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Blood Coagulation Factor IX: Purification, Isolation, Activation

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Abstract

This paper attempts to provide an optimized strategy for the purification, activation, and isolation of blood coagulation Factor IX mutants. The goal of this work is to enable future biochemical and structural studies of Factor IX to a gain a better understanding of the structural-functional role this protein plays in the blood coagulation cascade. The orchestration and amplification of the blood coagulation cascade requires the binding of Factor VIII (FVIII) to an activated platelet surface, where it serves as a cofactor to a serine protease, Factor IX (FIX). Factor IX circulates the bloodstream as a catalytically silent multidomain protein[1]. Like many other coagulation factors, FIX requires cofactor-triggered and substrate-assisted modulations to become active[2]. Following activation of FIXa, formation of the FIXa/FVIIIa 'Xase' complex is enabled by structural rearrangements in the 99-loop and 60-loop of FIXa that allow for the proteolytic cleavage or turnover of FX to FXa[2,3]. The 200,000 fold activity enhancement that culminates from Xase formation is responsible for thrombin generation and blood clot formation[4]. A structural understanding of FIXa/FVIIIa activity and assembly in the Xase complex remains largely unknown and represents a fundamental challenge in the physiological understanding of blood coagulation disorders.

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Introduction

Hemostasis refers to the physiological process responsible for maintaining normal blood clotting in response to an injury. The delicate balance between blood clot formation and uncontrolled bleeding is achieved through the synergetic action of blood coagulation factors. One common feature of the coagulation factors is that they primarily circulate the blood in their inactive zymogen form to maintain hemostasis. These inactive precursors need proteolytic activation to develop enzymatic activity and often require a cofactor to reach full catalytic potential[5,6]. Blood coagulation is initiated through two converging pathways: the intrinsic and the extrinsic pathways [Fig1], both of which lead to the activation of factor X to factor Xa (FXa). Upon vessel injury, the cascade is initiated by the exposure of tissue factor protein to the bloodstream which forms a membrane-associated complex with Factor VIIa to form the extrinsic Xase complex. Sustained blood coagulation further requires activated factor IX (FIXa) to complex with activated Factor VIII (FVIIIa) on the surface of an activated platelet, forming the intrinsic Xase complex[2] .FXa propagates the cascade, creating a higher amount of thrombin for fibrin clot formation, which forms a mesh over the wound and allows for successful clotting[6]. Thrombin also induced the activation of Factor XI, which converts more FIX into FIXa. The intrinsic Xase complex, formed by FVIIIa and FIXa, plays an instrumental role in amplifying the final stages of the blood coagulation cascade, and the effect of the protein complex is illustrated by a 200,000- fold increase in FX turnover by FIXa upon assembly of the Xase[4,7].

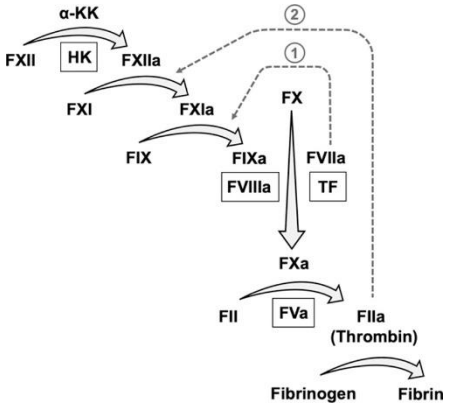

Figure 1: Blood Coagulation Cascade

Mutations or deficiencies in FVIII or FIX can disrupt the formation and efficiency of the intrinsic Xase complex among other things, and lead to blood coagulation disorders, as seen in patients with hemophilia A and B, respectively. Like many other blood coagulation factors, FIX circulates the bloodstream as a catalytically silent multidomain protein, requiring cofactor-triggered and substrate-assisted modulations to form the highly potent Xase complex[2]. FIX consists of a calcium-binding Gla domain, two EGF (epidermal growth factor)-like domains, an activation peptide, and the protease domain[7]. FIX is first activated by FXI into FIXa by cleavage of the activation peptide, but the activity of FIXa alone remains poor in the absence of cofactor FVIII. Thus, the Xase complex formation on the surface of an activated platelet with cofactor FVIIIa represents a critical second phase of activation. Despite the key physiological relevance, a structural understanding of the intrinsic Xase complex and the mechanism for enhanced FX turnover by activated FIXa remains only partially understood.

