History of Discovery

Taking the Thrombin “Fork”

Kenneth G. Mann

Abstract—The proverb that probably best exemplifies my career in research is attributable to Yogi Berra (http://www.yogiberra.com/), ie, “when you come to a fork in the road … take it.” My career is a consequence of chance interactions with great mentors and talented students and the opportunities provided by a succession of ground-breaking improvements in technology. (Arterioscler Thromb Vasc Biol. 2010;30:1293-1299.)

As an undergraduate physics major at Manhattan College, I encountered C. W. Batt who hired me as a research technician for his studies involving the enzymology of lipases. I became fascinated with enzyme catalysis and started my slide from “pure” physical science to biology and medicine. After my Biochemistry PhD studies with Carl Vestling at the University of Iowa, I pursued biophysical chemistry with Charles Tanford at Duke as an NIH postdoctoral fellow. I aimed at having a career dealing with the mechanisms of protein folding. In the meantime, Bill Batt’s research program had become involved with the isolation of thrombin from the pharmaceutical “topical thrombin,” and he asked me to characterize the product he had isolated. Curiously, the preparations had constant specific activity toward a synthetic substrate (tosyl arginine methyl ester) but were highly variable in clotting activity. Utilizing newly developed techniques for molecular weight,1 and with the generous support of Charles Tanford, I aimed at having a career dealing with the mechanisms of protein folding. In the meantime, Bill Batt’s research program had become involved with the isolation of thrombin from the pharmaceutical “topical thrombin,” and he asked me to characterize the product he had isolated. Curiously, the preparations had constant specific activity toward a synthetic substrate (tosyl arginine methyl ester) but were highly variable in clotting activity. Utilizing newly developed techniques for molecular weight,1 and with the generous support of Charles Tanford, I characterized these preparations and concluded that there were multiple forms of thrombin related by proteolytic cleavage,2 subsequently designated α, β, and γ thrombin.3

Prothrombin Activation and Structure

I accepted an assistant professorship in Biochemistry at the University of Minnesota in the College of Biological Sciences, where I pursued protein folding as my primary objective. As a side “hobby,” and with the enthusiastic encouragement of Roger Lundblad, I elected to also pursue prothrombin activation using the biophysical techniques that had enabled our studies of thrombin and the new technique of SDS gel electrophoresis.4 At the time, the most prominent laboratories utilizing quantitative protein chemistry to study coagulation reactions were those of Earl Davie,5,6 Russell Doolittle,7,8 Harold Scheraga,9 Donald Hanahan,10 and Stephan Magnusson.11 I sent a research grant to the NIH requesting support for my studies and, with my sense of youthful hubris, I anticipated I would start those studies when the grant money came in. In the meantime, I was befriended by Walter Bowie and Charles Owen at the Mayo Clinic who helped me in interpreting the somewhat confusing coagulation literature.

In July 1970, I received a letter from the NIH signed by Fann Harding that indicated that my grant application was not only not to be funded but also it was disapproved based on my inadequate scientific credentials and my naive biophysical approaches to the problem. In those days, before the availability of “pink sheets,” one contacted the NIH study section executive secretary who could provide excerpts from the reviewers’ comments. Dr Harding provided a description from her notes detailing the reviewers citing my lack of appropriate scientific training and the ridiculousness of my approach. My response was determination to show my new colleagues in the Hematology Study Section that they were incorrect.

My colleagues in the laboratory at the time were David Fass, who had come to Minnesota as a postdoctoral fellow to work on Neurospora genetics but was abandoned by his preceptor who was more interested in protesting the Vietnam War, and Charles Heldebrandt, a first-year graduate student from Berkeley who overslept his meeting with his primary choice of preceptor and ended up with me. Because the techniques I anticipated using were material-intensive and my budget was limited, at the suggestion of my wife Jeanette, I acquired an antique ($25) cream separator to prepare plasma. We separated bovine citrate blood into plasma and cells using the modified, antique, hand-crank cream separator and developed isolation procedures for prothrombin and thrombin. We then initiated the first SDS4 mass-based analysis for conversion of a zymogen to a protease in studies of prothrombin activation.12 These initial studies were presented at the 1970 Federation of American Societies for Experimental Biology meeting and were sufficiently well-received by the Hematology community that the previously disapproved grant application entitled the “Activation of Prothrombin” was approved and funded, and it has been continuously supported since 1972 as an R01, a Specialized Center of Research, and a Program Project Grant. Our studies and contemporaneous reports from the Jackson13 laboratory led to the evolution of sequences of bond cleavage during prothrombin activation described by pathway 1 to 3 (Figure 1); meizothrombin was not found, and thus pathway 2 to 4 was excluded (but see later).
Influence of factor Va on the reaction mechanism. Activation of human prothrombin by human prothrombinase.

