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INHIBITION OF HUMAN BLOOD COAGULATION FACTOR Xa BY α_2-MACROGLOBULIN. Joost C.M. Meijers, Pim N.M. Tijburg, Bonno N. Bouma. University Hospital Utrecht, Dept. of Haematology, P.O. Box 16250, 3500 CG Utrecht, The Netherlands

The inactivation of activated factor X (factor Xa) by α_2-macroglobulin (α_2M) was studied. Irreversible inhibition was observed with the initial formation of a reversible enzyme-inhibitor complex. The second-order rate constant for the reaction was 8.4×10^4 M^{-1} $min.^{-1}$. The binding ratio was found to be 2 mol factor Xa/ mol α_2M. Interaction of factor Xa with α_2M resulted in the appearance of four thiolgroups/molecule α_2M. The apparent second-order rate constants for the appearance of thiolgroups were dependent on the factor Xa concentration. Sodium dodecyl sulphate gradient polyacrylamide gel electrophoresis was used to study complex formation between α_2M and factor Xa. Under non-reducing conditions four factor Xa - α_2M complexes were observed. Reduction of these complexes showed the formation of two new bands. One complex (Mr 225000) consisted of the heavy chain of the factor Xa molecule covalently bound to a subunit of α_2M, while the second complex (Mr 400000) consisted of the heavy chain of factor Xa molecule and two subunits of α_2M. Factor Xa was able to form a bridge between two subunits of α_2M, either within one molecule of α_2M, or by linking two molecules of α_2M. The role of the light chain of factor Xa in this process remains to be elucidated. For this purpose, monoclonal antibodies specific for the light chain of factor Xa were prepared. Sodium dodecyl sulphate agarose electrophoresis studies showed that complexes involving more than two molecules of α_2M were not formed.

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THE EFFECT OF PROCOAGULANT PHOSPHOLIPID VESICLES WITH NET POSITIVE CHARGE ON THE ACTIVITY OF PROTHROMBINASE. J. Rosing, H. Speijer, J.W.P. Govers-Riemslag and R.F.A. Zwaal. Dept. of Biochemistry, University of Limburg, Maastricht, The Netherlands

It is generally thought that procoagulant phospholipid surfaces that promote the activation of vitamin K-dependent coagulation factors should have a net negative charge in order to promote calcium-dependent binding of the enzymes (FVIIa, FIXa and FXa) and substrates (prothrombin and FX) of the coagulation factor-activating complexes. Two models have been proposed to explain calcium-mediated association of vitamin K-dependent proteins with phospholipid: a) an electrostatic model, in which a positively-charged protein-calcium complex is attracted by a negatively-charged phospholipid surface and b) a chelation model in which a coordination complex is formed between calcium ions, γ-carboxyglutamic acids of the proteins and negatively-charged membrane phospholipids. To study the effect of the electrostatic potential of phospholipid vesicles on their activity in the prothrombinase complex the net charge of vesicles was varied by introduction of varying amounts of positively-charged stearylamine in the membrane surface. Introduction of 0-15 mole% stearylamine in phospholipid vesicles that contained 5 mole% phosphatidylserine (PS) hardly affected their activity in prothrombin activation. Electrophoretic analysis showed that vesicles with > 5 mole% stearylamine had a net positive charge. The procoagulant activity of vesicles that contained phosphatidic acid, phosphatidylglycerol, phosphatidylinositol or phosphatidyl-β lactate (PLac) as acidic phospholipid was much more effected by incorporation of stearylamine. Amounts of stearylamine that compensated the negative charge of acidic phospholipid caused considerable inhibition of the activity of the latter vesicles in prothrombin activation. The comparison of vesicles containing PS and PLac as acidic phospholipid is of special interest. PS and PLac only differ by the presence of NH_3^+-group in the serine moiety of PS. Thus, in spite of the fact that vesicles with PLac are more negatively charged than vesicles with PS, they are less procoagulant. Our results show that a) although procoagulant membranes have to contain acidic phospholipids there is no requirement for a net negative charge, b) the amino group of phosphatidylserine has an important function in the interaction of procoagulant membranes with vitamin K-dependent proteins and c) the chelation model can satisfactorily explain calcium-mediated lipid-protein association.