To understand the low-activity state of FIXa, previous work has been aimed at investigating whether and how selected recombinant amino acid substitutions in FIXa sequence led to increased catalytic activity. Indeed, Zogg *et al*[2] identified several Xase-like FIXa variants that illustrate a synergistic conformational activation mechanism. The structural and biochemical analysis of these variants reveals conformational changes in the 99-loop that are known to restrict substrate binding. Strikingly, the Xase-like mutants have improved substrate affinity (K_m) and turnover (k_{cat}), mimicking a Xase-like FIXa and revealing underlying mechanisms of FVIII-driven changes to FIXa in the Xase complex. Moreover, Knight *et al*[8], identified FIX variants with enhanced activity through ancestral sequence reconstruction. This variant, termed FIX An 96 demonstrated 12-fold greater FIX activity than human FIX, and displayed remarkable potency when administered to hemophilia B mice in vivo[8]. Together, this work represents an avenue for

future structural characterization of FIX activity and assembly in the Xase complex, as well as a platform for the identification of potential FIX therapeutics.

Drawing from recent work, the goal of this study was to optimize a strategy for expressing, purifying, and activating FIX mutants to enable biochemical and structural characterization. Human FIX is a highly post-translationally modified protein and consequently forms inclusion bodies when expressed in *E. coli*. Traditionally, refolding experiments have been employed to overcome this issue. Unfortunately, low yields coupled with the tedious nature of refolding experiments make it alluring to investigate alternative approaches. Overexpression of recombinant proteins that yield aggregates is fortunately not a new problem in biochemistry and many techniques have been used to facilitate the expression of soluble, correctly folded proteins. One such technique draws on the machinery of molecular chaperones. By co-transforming a chaperone containing plasmid together with a plasmid containing a desired protein of interest, proteins have reportedly been expressed in higher yields while avoiding undesired aggregation[9]. Moreover, once FIX is expressed in the soluble form, it requires cleavage of the activation peptide either by FXIa or Russell's Viper Venom (RVV-X), a procoagulant enzyme. In this study, we tested several methods for the expression, purification, and activation of FIX. The methods, results, as well as the intermediate obstacles of Part 1, are as follows: expression of FIX mutants with chaperones, refolding approach one, refolding approach two, activation of FIX An 96 variant with FXIa, activation of FIX mutant with RVVX, and finally a summary of the optimized purification, isolation, and activation scheme.

PART 1: Purification and Isolation

Expression of FIX Mutants with Chaperones

pET22b+ plasmids were obtained by Dr. Childers following correspondence with researchers at the University of Salzburg[2], one with FIX_2 mutant (K98T-Y177T) and one with FIX_3 mutant (Y94F-K98T-Y177T). A chaperone plasmid set was ordered from *Takara* (cat # 3340) to investigate how chaperones may help in the folding and solubilizing of FIX_2. Each of the three chaperone plasmids (10ng/µL) were individually co-transformed with FIX_2 plasmid (16.1ng/µL) into BL21 cells [Table 1]. To transform the cells, 50ng of chaperone plasmid and 50 ng of fiXa_2 plasmid were added to BL21 cells, incubated on ice for 30 minutes, and heat pulsed at 42° C for 30 seconds then cold shocked on ice for 5 minutes. Following the transformation, 950 µL of room temperature SOB was added to each of the three Eppendorf tubes and incubated for an hour at 37°C at 250 rpm. Then, 200 µL of the sample was plated onto agar plates containing 50 µg/ml ampicillin and 20 µg/ml chloramphenicol to select for cells containing both plasmids of interest, and the agar plates were incubated at 37°C overnight. Overnight growths were prepared and the following day 2XYT broth was prepared with appropriate antibiotics and inoculated with the 10 mL overnight

Table 1: Summary of Proteins for each transformation

	FIX_2	GroES	GroEL	dnaK	dnaJ	GrpE	tig
FIX_2 pGro7	43 kDa	10 kDa	60 kDa				
FIX_2 pKJE7	43 kDa			70 kDa	40 kDa	22 kDa	
FIX_2 pG-Tf2	43 kDa	10 kDa	60 kDa				48 kDa

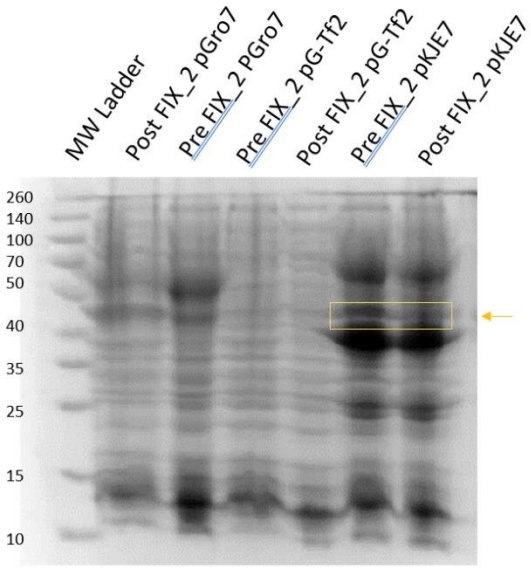

Figure 2: Solubility test performed pre/post centrifugation for each of the three samples