- Arg286-Thr287.

Figure 1. Schematic illustration of the pathways for the activation of human prothrombin. Cleavage of prothrombin at Arg273-Thr274 (Reaction 1) yields Fragment 1-2 and prothrombin 2. Further cleavage of prothrombin 2 at Arg323-Ser324 (Reaction 3) yields a disulfide-linked two-chain form of α-thrombin. Cleavage of prothrombin in the opposite order (cleavage at Arg273-Thr274; Reaction 4) yields meizothrombin. Meizothrombin is composed of the Fragment 1-2 and α-thrombin chain that are covalently linked by a disulfide bond. Further cleavage at Arg273-Thr274 produces Fragment 1-2 and α-thrombin. The arrows labeled a and b indicate bonds that are susceptible to cleavage by the feedback action of thrombin. a = Arg155-Ser156, b = Arg286-Thr287.


Our success led to an inquiry from Walter Bowie as to whether I would consider joining the research group that he had formed at the Mayo Clinic/Foundation as a member of the Department of Medicine. The notion of joining a Department of Medicine was completely foreign to me because I had always presumed that my research career would be in an academic Biochemistry Department. However, Walter’s enthusiasm coupled with the generosity of the Mayo recruitment package led me to take the Medicine “fork.” My immersion in the medical community at Mayo was an epiphany for me. I recognized the power of subjective clinical interpretation in understanding human pathology and the need to integrate the data from fundamental laboratory science with the observations of experts in clinical medicine to produce internally consistent descriptions of human physiology and pathology. The mentorship by members of the internal medicine community at Mayo had a profound effect on my intellectual horizons.

Five years later, I became Vice Chairman of Medicine for Research at Mayo. In this role, I was able to catalyze the development of research teams in most medicine divisions that incorporated both clinical and basic investigators.

At Mayo, our laboratory initially focused on metal ion and phospholipid binding by prothrombin and factor Xa with Paul Bajaj. With the advent of the revolutionary Beckman spinning cup sequencer, we elected to pursue amino acid sequence studies on the human protein with Ralph Butkowski, Michael Downing, and Jacques Elion. Frank Pendersgast, another “refuge” who migrated to my laboratory, found time between his green fluorescent protein/aequorin PhD studies to investigate the conformational changes in prothrombin associated with metal ion binding. In these studies we noted, but could not explain, a synergy between Mg2+ and Ca2+ binding to prothrombin fragment 1 that has since been resolved by x-ray crystallographic studies from the Bajaj laboratory demonstrating that magnesium ions occupy some of the sites in the GLA domains. The identifier (GLA) of the vitamin K-dependent proteins was a consequence of the brilliant work of Stenflo, who showed that the effect of vitamin K, essential to the membrane binding qualities of the vitamin K-dependent proteins, involved the posttranslational biosynthesis of γ-carboxyglutamates from pre-existent glutamate residues in the nascent prothrombin molecule. This observation led to the expansion of the vitamin K metabolic pathway by Suttie and Stafford.

Factor V and Prothrombinase

Factor V remained an elusive piece of the prothrombinase puzzle. Although identified in 1943, its isolation proved difficult. A fortuitous event occurred when Michael Neshem’s graduate student support was terminated by his preceptor. I hired him as a temporary technician so he could complete his thesis and find an appropriate postdoctoral position. While I was writing a renewal application during the Christmas break, Michael showed me an electrophoresis gel with a single band (Figure 2) that he identified as factor V. In response, I indicated he had found his postdoctoral position. Soon thereafter, Esmon isolated factor Va and identified it as a 2-chain molecule with a divergent metal ion requirement for association. Michael characterized factor V, its conversion to factor Va, and its incorporation into the prothrombinase complex using biophysical and quantitative enzymatic studies.

