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A HYDROPHOBIC SITE IN HUMAN PROTHROMBIN PRESENT IN A CALCIUM-STABILIZED CONFORMER

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Human prothrombin and prothrombin fragment 1 were demonstrated to bind to Phenyl-TSK columns in the presence of 5.0 mM calcium ions but not in the presence of either magnesium ions or manganese ions. The calcium-dependent interaction of prothrombin fragment 1 is markedly reduced upon oxidation of approximately one mole of tryptophan per mole of protein. The ability of prothrombin fragment 1 to inhibit prothrombin activation by factor Xa in the presence of calcium ions and phospholipid is also markedly reduced by reaction with N-bromosuccinimide. These results provide the first demonstration of a calcium-specific site in prothrombin outside of the "GLA domain".

While the activation of prothrombin(FII) by factor Xa in the presence of factor Va and phospholipid has strict specificity for Ca^{++}(1,2), other metal ions such as Mn^{++} and Mg^{++} have been observed to stabilize conformational states of FII in a manner similar to calcium ions(3,4). These observations suggested unique interaction of Ca^{++} with FII. The present study was initiated to explore the possible existence of a specific Ca^{++}-dependent functional domains outside of the "GLA domain."

MATERIALS AND METHODS

Human FII was purified from Cohn III paste(generously provided by Dr. Christine Vogel Sapan of North American Biologicals, Inc.) as previously described(5). Human prothrombin

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fragment 1(F1) was prepared by proteolysis of purified FII with thrombin followed by chromatography on DEAE-Cellulose(6). The phenyl-TSK column (Spherogel TSK-Phenyl-5 PW, 7.5 x 75 mm) was obtained from Altex. N-2-Hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) was obtained from Research Organics. All other chemicals were of reagent grade.

Hydrophobic interaction chromatography (HIC) was performed using an ISCO High-Performance Liquid Chromatographic System. The basic solvent was 20 mM HEPES - 150 mM NaCl, pH 6.8 with divalent cations as indicated. Effluent was monitored at 280 nm using an ISCO V4 detector. Data reduction was accomplished using a Chemsearch program provided by ISCO. Modification of F1 with N-bromosuccinimide(NBS) was performed as previously described(7). The effect of F1 on FII was assessed essentially as described by Tarvers et al.,(8) except that defined phospholipid vesicles (75 phosphatidyl choline - 25 phosphatidyl serine) were prepared by the technique of Barenholz et al.,(9) as described by Nesheim et al.,(10). The formation of thrombin was monitored by the hydrolysis of Tosyl-Gly-Pro-Arg-p-nitroanilide(11).

RESULTS AND DISCUSSION

Previous work from our laboratory on the high performance size-exclusion chromatography(HPSEC) of FII and F1(12) showed an apparent decrease in the M_r for both proteins in the presence of Ca^{++}. Since it was not possible to exclude matrix-protein interactions(13) during this study, it was possible that such interactions were responsible for the observed changes. The effect of Ca^{++} on the chromatographic behavior of FII on a Phenyl-TSK column is shown in Figure 1. In the absence of Ca^{++}, there was a major peak(elution time = 2.45 min) and a slight trailing shoulder(elution time of midpoint = 2.85 min) suggestive of interaction with the matrix. In the presence of Ca^{++}, approximately 80% of the protein eluted with a midpoint of 2.85 min. This retardation is consistent with the presence of a "hydrophobic patch" on a Ca^{++}-stabilized conformer of FII. The presence of two chromatographic peaks is compatible with an equilibrium between major conformers of FII(14). Identical results were obtained with F1 showing that the "hydrophobic patch" is in this portion of the FII molecule.