culture and 10 mL of ethanol. The cells were grown at 37°C, shaking at 200 RPM and when the sample reached O.D. of 0.4 chaperone expression was induced with either L-Arabinose or Tetracycline. The culture was turned down to 15°C for an hour until reaching O.D. of 0.6, and FIX_2 expression was induced with IPTG. The cell cultures were spun down at 6,371 g for 15 minutes. The cell pellets were lysed (0.5 mg/ml lysozyme, 20 mM PMSF, 20 mM Tris pH 8, 300 mM NaCl, 10 mM Imidazole, 10 % glycerol, and 2 % Triton X-100) and sonicated three times for 30 seconds on/off. The sample was spun down for 45 minutes at 40,000g and samples were taken pre/post-centrifugation for the purpose of an expression test (Fig 2). The results indicate that expression of FIX with chaperone pkJE7 increased solubility of FIX, but further purification experiments are required to separate FIX from plasmid proteins.

Expression of insoluble FIX Mutant: Refolding Approach # 1

FIX_2 constructs were expressed and pelleted. Lysis buffer was added to the 38 g pellet until solubilized. The lysate was sonicated for 3 cycles of 30 seconds on/ off, then centrifuged for 40 minutes at 37,000g. The supernatant was disposed and the pellet containing inclusion bodies was stored. Following Zogg paper[2], changes were made in the refolding procedure given that drop-wise dilution was not an option due to differences in equipment, and instead, the protein refolding experiment utilized gradual changes in dialysis buffer. The sample containing FIX_2 was resuspended and washed twice in 100 ml of inclusion body wash 100mM Tris pH 7.2, 20 mM EDTA, 0.5 M NaCl, 2% Triton X-100). After complete solubilization overnight (7 M guanidinium HCl, 100mM Tris pH, 20 mM EDTA, 100 mM DTT, pH 8.5), the sample was again centrifuged at 40,000 g for 15 minutes. The 100 ml of supernatant, after precipitates were removed, was saved to undergo a two-week refolding process. The initial 1-liter dialysis buffer contained 6M Urea, 0.3 M NaCl, 0.1 M arginine, 100 mM Tris-HCl, and 1 mM DTT, pH 8.5.

For 6 days, Urea was titrated out of the sample while increasing arginine and holding other components constant. Then DTT was gradually titrated out with small increments in $CaCl_2$, to a final concentration of 20 mM. Finally, the allegedly refolded sample was spun down at 40,000 g for 30 minutes to ensure that any insoluble protein was removed, the supernatant was passed over a QHP column and the components of each fraction were assessed using SDS Gel Electrophoresis [Fig 3].

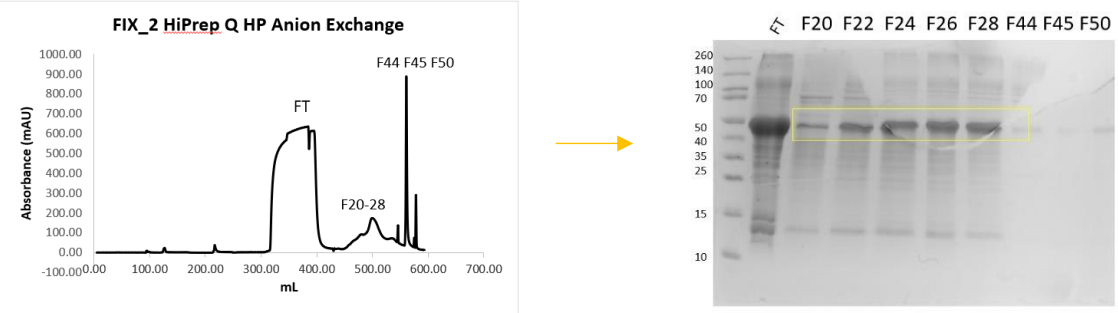

Figure 3: Column chromatogram with fractions assessed using gel electrophoresis

Fractions containing FIX_2 were pooled and concentrated, yielding 2 mg of FIX_2. FIX_2 was then activated to FIXa by RVV-X in a 1:50 molar dilution for 40 hours. A sample was run on a reducing gel to confirm the percent activated, given that the heavy and light chain will separate after activation in reducing environments. After the release of the activation peptide, FIXa_2 was active-site inhibited with 5 mM FPR-CMK. The catalytic, also known as the heavy chain of FIXa contains a heparin-binding site, using a Heparin Sepharose column, affinity purification was performed to isolate any not activated factor IX. Unfortunately, all of FIX eluted immediately, perhaps due to a problem with the Heparin column [Fig 4]. The sample was buffer exchanged to investigate if the binding was due to high salt, but the same results were found. A different