Human factor V (“labile factor”) isolation proved to be much more difficult because fragmentation of the protein was clearly occurring during isolation. The development of monoclonal antibodies by Kohler and Milstein appeared to offer an option for an immunochromes isolation. Jerry Katzmann was familiar with hybridization technology and, with Neshem, made the first monoclonal antibody to a coagulation protein, factor V. These monoclonal isolation techniques were subsequently expanded to all the proteins of the coagulation proteome.

The prothrombinase studies were facilitated by the development of 2 new technologies, ie, the use of fluorescent argininepeptidodichloromethylketones to label the active site of factor Xa and the development of “DAPA,” a fluorescent, reversible, specific inhibitor of thrombin that permitted studies of prothrombin activation without the complication of feedback cleavage of prothrombin by thrombin. We utilized argininepeptidodichloromethylketone-modified factor Xa to evaluate the incorporation of factor Xa into factor Va membrane complexes, showing a 1:1 stoichiometry. The same stoichiometry was observed on platelets by Paula Tracy, who brought cell biological experience and initiated our transition into the cell biology realm. Utilizing doubly labeled factor Va and factor Xa simultaneously added to activated platelets, Paula was able to establish that the prothrombinase complex on platelets was also a 1:1 complex with activity equivalent to that seen on phospholipids (Figure 3).
Another major problem associated with identifying the contributions of the components to the prothrombinase complex were the complex interdependent interactions between factor Va–membrane, factor Xa–membrane, prothrombin–membrane, protein–protein binding interactions, and the catalytic process itself, all of which are altered when any one component's concentration is altered. The independent contributions of the 5 components to the assembly of the prothrombinase complex utilizing the traditional means of altering the concentration of each component while holding the others constant thus was not achievable because component concentration changes altered protein–protein and protein–membrane occupation. This problem was overcome by Russell Tracy and Michael Nesheim, who developed the program "Clot speed," a numeric model solved on the newly available Apple computer (Apple, Cupertino, Calif) that could explain the complex interactions between the different proteins and membrane (Figure 4). These models have been improved and expanded as more data and faster computers became available.

Studies of the stoichiometric prothrombinase complex created a paradigm shift. Most coagulation experts held that the cofactors were important accessories to the coagulation process. In contrast, the prothrombinase kinetic data showed that the membrane-bound 1:1 complex of factor Xa and factor Va was the only biologically relevant enzyme with an activity over 300,000 times that of the free factor Xa (Table). Although explicit data were not available for the other vitamin K-dependent enzymes, the existing information suggested that the factor VIIIa–factor IXa complex (intrinsic factor Xase) was a homolog to prothrombinase. We hypothesized this equivalence (Figure 5) and subsequently expanded the concept to the tissue factor–factor VIIa complex with the reports from the Nemerson and Edgington laboratories that isolated tissue factor and expressed the recombinant protein. Subsequently, the elegant work of Esmon, Owen, and Esmon identified and isolated thrombomodulin, the essential cofactor for thrombin in protein C activation. This addition established the quartet of 4 vitamin K-dependent complexes performed their functions in a homologous fashion.

Figure 3. Double reciprocal plot of the binding of Factor Xa to unstimulated platelets derived from equilibrium binding measurements of radiolabeled Factor Va and factor Xa to platelets. The binding of Factor Xa to platelets was modeled as Factor Xa binding to platelet-bound Factor Va and can be described by Equation 1 previously detailed under "Methods" (Tracy PB, Nesheim ME, Mann KG. Coordinate binding of factor Va and factor Xa to the unstimulated platelet. J Biol Chem. 1981;256:743–751.). Accordingly, the ratio of molecules of platelet-bound Factor Va to platelet-bound Factor Xa is plotted as a function of nominal Factor Xa concentration. The plot gave a dissociation constant of $6 \times 10^{-10}$M and a binding stoichiometry of 1.04 molecules of platelet-bound Factor Va bound per molecule of bound Factor Xa.