In order to further define the significance of a Ca^{++}-dependent hydrophobic site, the effect of other divalent cations on the HIC of FII was investigated. As shown in Figure 2, while the presence of Ca^{++} results in retardation of FII on Phenyl-TSK, the presence of 5.0 mM Mg^{++} had no effect. Similar results were obtained for Mn^{++}. Thus, the observation of a hydrophobic site

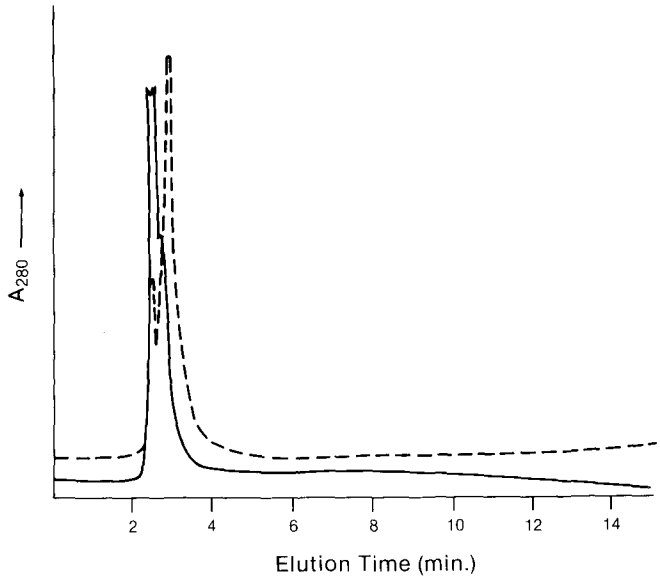

Figure 1. The Effect of Calcium Ions on the Hydrophobic Interaction Chromatography of Prothrombin on Phenyl-TSK. The column was equilibrated with either 20 mM HEPES-150 mM NaCl, pH. 6.8(————) or 20 mM HEPES-150 mM NaCl-5 mM $CaCl_2$(—— ——). A 25 uL portion of FII was applied via sample injector to a 7.5 x 75 mm column of Phenyl-TSK 5 PW. The column was developed at a rate 1.0 mL per minutes and absorbance at 280 nm monitored using a ISCO V4 detector.

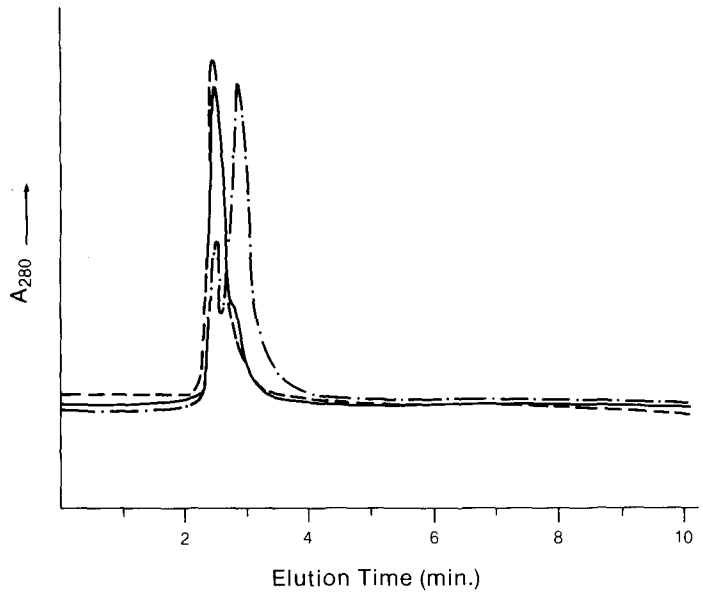

Figure 2. The Effect of Calcium Ions or Magnesium Ions on the Hydrophobic Interaction Chromatography of Prothrombin on Phenyl-TSK. The experiment was performed as described under Figure 1 in either 20 mM HEPES-150 mM NaCl, pH 6.8(————), 20 mM HEPES-150 mM NaCl - 5 mM $CaCl_2$(—•—) or 20 mM HEPES-150 mM NaCl-5.0 mM $MgCl_2$, pH 6.8(—— ——).

in FII using HIC has the same specific divalent cation requirement as does the biological activation of this protein.