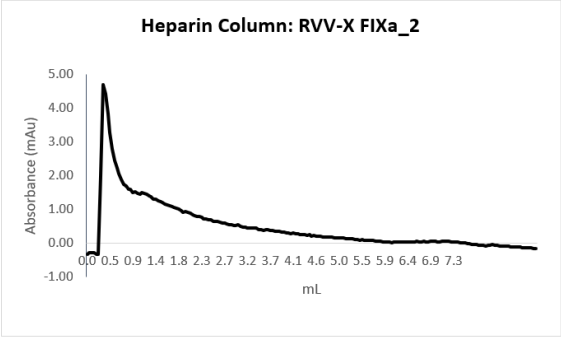

Figure 4: Heparin column chromatogram

approach was attempted following Brandstetter's 1995 paper[10], "RVV-X and not fully activated proteins were removed by negative chromatography on Q-Sepharose ff, making use of a shift in the isoelectric point upon removal of the acidic activation peptide. Only correctly activated proteins passed the column" the protein was loaded onto a Q column and expected to flow through[10]. Unfortunately, it did not and was finally eluted with 1 M NaCl in 20 mM Tris (pH 7.4), 5 mM $CaCl_2$. The protein was pooled, and dialyzed in a low salt solution (20 mM Tris, 2.5 mM $CaCl_2$, 25 mM NaCl pH 7.4) overnight, then concentrated on a 10 kDa MWCO to 0.5 mL with a final A_{280} of 1.6 mg/ml. Finally, size exclusion chromatography was performed on a Superdex 75 in 20 mM Tris, 2.5 mM $CaCl_2$, 20 mM NaCl pH 7.4. The chromatogram was different from expected, perhaps evidence of soluble aggregates and improper refolding technique. We concluded that the refolding experiment needed to be optimized, so as to not yield soluble aggregates. Areas of improvement include additional sonication between the inclusion body wash to resuspend and solubilize sample, the addition of 0.3 mM cystine, and 3 mM cysteine in the refolding buffer to drive the formation of disulfide bonds by creating a net oxidizing environment and allowing wrongly formed disulfide bonds to be reduced again, and finally, a correction should be made to shift the pH of the solubilization buffer to 3.5 to completely denature the protein of interest before refolding the denatured sample.

Expression of insoluble FIX Mutant: Refolding Approach # 2

Following a more recent protocol[7], the refolding scheme was optimized. FIX_2 constructs were expressed and pelleted. 100 mL of Lysis buffer (100mM Tris pH 7.4, 500mM NaCl, 20mM EDTA, 2% Triton X-100 and 1 mg/ml Lysozyme) were added to the 25 g pellet while stirring. The sample was sonicated for 30 seconds on/off 3x, then centrifuged for 40 minutes at 4°C, 40,000g. The supernatant was disposed of and the pellet containing inclusion bodies was washed

3 times in inclusion body wash (50 mM Tris pH 7.4, 50 mM NaCl, 20mM EDTA) with an additional round of sonication, and spun down at 40,000 g. FIX_2 was solubilized overnight in denaturant (50 mM pH 8.5, 50 mM NaCl, 20 mM EDTA, 8.5 M guanidinium chloride and 100 mM BME). _The 50 ml sample was dialyzed twice in 4 L of 20 mM EDTA at pH 4, quickly removing denaturant and the sample was once again spun down and dissolved in 25 mL of 50 mM NaCl, 8.5 M guanidinium HCl, 20 mM EDTA pH 3.5. For 2 days the sample was dialyzed in 4 L of refolding buffer (50 mM Tris pH 8.5, 150 mM NaCl, 20 mM $CaCl_2$, 500 mM L-arginine, 0.3 mM cystine, 3 mM cysteine). Refolded FIX_2 was dialyzed 3x in 4L of low salt buffer (20 mM Tris pH 7.4, 40 mM NaCl, 5 mM $CaCl_2$). Incorrectly folded protein was removed

