in 1981 GRIFFIN found the first family with a hereditary deficiency of this protein and its association with a high risk of thromboembolic diseases or thromboembolic complications.

Since then there has been increasing interest and research regarding Protein C. It belongs to a complex system of interacting proteins, it needs vitamin K for the synthesis of its biologically active form, it needs a vitamin K dependent protein as cofactor and gains its full activity only after activation by thrombin and thrombomodulin. Moreover a specific inhibitor exists for Protein C.

It is imposing that only a few years were necessary to clarify the complex basic biochemistry of Protein C and related proteins. New insights into the regulation of coagulation by plasmatic inhibitors arose. Many families have since been found with a congenital deficiency of this protein and many cases of spontaneous or recurrent thrombosis could be elucidated as a deficiency of Protein C or its cofactor Protein S.

Despite advanced knowledge there are still many problems in determining Protein C functionally. Most of them arise from its characeristic features. The difficulties of the functional assay in routine use are presumably the reason why little information is available about the changes in Protein C activity during the course of various diseases.

The aim of the workshop on Protein C, which was held form July 9th - 11th, 1984, in Titisee near Freiburg i.Br., was to sum up present knowledge in the biochemistry, physiology and pathophysiology of the inhibitor and related proteins. Important topics were the possibilities of determining Protein C concentration immunologically or the functional activity by chromogenic or clotting assays.

The basic information on Protein C and connected proteins was followed by a number of clinical reports on inherited and acquired disorders. Multifarious open questions were outlined in the lectures and subsequent discussions.

I hope the proceedings of the workshop which are documented in this book will reflect the current status of the complex Protein C system and spread information on this new field of cagulation.

I wish to express special thanks to Dr. Ernst Zimmer, Mannheim, for his thorough help with the editing of the manuscripts. Sincere gratitude is extended to Mrs. Monika Krumnow, Mrs. Evelyne Glowka and Dr. Rudolf Weber of Walter de Gruyter Publishers, whose helpful cooperation has made the task of editing this book a pleasant one.

Freiburg i. Br., July 1985 Irene Witt

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BIOCHEMISTRY AND PHYSIOLOGY OF PROTEIN C

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Identified originally as an anticoagulant activity elicited by treating prothrombin preparations with thrombin (1), protein C was purified as a by-product of prothrombin purification (2), after having been identified as a third, unknown, component in an ion-exchange chromatogram (hence, "C"). As a member of the family of vitamin K-dependent zymogens of serine proteinases, protein C is γ-carboxylated at the first eleven glutamate residues (3, 4), and has the core structure of the chymotrypsin family. The zymogen is comprised of two non-identical polypeptides, of 155 and 260 amino acid residues, linked by a single disulfide. Of yet unknown significance, aspartate-71 is ß-hydroxylated (5). Removal of an N-terminal tetradecapeptide from the heavy chain activates protein C to an arginine-specific endopeptidase, with the catalytic center confined to the heavy chain.

The only natural substrates of activated protein C so far identified are the active forms of factors V and VIII (6-8). In a reaction dependent on phospholipid (or platelets), factors Va and VIIIa are inactivated rapidly by catalytic concentrations of activated protein C, owing to cleavage of specific peptide bonds. Cleavage of procoagulant cofactors appears to account for the anticoagulant activity of activated protein C, which is comparable *in vivo* and *in vitro* in blood or plasma (9, 10). The anticoagulant activity of activated protein C is species-specific, a finding that has led to identification of protein S, discussed elsewhere in this volume, as an obligate cofactor for activated protein C (11-13).

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A fibrinolytic action of activated protein C has been observed in dogs (9, 10, 14). Intravascular activated protein C elicits a transient but profound fibrinolytic state that arises from circulation of a tissue-type plasminogen activator. The response requires both humoral and cellular elements, and is likely to proceed via multiple steps. However, as discussed in detail elsewhere in this volume, the response is not observed in squirrel monkeys given anticoagulant doses of human or endogenous (monkey) activated protein C (15). Whether the response in dogs is unique or much more profound than in other species has not been ascertained.

