Crystal Structure of the Antihaemophilic Clotting Factor IXa : Explanation for Its Activity Enhancement towards Factor X upon Complex Formation with Factor VIIIa

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ABSTRACT

Hereditary deficiency of factor IXa (fIXa), a key enzyme in blood coagulation, causes haemophilia B, an X-linked severe bleeding disorder occurring in approximately 1 in 30,000 males. About 500 different mutations, more than for any other human gene, have been described for fIX. The X-ray crystal structure of full-length porcine fIXa β determined in the absence of calcium reveals a fIXa-like catalytic module, with D-Phe-Pro-Arg-chloromethyl-ketone (PPACK) covalently bound to an active site of thrombin-like geometry. The entrance frame to the specificity pocket, however, displays a considerable trembling motion imposed by the high-energy conformation of residue Glu388 (C219), which is strictly conserved in fIX species but replaced by glycines in all other serine proteinases of trypsin-like specificity. Dynamic simulations suggest a cryptic pocket in unliganded fIXa β and the requirement for its opening upon substrate binding, in agreement with the very low activity of free fIXa β towards chromogenic substrates. The two EGF-like domains interact at an angle of 110°, stabilized by a strictly conserved and fIX specific inter-domain salt bridge. The Gla domain, only defined for its hydrophobic stack, has been modeled in its calcium form using prothrombin fragment 1. Due to the inter-EGF bend, the distance from the active site residues to the expected membrane plane is almost 90 Å, in fair agreement with results obtained from fluorescence energy transfer experiments. Most natural mutations, which affect conserved fIX-specific surface residues, map to the concave surface strip of the bent molecule, probably indicating its involvement in intrinsic fIXase complex formation. In the membrane-bound complex, fIXa β and its substrate fX seem to envelope parts of fVIIIa from opposite sides with their catalytic modules pushed together, so that the opening of fIXa's active site is enforced. This model could explain the tremendous activity enhancement of fIXa upon complex formation.

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Human fIX¹⁾, after intracellular processing, is secreted as a 415 residue single chain molecule into the blood, where it is activated to fIXa β upon proteolytic cleavage at Arg145-Ala146 and Arg180-Val181 (Ile in porcine fIX). The N-terminal light chain (146 residues) and the C-terminal heavy chain (235 residues) of porcine fIXa β ²⁾ are disulfide connected through Cys132-Cys289 (C122) (fIX residues will be numbered according to the human fIX sequence³⁾, with heavy chain residues also designated by the chymotrypsinogen numbering assigned with a prefix 'C'). The light chain consists sequentially of the Gla/hydrophobic stack module (residues 1-38 and 39-46), two EGF-like domains (47-84 and 85-127), and a linker-peptide (128-145), whereas the heavy chain (181-415) forms the catalytic module (with the domain boundaries used here corresponding to the intron/exon boundaries⁴⁾). Free fIXa β is an extremely poor proteinase⁵⁾, but becomes a potent fX activator upon calcium-dependent complex formation with its cofactor, fVIIIa, on anionic phospholipid membranes or on the surfaces of endothelial cells or activated platelets⁶⁾. This so-called intrinsic fXase complex converts fX to fXa on the intrinsic blood clotting pathway by catalyzing hydrolysis of a single bond at Arg194-Ile195.

Of the related blood clotting proteinases, X-ray structures are available for thrombin and several thrombin complexes⁷⁾, of prothrombin fragment 1 (consisting of a Gla-domain and kringle 1⁸⁾), and of Gla-domainless fXa, in which the EGF1 module is disordered⁹⁾. NMR-structures have been reported for the EGF1 and EGF2 domains of fIX and fX¹⁰⁾, and for the calcium-free and the calcium stabilized Gla domain of human fIX¹¹⁾. Our structure of a porcine fIXa β represents the first reported spatial structure of a full-length, membrane-dependant clotting fac-

tor.

This PPACK-fIXa β structure¹²⁾ reveals a tulip plant-like shape, with the catalytic module and the active-site residues representing the blossom and the pistil, the short linker peptide the perianth, the two interlaced EGF-domains the bent stalk, and the N-terminal Gla-domain the bulb (Figs. 1, 2). The overall length and maximal spatial extension of the molecule are 130 Å and 105 Å, respectively. The catalytic module exhibits the typical fold of (chymo)trypsin-like serine proteinases, i.e. essentially consists of two interacting six-stranded barrel-like domains, covered by three helical segments and several surface located polypeptide loops; the Ile181 (C16) ammonium group forms with the Asp364 (C194) carboxylate the buried salt bridge characteristic for an "active" state, in agreement with the unfavourable effect of mutations in both fIXa residues on clotting activity¹³⁾. Significant main chain deviations to the fXa catalytic module are only observed in the loops around residues 201 (C36) and 298 (C129), 224 (C60), and 260 (C95), where fIXa exhibits single residue deletions, an identical number of residues, and a two residue insertion, respectively. Like fXa, fIXa carries a more acidic surface patch, where the strongly basic fibrinogen recognition exosite extends in thrombin⁷⁹⁾; the underlying acid-rich Glu235 (C70)-Glu245 (80) calcium binding loop, the integrity of which is essential for clotting activity¹³⁾, is ordered, with the GluC70 and GluC80 side chains and the 237 (C72) and 240 (C75) carbonyls liganding to a spherical electron density, which in the absence of calcium might be accounted for by a water molecule. Different from fXa, the Glu224 (C60)-Pro225 (C60A) segment and the exposed surface loop following Asn26 (C95) are widely separated leaving between a furrowed exit from the active site. In general,

most structural differences between the fIXa and the fXa catalytic domains show up in the specificity pocket (see below) and in several distinct surface residues, rather than in the overall shape and fold.