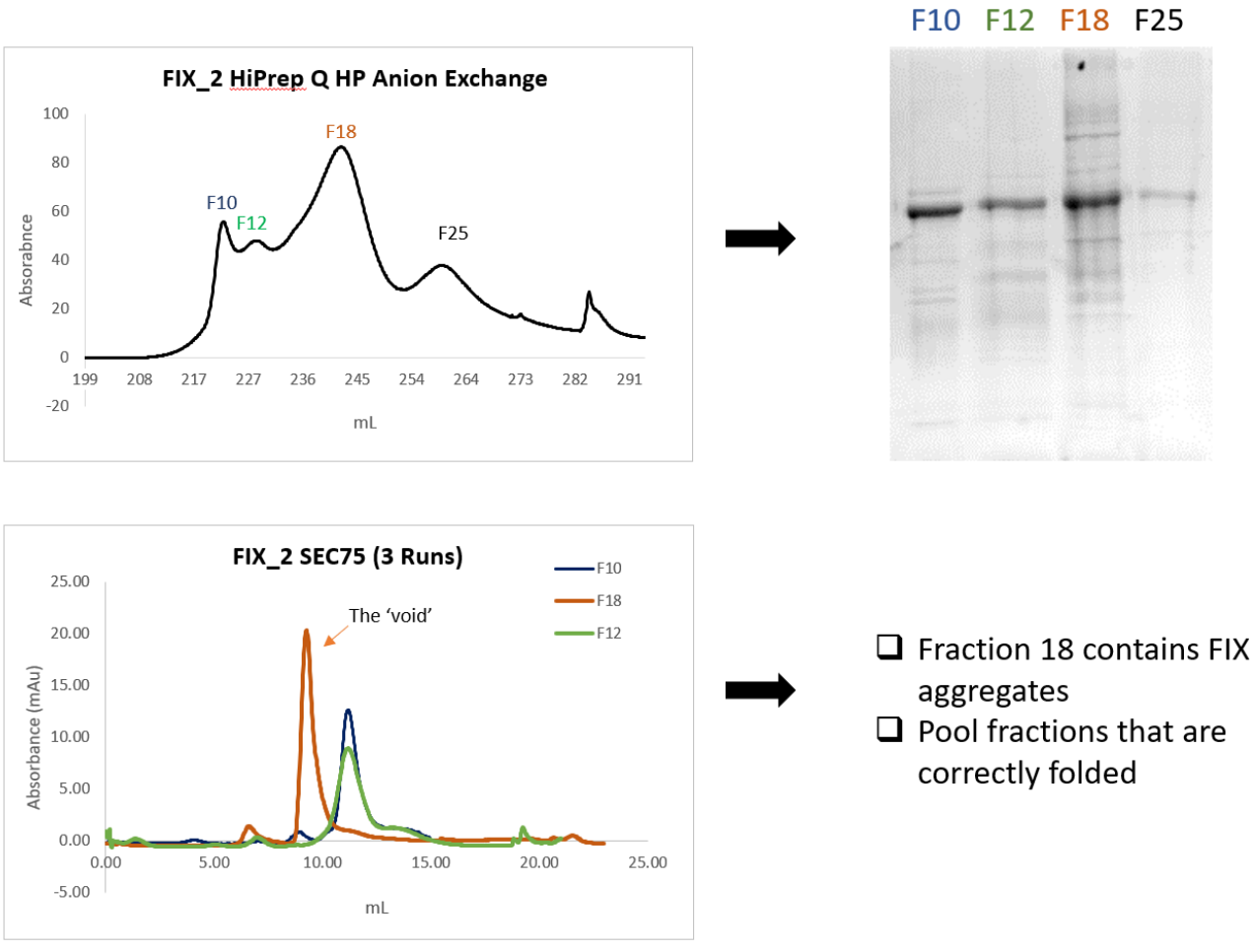

Figure 5: Optimized Purification Scheme to Account for Incorrectly Folded FIX

by centrifugation and the supernatant was saved for purification. FIX_2 with a PI (5.81) lower than pH of Buffer A (7.4) was purified using a QHP column flowing at 2ml/min, 80 mL of the sample at a concentration of 0.4 mg/ml was expected to pass in the flow-through according to previous work .[3,5] Conversely, the protein was eluted on a 40 mL gradient of buffer containing 1.0 M NaCl. Fractions 10, 12, and 18 were further purified with an SEC Superdex 75, revealing that fractions that eluted from QHP earlier were correctly folded [Fig 5].