Although protein C is activated slowly by thrombin, rapid activation of the zymogen, like that of other vitamin K-dependent zymogens requires a cofactor. Termed thrombomodulin, the cofactor was identified in perfused heart and cultured endothelium preparations (16, 17) and was purified from detergent extracts of rabbit lung (18). The purified protein is a single polypeptide having a molecular weight of 75,000, contains about 10 % proline, and 38 half-cystines. Seemingly unique to vascular endothelium, thrombomodulin is an integral membrane protein that binds thrombin stoichiometrically with high affinity (K_D = 0.5 nM) and specificity.

The bound enzyme is a potent activator of protein C, but is inactive toward other biological (procoagulant) substrates (19, 20), heparin cofactor II and α_2-macroglobulin. This change of specificity is reversible upon dissociation of thrombin from thrombomodulin. Studies of the Ca^{2+}-dependence of activation of native and modified derivatives of protein C indicate that membrane components other than thrombomodulin also participate in thrombin-catalzyed protein C activation (21, 22).

From the viewpoint of biochemistry, protein C fits well into the family of cofactor-regulated vitamin K-dependent zymogens. From the viewpoint of physiology, we may expect that protein C and thrombomodulin are but two members of a mostly unknown regulatory system that should come to be as complex as the

coagulant system. The pathology of protein C has already indicated that participation of protein C in the strict control of procoagulant expression is requisite to homeostasis.

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MECHANISM OF ACTION OF PROTEIN C

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Introduction

Human protein C, a vitamin K-dependent glycoprotein consisting of disulfide-linked heavy and light polypeptide chains, circulates as an inactive zymogen in plasma at a concentration of 4 µg/ml (1). Recent data suggests that protein C is initially synthesized in the liver as a single chain molecule that undergoes processing into a two chain molecule by proteolytic cleavage (2). The heavy chain constitutes the serine protease part of the molecule whereas the vitamin K-dependent structure is located in the NH_2-terminal part of the light chain containing 11 γ-carboxyglutamic acid residues. During activation of human protein C by thrombin, the Arg_{12}-Leu_{13} bond is cleaved at the amino-terminal end of the heavy chain and a dodecapeptide of 1,400 molecular weight is released (3).
This protein C activation peptide is measurable in humans, reflecting the degree of protein C activation in the vascular system (4). The rate of the activation of protein C by thrombin, the only known physiological activator, is catalysed several thousand fold after thrombin complexes with thrombomodulin, an endothelial cell cofactor (5). Human thrombomodulin has been purified recently from placenta and some striking differences between human placenta thrombomodulin and rabbit lung thrombomodulin have been reported (6). Human thrombomodulin bound to thrombin does not neutralize the pro-

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coagulant properties of thrombin in contrast to rabbit thrombomodulin (Majerus et al., American Heart Association Meetings, Miami, 1984).

Human protein C in its activated form has powerful antithrombotic properties by virtue of at least three effects on hemostasis. Protein C is an anticoagulant by inactivating the coagulant cofactors, factors Va and VIIIa, that mediate activation of factor X by factor IXa and activation of prothrombin by factor Xa (7, 8, 9, 10).

Activated protein C (APC) destroys platelet prothrombin-converting activity by inhibiting the binding of factor Xa to platelets through inactivation of platelet factor Va, the Xa receptor, thus resulting in a reduction of thrombin formation (11, 12).

Activated protein C has fibrinolytic properties both in vivo and in vitro (13, 14). The fibrinolytic potential of cultured endothelial cells is increased by APC and an inhibitor of plasminogen activator (antiactivator) is neutralized by APC in in vitro blood clot lysis assays (15, Sakata, Loskutoff and Griffin, unpublished results). The intravenous infusion of bovine APC into dogs apparently elevates circulating plasminogen activator (13).

Protein C as an anticoagulant

In 1960 Mammen et al. detected a thrombin-dependent anticoagulant in a preparation of bovine vitamin K-dependent proteins (16, 17). After purification of this anticoagulant, named by these authors autoprothrombin II-A, the identity of APC to it was demonstrated (18). In spite of initial suggestions that APC functions as an competitive inhibitor of prothrombin activation (17, 18), it is clear now that the anticoagulant activity is mediated through the selective proteolytic inactivation of factor VIII and factor V.

Infusion of thrombin (1 U/kg/min) into dogs resulted in the generation of anticoagulant activity within 5 min of starting

the infusion as shown by prolonged partial thromboplastin times and factor Xa clotting times (5). The nature of the anticoagulant was identified as APC by partial purification of the anticoagulant from the dog plasma and by the observation that antibodies to APC added to the plasma before measuring the clotting time abolished the anticoagulant effect (5). In retrospect, the anticoagulant effect of APC was also probably demonstrated during other experiments in which low levels of thrombin were infused into animals resulting in the appearance of anticoagulant activity (19).