Figure 4. Concepts employed in the development of the model of prothrombinase. The circle in the center represents a spherical phospholipid vesicle. This is surrounded by a region designated the interface shell (Nesheim ME, Eid S, Mann KG. J Biol Chem. 1981;256:9874–9882). and is defined on the basis of the increase in hydrodynamic radius of the scattering particles that occurs upon binding of prothrombin or Factor Va to PCPs vesicles (Lim TK, Bloomfield VA, Nelsestuen GL. Biochemistry. 1977;16:4177–4181; Higgins DL, Mann KG. J Biol Chem. 1983;258:6503–6508.). This region is surrounded by the bulk solution. Catalysis occurs by a mechanism that conforms to the Michaelis-Menten equation in both regions, with Km = 131 µm. In solution, the enzyme is considered Factor Xa with kcat = 0.61 min⁻¹, whereas in the interface shell the enzyme is considered the Factor Xa-Factor Va complex with kcat = 2100 min⁻¹ (Nesheim ME, Taswell JB, Mann KG. J Biol Chem. 1979;254:10952–10962; Rosing J, Tans G, Govers-Riemslag JWP, Zwaal RFA, Hemker HC. J Biol Chem. 1980;255:274–283). The rate of the reaction is given by the sum of velocities in both regions, as indicated by the equation on the figure.

The last term on the right has been multiplied by ΔL[L]0 in order to express rates in the interface shell with respect to the total volume of the system. The term ΔL[L]0 is the total volume occupied in the system by the interface shell region, where [L]0 is the total phospholipid concentration and 6 is a constant of proportionality. The volume occupied by the total interface shell region is typically a very small fraction (0.01%) of the total volume of the system. Although the region is “small,” it constitutes the locus in which the vast majority of prothrombin conversion occurs. The application of the model involves determination of the distribution of enzyme and substrate between bulk solution and the interface shell for any given set of initial concentrations of enzyme, substrate, and PCPs. From the distribution, bulk and interface shell concentrations are calculated, which in turn permit the calculation of expected reaction rates.


Subsequent to my move to Vermont, using a combination of fluorescence and light scattering stop-flow experiments, Sriram Krishnaswamy and Ken Jones45 described the kinetics of the assembly of the prothrombinase complex on synthetic membranes. These experiments showed the initial step in the formation of complex is the binding of each protein to the membrane with a subsequent rapid rearrangement to form prothrombinase (Figure 6).

Krishnaswamy,44 in collaboration with Nesheim, also reevaluated the pathway to thrombin catalyzed by prothrombinase and showed that prothrombinase activation of prothrombin, in contrast to the activation of prothrombin by factor Xa, proceeded through pathway 2 to 4 (Figure 1), with the initial product being meizothrombin. The mechanistic change associated with prothrombinase activation of prothrombin had been overlooked because the issue of the pathway to prothrombin activation in the absence of meizothrombin shown in earlier studies had been “settled.” Why these pathways diverge is still an area of active research.45,46

Michael Kalafatis47 initiated studies of the process of inactivation of factor Va by the activated protein C system.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Rate*</th>
<th>Relative Rate</th>
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<tbody>
<tr>
<td>Xa</td>
<td>0.0044 ± 0.0015</td>
<td>1.0</td>
</tr>
<tr>
<td>Xa, PCPS</td>
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<td>1.0</td>
</tr>
<tr>
<td>Xa, Ca²⁺</td>
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<td>2.3</td>
</tr>
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<tr>
<td>Xa, Ca²⁺, PCPS, Va</td>
<td>1210 ± 70</td>
<td>278 000</td>
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<tr>
<td>Xa, Ca²⁺, PCPS, Va</td>
<td>2100 ± 120</td>
<td>480 000*</td>
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*Rates expressed as mol of thrombin per minute per mol of factor Xa ± SD of at least 3 measurements.

Velocity at Vmax.

All reactions were performed at 22°C in 0.02 mol/L Tris.HCl and 0.15 mol/L NaCl (pH 7.4). Prothrombin was preincubated in Ca²⁺ (when used) at least 10 minutes before the initiation of the reactions with factor Xa. Final concentrations of the various components were: prothrombin, 1.39×10⁻⁶ M (0.1 mg/mL); factor Xa, 5.0×10⁻⁸ to 1.0×10⁻⁶ M (275 to 550 ng/mL); factor Va, 1.5×10⁻⁸ M (5 µg/mL); Ca²⁺, 2.0 mmol/L and PCPS (75% phosphatidyl choline, 25% phosphatidyl serine), 1.15×10⁻⁵ M.