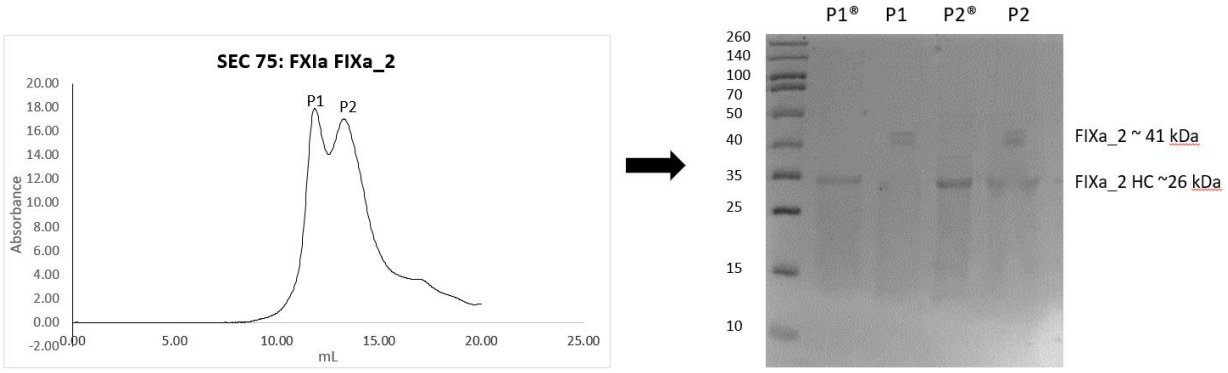

Figure 6: Size- exclusion chromatogram showing two overlapping peaks

After buffer exchanging and concentrating, activation of FIX_2 was achieved by incubation with 0.007 u of FXIa per µg FIX (*PROLYTIX)* and shaken gently at 20°C overnight. Activated FIXa_2 was inhibited with D-FPR-chloromethylketone (PPACK) to a final concentration of 5 mM. Further purification was performed using size-exclusion chromatography, with the intent to remove any FXI and inactive FIX_2. Two overlapping peaks were observed, suggesting that the earlier peak with a slightly higher molecular weight would contain FIX_2, and the later peak would contain FIXa_2 [Fig 6]. This reasoning stems from the fact that activation of FIX results in the removal of the 2.4 kDa activation peptide. To test our hypothesis, samples from peak one and peak two were analyzed using gel electrophoresis in reducing (Indicated by ®) and non-reducing conditions. The presence of the 26 kDa heavy chain in lanes one and three indicate that the two peaks observed in the chromatogram cannot be explained by the presence of inactive FIX. Following the results indicated in figure 6, the fractions were further analyzed using QTOF

mass spectrometry. Unfortunately, these results were inconclusive, peak two showed relatively low-resolution. Peak one contained two prominent peaks, both of which were not expected and may be a result of protease activity, that should be further investigated. Inactivated FIX_2 has a molecular weight of 43.8 kDa, and FIXa_2, lacking an activation peptide, has a molecular weight of 41.4 kDa. The inconclusive results from mass spec analysis are shown in Figure 7 and should serve as the basis of future investigations into FIXa protease activity.