The final experiment was designed to further define this 'hydrophobic site' in FII. The presence of CA^{++} or other divalent cations results in the observation of various conformational changes in either FII or F1 including an increase in absorbance at 292 nm(15) with a concomitant decrease in intrinsic fluorescence(16). Although these observations, which reflect changes in the microenvironment around a tryptophanyl residue or residues, are not specific for Ca^{++}, the ease of the site-specific modification of this residue in proteins(17) made this a logical point to start in defining the amino acid residue(s) responsible for a hydrophobic site in FII. We chose to study the NBS modification of F1 since F1 contains only 3 Trp residues compared to 12 in FII and since the site in question is in the F1 domain. At a four-fold excess of reagent to protein, spectrophotometric analysis showed the modification of one mole of Trp per mole of protein. Figure 3 shows that this modification of F1 fragment 1 greatly reduced retardation on

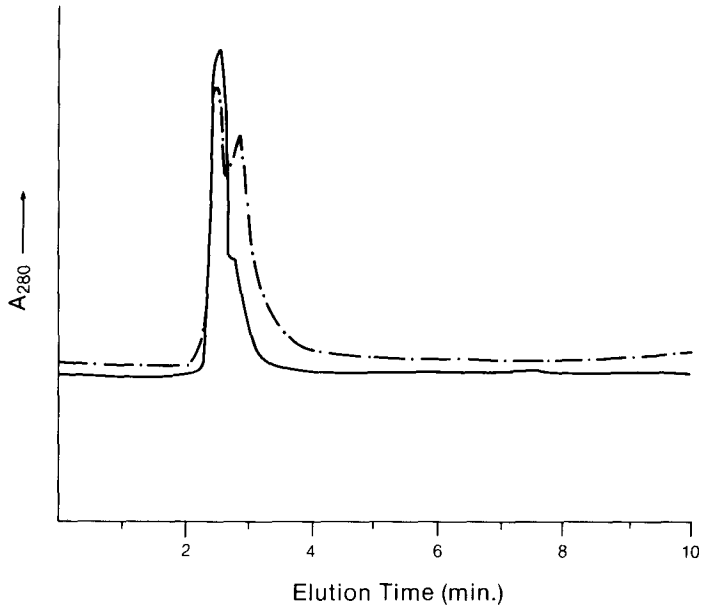

Figure 3. The Effect of Tryptophan Modification on the Hydrophobic Interaction Chromatography of Prothrombin Fragment 1 in the Presence of Calcium Ions. The experiments were performed essentially as described under Figure 1 in the absence(———) or presence(—•—) of 5.0 mM $CaCl_2$ in 20 mM HEPES-150 mM NaCl, pH 6.8.

Phenyl-TSK in the presence of Ca^{++} suggesting the elimination of one component of the Ca^{++}-stabilized hydrophobic site in FII. We(8) and others(18) have shown that F1 inhibits the activation of FII by factor Xa in the presence of Ca^{++} and phospholipid. In data not shown, oxidation of one mol of Trp per mol F1 with NBS as described above destroyed the ability of F1 to inhibit FII activation under these conditions. This data, when combined with the metal ion specificity studies described above, suggests that this Ca^{++}-stabilized "hydrophobic patch" in FII is of physiological significance.

Hydrophobic interaction chromatography is proving to be increasingly useful in the study of proteins(19). It is becoming apparent that, with most native proteins, HIC is based on the presence of relatively small "hydrophobic patches" on the surface of the proteins(20-22). There are several recent examples from other enzyme systems of situations where modest changes in structure result in markedly differently behavior with HIC(23,24). As a results of the present study, we would suggest that FII is a amphitropic protein(25) demonstrating specific hydrophobic binding properties only in the presence of a functional divalent cation. This site is probably related to the Ca^{++}-specific epitope in human FII recently described by Borowski and coworkers(26). These results also provide an additional method for studying the structure of the vitamin K-dependent coagulation factors.

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