Velocities of the first 5 reactions were measured by discrete thrombin assays with S2238. The last 2 reactions (Xa, Ca²⁺, PCPS, Va) were monitored with DAPA.


Figure 5. Model of two collected blood coagulation reactions: conversion of factor X to factor Xa; conversion of prothrombin to thrombin. Their respective catalysts are bound adjacent to one another on their respective cofactors on the platelet membrane.

The development of the gas phase sequencer and the technology of cDNA sequencing advanced knowledge in an exponential fashion.

The Tissue Factor Pathway Coagulation Proteome

Osterud and Rapaport identified that factor IX was a substrate of factor VIIa tissue factor; however, the rate appeared too slow to be biologically relevant. Jeffrey Lawson made the discovery that both substrates were activated at approximately the same rate because factor Xa feedback participated in factor IX activation. These studies provided an important step in the linkage between the tissue factor-initiated coagulation and hemophilia A and hemophilia B. More importantly, this result and his access to the complete inventory of the related procoagulant proteome components led Jeffrey to reconstruct and analyze the tissue factor proteome at biologically relevant concentrations using isolated proteins. These data caused Ken Jones to temporarily halt his interest in environmental politics to develop a numeric model to explain the steps of cleavage and loss of activity in factor Va on activated protein C cleavage, including 506 and 306 cleavages and the dissociation of the A2 domain fragments from the molecule. In his spare time, Matthew also crystallized factor Va, with Stephen Everse, and Ty Adams produced a crystal structure for this protein.

Corn Trypsin Inhibitor, Tissue Factor, and Blood

The in vitro reconstructions of the tissue factor pathway were extended to studies by Matt Rand of whole blood enabled by the use of corn trypsin inhibitor, which inhibits the contact pathway and the Western blot technique (Figure 7). These studies were extended by Kevin Cawthern and Kathleen Brummel-Ziedins. Tom Orfeo subsequently combined the corn trypsin inhibitor blood model, the proteome model, and the numeric model to show that in hemorrhage control, the steps of reaction is largely governed by the supply of prothrombinase assembly. Prothrombinase assembly on the PCPS vesicle surface proceeds after initial reactions that yield separate Xa-PCPS and Va-PCPS binary complexes on the surface of the same vesicle. The PCPS-bound proteins then react rapidly on the vesicle surface to form prothrombinase.

From: Krishnaswamy S, Jones KC, Mann KG. Prothrombinase assembly by the PCPS vesicle surface proceeds after initial reactions that yield separate Xa-PCPS and Va-PCPS binary complexes on the surface of the same vesicle. The PCPS-bound proteins then react rapidly on the vesicle surface to form prothrombinase.
Summary

The somewhat schizophrenic existence of the laboratory is illustrated by dual interests in fundamental molecular architecture and mechanism and the translation of information relevant to human physiology and pathology. My experience illustrates the significance of chance in identifying the direction a research program takes. Chance includes both the unanticipated acquisitions of talented collaborators and the opportunities provided by development of new technologies that dramatically expand research horizons. The interactions of individuals with different skills, talents, and goals provide the combinations of imagination, technical competence, and ambition that occur when multiple individuals work in synergy to address important problems.

Technological advances were clearly fundamental to our success. The developments of SDS electrophoresis, automated protein sequencers, molecular biological techniques, and monoclonal antibodies were essential. These technical advances occurred at "the right times."

As academic scientists, we produce 2 products. One is represented by papers that have some temporal utility. The other is represented by the individuals who extend, as independent investigators, their contributions to research and the training of new generations of scientists. Among the best life experiences in academic research are the pleasure of making new discoveries and the immense pleasure of observing the success of your protégés making new discoveries and developing their protégés.

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Disclosures

None.

References


Figure 8. Scheme of a two-compartment model of the regulation of TF-initiated blood coagulation. A cross-section of a blood vessel showing the luminal space, endothelial cell layer, and extravascular region is presented at the site of a perforation. The blood coagulation process in response is depicted in four stages. Tissue factor-factor Villa complex, TF-Villa; prothrombinase complex, Xa-Va; intrinsic factor Xase, Villa-Xa; ATIII-endothelial cell heparan sulfate proteoglycan complex, bound to thrombin or factor Xa, HS-ATIII-Xlla or Xa; protein C bound to thrombomodulin-thrombin, TM-lla-PC.


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