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PENETRATION OF FACTOR Xa INTO PHOSPHOLIPID LAYERS.

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Negatively charged phospholipids (PL) at the platelet surface play a crucial role in the conversion of prothrombin into thrombin in the prothrombinase complex. Interactions of prothrombin and factor Xa (FXa) with PL are generally thought to occur through ionic interactions via Ca^{++} bridges. However, we have shown by various independent approaches that prothrombin (as well as FV and FVa) actually penetrates into the PL layer. We show that this is also the case, in the presence of Ca^{++}, for the other vitamin K-dependent, Gla-containing protein of the complex, FXa.

Electrochemical surface measurements indicate a reversible increase in the capacitance of condensed PL monolayers (containing various concentrations of phosphatidylserine (PS)), upon interaction with bovine FXa (kindly provided by Dr. C.M. Jackson). Reduction of the FXa disulfide bridges at the contact of the electrode further demonstrates penetration. Parallely, radioactive surface measurements allow to follow the adsorption of FXa which is quite reversible on PS monolayers and to determine the association constant, 6.10^6 1/mol, very similar to the Ka of FX, obtained by various different techniques.

Finally, penetration of FXa with PS containing PL vesicles was tested using the radiolabelled apolar probe (INA) which binds selectively to the lipid embedded domains of protein. FXa was radioactively labelled and the label preferentially found on the active site containing FXa heavy chain. Moreover, interaction of FXa with PS vesicles decreases its K_m for S 2222.

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BINDING OF CALCIUM TO HUMAN AND BOVINE FACTOR X. D.M. Monroe#, D.W. Deerfield II*, D.L. Olson*, T.N. Stewart*, H.R. Roberts#, R.G. Hiskey*, and L.G. Pedersen*. #Center for Thrombosis and Hemostasis and *Department of Chemistry, University of North Carolina, Chapel Hill, NC 27514 USA

Human and bovine factor X contain 11 and 12 glutamyl residues respectively within the first forty amino terminal residues that are posttranslationally modified to γ-carboxyglutamyl (Gla) residues. Calcium binding to these Gla residues and at other sites is critical for activity in factor X. We have measured calcium binding to human factor X by equilibrium dialysis for the first time. We have also re-examined calcium binding to bovine factor X in order to compare the two species. Factor X (10 μM) was incubated with ^{45}Ca in 20 mM Tris (pH 7.5), 100 mM NaCl in a half cell separated by a 12-14000 molecular weight fast-equilibrium disk membrane at 25°C for 24 hours. Four aliquots (100 μL each) were removed from each side of the cell and counted. Data were analyzed with a variety of models that allow for more than one class of binding site and for cooperativity among binding sites. Calcium binding to bovine factor X was best simulated by a model that assumes 1 very tight site, 3 cooperative tight sites, and 18 equivalent, non-interacting sites. Based on data from des(Gla)factor X, the first site is probably a high affinity non-Gla binding site. Our results differ from two previously published reports that indicated either 1 tight and 39 loose non-cooperative sites (R.H. Yue & M.M. Gertler (1978) **Thrombos. Haemostas. (Stuttg.) 40,** 350) or 20 calcium binding sites with the first 4 being cooperative (M.J. Lindhout & H.C. Hemker (1978) **Biochimica Biophysica Acta 533,** 318). Our data on human factor X fit the same model as used for bovine factor X; however, cooperativity is less in the 3 cooperative sites. Shown below are the first six thermodynamic equilibrium constants derived from a Scatchard analysis of binding data (values are M^{-1}).

factor X	K_1	K_2	K_3	K_4	K_5	K_6
bovine	18048	7046	8406	6415	4017	2672
human	16448	8224	7908	5914	5121	4411

Both proteins demonstrate the same total number of binding sites and essentially the same value for the first, tight binding site. Bovine factor X exhibits cooperativity, whereas human factor X has reduced cooperativity.

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POLYMORPHIC SITES IN FACTOR X GENE. C. Chelucci, H.J. Hassan, R. Guerriero, A. Leonardi, G. Mattia and C. Peschle. Department of Hematology, Istituto Superiore di Sanità, Rome, Italy.

The structure of factor X gene has been analyzed by Southern blot in 5 subjects with factor X deficiency.

Genomic DNA was digested with 8 different endonucleases and hybridized with a cDNA probe. The congenital deficiency observed in these patients is not apparently due to a major deletion or rearrangement. Since the gene locus is grossly intact, the disease presumably results from point mutation(s) not identified by the utilized endonucleases.

Our study was also focused on the presence of polymorphic site(s) in the factor X gene locus. Analysis of 50 normal subjects allowed to identify several polymorphic restriction sites after digestion with EcoRI, Hind III and Pvu II.

The restriction pattern obtained after Hind III digestion showed two bands of 7.3 and 6.0 Kb, while in two families an additional 7.6 Kb band was observed. Genomic DNA digested with EcoRI showed a 7.1 and 5.1 Kb fragments, and also a 6.6 Kb band with a 10% frequency.

After DNA digestion with Pvu II 5.6, 2.7 and ~1.0 Kb bands were observed. In three unrelated subjects we observed an additional 3.0 Kb fragment, in two other subjects a 3.5 kb band. Interestingly hybridization with a 178 bp cDNA subclone allowed to map the polymorphic sites in a the 3' region of the gene locus.

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ASSOCIATION OF HEPARIN AND FACTOR Xa: INFLUENCE ON THE RATE OF INHIBITION OF FACTOR Xa BY ANTITHROMBIN III-HEPARIN. B.A. Owen and W.G. Owen. Mayo Clinic/Foundation, Rochester, MN U.S.A.

Association of heparin non-covalently with bovine factor Xa was analyzed by Superose-12 gel chromatography. In 0.05 M NaCl, 0.02 M Tris, pH 7.5, DEGR-Xa (factor Xa inactivated by dans-Glu-Gly-Arg-CH_2Cl) was eluted as a single, sharp peak at V_e/V_t=0.65 (elution volume/internal volume). Mixtures of heparin and DEGR-Xa were eluted as two partially resolved peaks of protein at V_e/V_t=0.59 and 0.65. The fraction of DEGR-Xa in the leading peak was directly proportional to [heparin], and at 100 μM heparin the leading peak contained more than half the total protein. When 0.02 M HEPES was substituted for Tris a single, slightly broadened peak at V_e/V_t=0.64 was obtained on chromatography of 100 μM heparin and 10 μM DEGR-Xa. In a buffer system comprising 0.02 M Tris, 0.02 M HEPES, 0.03 M NaCl, pH 7.5, two peaks were eluted at V_e/V_t=0.59 and 0.65. Therefore, Tris increases the affinity of DEGR-Xa for heparin.

Solutions buffered with Tris or HEPES were compared for effects on the kinetics of inhibition of factor Xa by antithrombin III-heparin. Reaction mixtures containing 1 nM factor Xa, 30 nM heparin and 600 nM antithrombin III were assayed with S-2222 at intervals of 2-10 sec. Reagent concentrations were chosen (a) to assure pseudo-first-order kinetics, (b) to have [heparin] << K_D for factor Xa-heparin, and (c) to bind virtually all available heparin to antithrombin III. The same second-order rate constant, Kobs=2.5×10^7 $M^{-1}s^{-1}$, was obtained in both buffer systems. We conclude that the association of factor Xa with heparin observed directly by gel chromatography does not contribute to the reaction rate of factor Xa with antithrombin III-heparin.