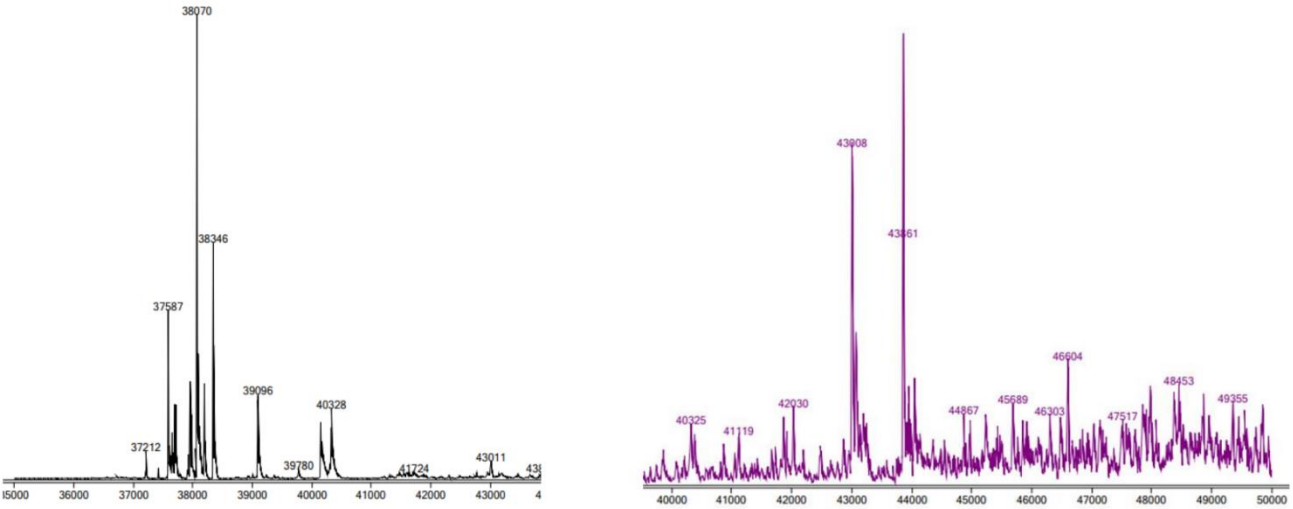

Figure 7: Mass Spectrometry analysis of two prominent signals in peak 1

Summary of FIX Purification, Activation, Isolation

The purification scheme for FIX mutants thus far, is best done through refolding experiments. Our results suggest that further optimization of the refolding scheme should be pursued and that key elements should be emphasized: 1) during inclusion body wash sonication should be performed to ensure homogenization, 2) the pH of the buffer prior to overnight denaturation should be low (pH=4) and the purpose of this intermediate step between inclusion body wash and overnight denaturation should be scientifically rationalized, 3) when the sample is denatured overnight excess buffer should be used, while the

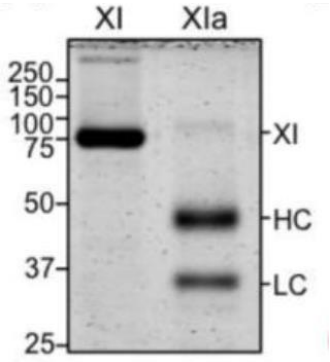

Figure 8: Gel electrophoresis showing HC & LC of FXIa[1]

literature suggests 25 ml per 2g of purified IBs, that is not enough to yield complete homogenization. In terms of isolating correctly folded FIX, the following should be recognized: 1) During the purification process, the literature notes that FIX will pass in the flow-through of a QHP column, although FIX has been observed to pass in the flow-through, it is quite possibly a mistake given the predicted PI of FIX and the pH of the buffers used, 2) 100% of the sample will not be correctly folded, it appears that modifications to the refolding process can increase the percent of the correctly folded protein recovered, but size-exclusion chromatography can be used to separate correctly folded FIX from FIX aggregate, 3) use of a heparin column has been under-investigated and utilized for the purification of both FIX and FIXa. For the activation of FIX, either RVV-X or FXIa can be utilized. However, Precautions should be taken depending on the approach: 1) under reducing conditions the 160 kDa homodimer FXIa, has two 80 kDa subunits which separate into a 50 kDa heavy chain and 30 kDa light chain [Fig 8], thus separation of FXIa and FIXa will require SEC under non-reducing conditions prior to any attempt to isolate FXIa heavy and light chains. 2) In any event that FIX is activated, longer incubation times and higher concentrations of activating protein appear to be optimal in terms of the percent FIX cleaved, but this may yield undesired protein degradation and protease activity, as well as misinterpretation of bands observed following gel electrophoresis.

PART 2: Preliminary Work and Future Directions

FIX An 96

Ancestral sequence reconstruction can be used to facilitate the identification of therapeutic protein variants with enhanced properties. Recently, researchers at Emory University resurrected a panel of ancient mammalian coagulation factor IX with the goal of identifying improved pharmaceutical candidates[8]. One variant, FIX An 96 demonstrated 12-fold greater FIX activity

production than human FIX. FIX An 96 has 39 substitutions but, a mechanistic understanding of the amino acid substitutions limited. Crystalizing of the heavy chain will be pursued in future work, as the activated heavy chain of several mutants has already been shown to crystalize well[2]. Currently, our laboratory has a limited supply of FIX An 96 that was provided to us by researchers at Emory University and expressed in HEK293 cells. An alternative approach would be to identify mutations of high interest, obtain plasmids with the truncated version of FIX that was shown to crystalize well, and purify FIX An96 variants through refolding experiments following expression in *E.coli*.

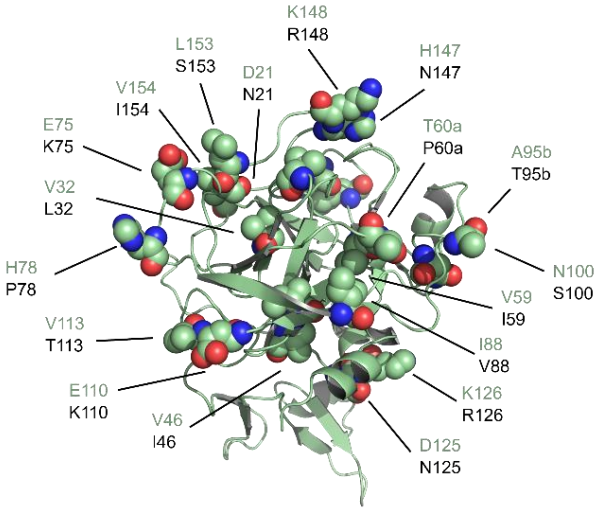

Figure 9: Model of point mutations between wild type FIX and FIX An96. Image courtesy of K. Childers.