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BIOLOGICAL CHARACTERIZATION OF AMINO-TERMINAL EXON DELETIONS OF T-PA. Glenn R. Larsen, Kim Henson, and Yitzak Blue, Genetics Institute, 87 CambridgePark Drive, Cambridge, Massachusetts

The secreted form of t-pa is proposed to be a mosaic protein which contains 4 different domain elements based on amino acid homologies with fibronectin finger elements, epidermal growth factor, kringle structures, and the active site of serine proteases. Of the 12 exons which encode these domains only the finger and epidermal growth factor are encoded separately by single exons. To investigate whether a single exon can encode a functional element or domain within a protein, the following precise exon alterations were made by loop-out mutagenesis techniques which deleted either the fibronectin finger, epidermal growth factor, or combination finger/growth factor domain(s). These mutant proteins were expressed in mammalian cells and characterized with respect to affinity for fibrin, fibrinolytic and fibrinogenolytic potential. All mutants demonstrated significantly lower affinity for fibrin with respect to the wild-type protein. We estimate the K_D of these mutants for fibrin to be at least 100-fold higher than the wild-type form which we determined to be approximately 0.3 uM. Each mutant retained characteristic activator stimulation by fibrin and were also shown to have the same approximate specific activity as the wild-type form. These mutants were further evaluated in citrated human plasma [^{125}-I]-fibrin clot lysis assays over a range of activator concentrations and shown to behave similarly to wild-type t-pa at therapeutic thrombolytic concentrations. At some lower concentrations, however, reduced fibrinolysis was observed for the mutant forms relative to wild-type. All mutants were evaluated for their fibrinogenolytic potential and demonstrated no significant decrease in coagulable fibrinogen over a five hour period. This was in dramatic contrast to an equivalent activator concentration of urokinase which showed a precipitous decline in coaguable fibrinogen in the first hour.

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IMPROVEMENT OF T-PA PROPERTIES BY MEANS OF SITE DIRECTED MUTAGENESIS N. Haigwood (1), E.-P. Pâques (2), G. Mullenbach (1) G. Moore (1), L. DesJardin (1), A. Tabrizi (1). Chiron Research Laboratories, Chiron Corporation, 4560 Horton Street, Emeryville, California, USA (1) and Behringwerke AG, Research department PF 1140, D-3550 Marburg, FRG. (2)

The clinical relevance of tissue-plasminogen-activator (t-PA) as a potent thrombolytic agent has recently been established. It has however been recognized that t-PA does not fulfill all conditions required for an ideal thrombolytic pharmaceutical agent; for example, its physiological stability and its short half life in vivo necessitate the use of very large clinical doses. We have therefore attempted to develop novel mutant t-PA proteins with improved properties by creating mutants by site-directed mutagenesis in M13 bacteriophage. Seventeen mutants were designed, cloned, and expressed in CHO cells. Modifications were of three types: alterations to glycosylation sites, truncations of the N- or C-termini, and amino acids changes at the cleavage site utilized to generate the two chain form of t-PA. The mutant proteins were analyzed in vitro for specific activity, fibrin dependence of the plasminogen activation, fibrin affinity, and susceptibility to inhibition by PAI.

In brief, the results are: 1) some unglycosylated and partially glycosylated molecules obtained by mutagenesis are characterized by several-fold higher specific activity than wild type t-PA; 2) truncation at the C-terminus by three amino acids yields a molecule with increased fibrin specificity; 3) mutations at the cleavage site lead to a decreased inhibition by PAI; and 4) recombinants of these genes have been constructed and the proteins were shown to possess multiple improved properties. The use of site directed mutagenesis has proved to be a powerful instrument to modulate the biological properties of t-PA.