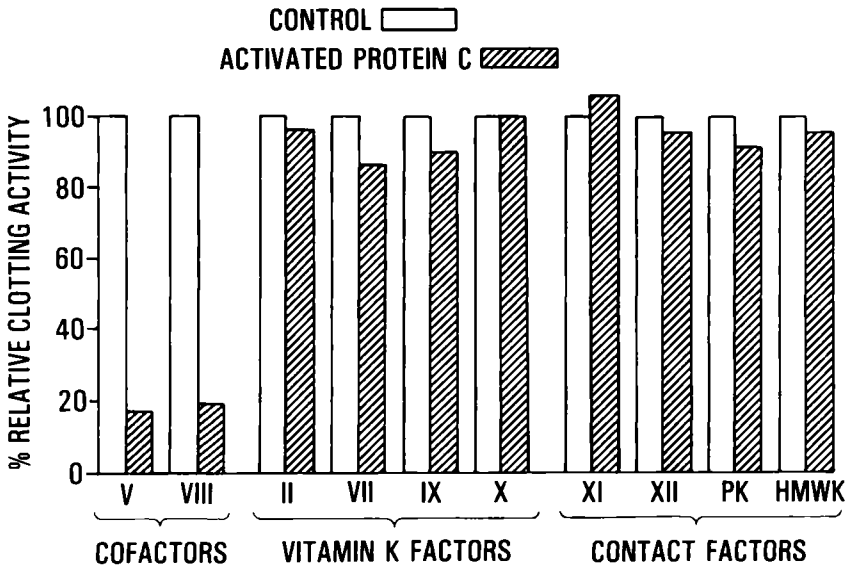

Fig. 1. Effect of activated protein C on coagulation factors in normal plasma. Activated protein C (3.0 µg/ml final) was combined with citrated normal human plasma (500 µl) in the presence of cephalin (58 µg/ml final) and calcium ions (4 mM final) in a total volume of 600 µl. After 3 min at 37°C, EDTA or purified antiprotein C antibodies were added, and dilutions of the mixture were assayed for individual coagulation factors. The height of each bar represents percent clotting activity compared to the control (open bars). Plasma plus activated protein C is represented by bars with diagonal lines. From Marlar, Kleiss and Griffin (20).

It was shown by Marlar et al. that human APC in the presence of phospholipids and calcium ions significantly prolongs the prothrombin time, the activated partial thromboplastin time and the partial thromboplastin time. No effect on thrombin time by APC was observed (20). As seen in Fig. 1, the anticoagulant activity of APC in plasma is due to the decrease of the clotting activity of factor V and factor VIII by APC since greater than 80 % of each activity were lost in a 3 minute incubation of APC with normal plasma in the presence of phospholipids and calcium ions (20). The inactivation of factors V and VIII in plasma due to APC showed a dose response relationship between the proteolytic destruction of factors V and VIII and the amount of APC present in the incubation mixture (20).

The inactivation of the activated form of factor VIII:C, factor VIIIa, and of factor Va by APC is much more rapid than that of the nonactivated forms. APC loses all anticoagulant activity when the active site is blocked with diisopropyl fluorophosphate (20). The contact system proteins, other vitamin K-dependent coagulation proteins, and fibrinogen are not affected by APC.

When the molecular changes during the inactivation of factor VIII and V by APC were studied, the polypeptide cleavage pattern of factor VIII:C caused by APC showed similarity to the proteolytic cleavage pattern of factor V (21).

During the inactivation reaction of purified human factor VIII:C by APC, a diminution of all factor VIII:C polypeptides of molecular weight between 92,000 and 188,000, leaving intact a doublet of molecular weight 79,000 to 80,000, occurs as shown on SDS polyacrylamide gel electrophoresis (21) (Fig. 2). The generation of a new major polypeptide of molecular weight 45,000 appears during this APC-induced proteolytic process. A time course study of the inactivation of purified factor VIII:C by APC revealed a progressive disappearance of factor VIII:C high molecular weight polypeptides as factor VIII activity decreased while the new peptide