MD Simulation

Molecular dynamics simulations on FIX variants will also be pursued in future work. Molecular dynamics simulations have become increasingly used in recent years to show the effect of a mutation, support experimental evidence, but most importantly generate enough representative conformations such that values of a property can be obtained. The structure Factor IX catalytic domain is available through the Protein Data Bank. Using PyMol, one could easily adjust this structure to include point mutations of interest and generate an initial atomic model for molecular dynamics simulations by utilizing GROMACS software. Exploratory work with GROMACS software has been conducted to examine FIXa_2 mutant with PPACK inhibitor bound [Fig 10], it may be advantageous to adjust the PyMOl structure such that PPACK inhibitor is absent, to explore the conformation dynamics between each state. Recently a 20 ns simulation, which is

highly feasibly with our current resources, was used to model FXa, a homolog of FIX, in the open and closed conformation[11]. Their findings emphasize the power of using MD simulations to analyze protein conformational changes in the nanosecond time scale and reveal dynamic interconversions between the open and closed states that are otherwise difficult to distinguish from the available static X-ray structures alone[11]. Molecular dynamics simulations have already proved useful in generating membrane-bound models of FX and FVIII[12–14]. Future computational work on FIX may aid in a better understanding of protein-protein interactions involved in the assembly of the Xase complex or provide a mechanistic understanding of the residues involved in the proposed induced-fit mechanism upon substrate binding[7]. Moreover, MD simulations provide insight into protein flexibility which is not only essential for understanding protein function but also advantageous for the identification of novel therapeutics in structure-based drug design efforts.

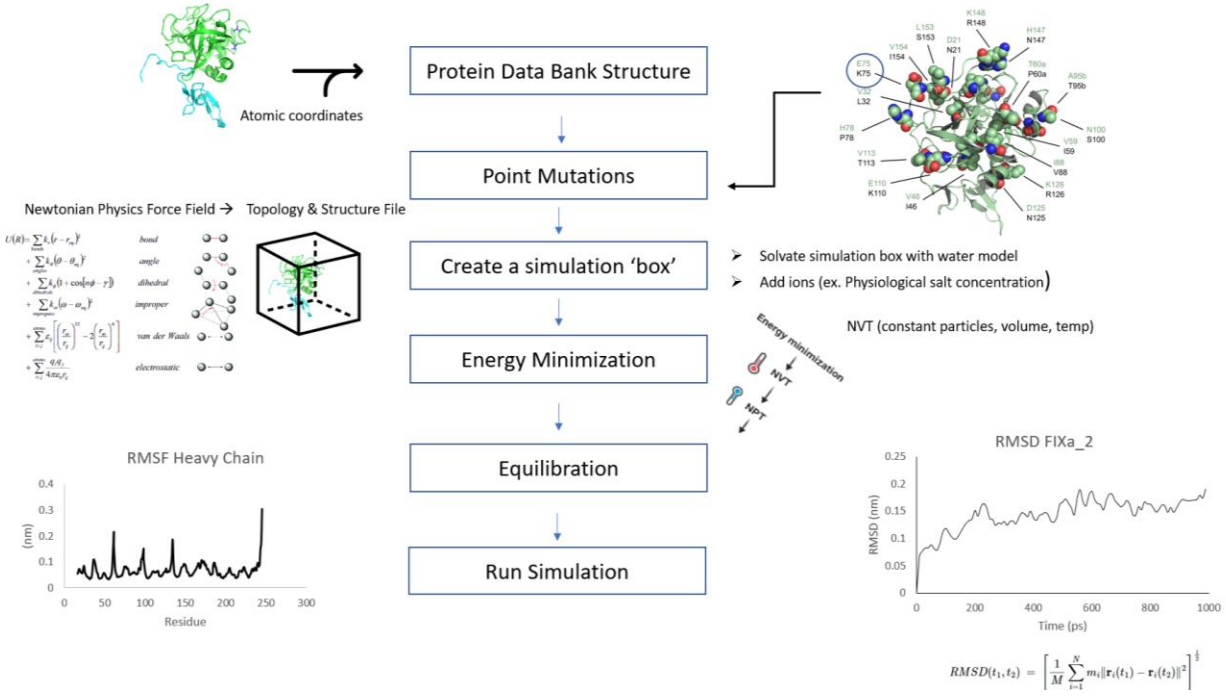

Figure 10: Example Schematic of MD Simulation Workflow

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