In the center of the active-site groove of fIXa β , which extends along the modular surface opposite to the linker/EGF2 attachment site, reside the activesite residues Ser365 (C195), His221 (C57) and Asp269 (C102). This active-site triad and the surrounding residues known to be essential for catalysis (such as the residues forming the oxyanion hole) display a geometry virtually indistinguishable from that found in PPACK-thrombin⁷⁾, due to an identical hemiketal linkage via SerC195 O γ and HisC57 N ϵ 2 to the carbonyl and the methylene group of Arg3I of the bound PPACK (with PPACK numbers bearing a suffix 'I'). The peptidyl moiety of PPACK juxtaposes fIXa segment Ser384 (C214)-Gly386 (C216) in a twisted antiparallel manner. The specificity pocket opening aside SerC195 accommodates the extended Arg3I side chain forming the usual charged hydrogen bonds with the Asp359 (C189) carboxylate group at its bottom. The Ser at position 360 (C190) renders the fIXa pocket more trypsin-like, presumably resulting in an equal acceptance of Lys and Arg as P1-side chains. Amino acid replacements in and around this pocket should impair its correct formation or productive substrate binding, in agreement with bleeding disorders observed in sush mutants (c.f.ref.14).

The Tyr266 (C99) phenolic group of fXa β is displaced from its location in fXa⁹⁾ leaving space between for accommodation of Pro2I or of Thr, Val, Phe or Trp-P2-residues of natural (fX) or synthetic substrates⁵⁾. As a consequence, the "pocket" left between TryC99, TrpC215 and residue Phe342 (C174) is too small for the benzyl group of D-phenI to

slot into. The clear electron density for residues C174 and C192, originally assigned by cDNA sequencing³⁾ to porcine fIXa as Val and Ile, allowed their identification as Phe and Gln, respectively; both crystallographic assignments were recently confirmed by reviewing the original sequencing data¹⁵⁾.

The entrance frame to the fIXa β specificity pocket, segment Ser384 (C214)-Cys389 (C220), exhibits (due to the strict deletion of a chymotrypsinogen-equivalent residue C218) a conformation typical for all active serine proteinases of trypsin-like specificity. It is tightly fixed only in its initial part till Trp385 (C215), however, while the consecutive kinked segment Gly386 (C216)-Glu387 (C217)-Glu388 (C219)-Cys389 (C220) is (in contrast to all other active trypsin-like enzymes) much more mobile; in particular the carbonyl group of GluC219 is only weakly defined by electron density, but refines unequivocally to an orientation towards the S1-pocket, where it forms a favourable hydrogen bond with the outermost guanidyl nitrogen of Arg3I.

GluC219 (like the equivalent Gly-residues in all other trypsin-like enzymes) exhibits a high energy main chain conformation ($\phi \approx 55^\circ$, $\Psi \approx -164^\circ$), which in proteins is generally only adopted by Gly residues, but is in rare cases also found for non-Gly residues. FIXa is the only serine proteinase of trypsin-like specificity, where this position C219 is occupied by such a non-Gly residue; in addition, GluC219 is strictly conserved in the seven fIX sequences known so far²⁾. Preliminary dynamics simulations with a fIXa β model, from which the PPACK inhibitor is removed, show a quite dynamic trembling of GluC219 including its carbonyl group and a twisting motion of this entrance frame segment, resulting in a partially collapsed pocket incompatible with normal substrate binding, in strong contrast

to similar runs on fXa.

This partially occluded specificity pocket might explain the several orders of magnitude reduction in catalytic efficiency (k_{cat}/K_m) of fIXa β towards chromogenic amide substrates and its poor binding capability for synthetic inhibitors compared, e.g., with fXa⁵. Recognition and binding of specific (i.e. Arg and Lys containing) peptidic substrates and chloromethylketones should be hindered, so that the scissile peptide bond and the reactive groups, respectively, should be less frequently and correctly presented to the active-site residues. Benzamidine, e.g., will have to fix the C219 carbonyl group and to widen the pocket upon binding, and will be rapidly expelled, reflected by a 15 and 100 fold lower affinity towards fIXa β compared with fXa and thrombin¹⁷). In the presence of heparin, the plasma inhibitor antithrombin III nevertheless can block the active center of fIXa at a medium rate, presumably due to more elaborated interaction sites in the vicinity. This cryptic state of fIXa β is somewhat reminiscent of the mobile and occluded S1-pocket, which in the trypsinogen-like zymogens preclude binding of substrates and inhibitors, if not overcome by a very high affinity for the active state-like binding sites¹⁸).

A depression on the catalytic module surface opposite to the active-site residues of fIXa β harbours its linker peptide. Downstream of Cys132, the linker chain is fixed till His139 and becomes then disordered in the last seven residues of the light chain; in N-terminal direction, it passes over to the laterally connected EGF2-domain via two interlaced turns. EGF2 and the catalytic module essentially sandwich this N-terminal part of the linker between, but interact also with one another via a common aromatic interface, similar to fXa⁹). Both EGF domains

of fXa β exhibit similar structures as the related EGF2 domain of human fXa and the isolated EGF1 domains of human/bovine FIXa^{9,10}). They have an axe blade-like shape and consist of 5 consecutive peptide strands pairwise linked through four β -hairpin loops and cross-connected via three disulfide bridges (see **Fig. 1**). Strand 1 always exhibits a quite curly conformation, while strands 2 and 3, and strands 4 and 5, respectively, form a "major" and a "minor" twisted β -pleated sheet.

The new feature in this structure, probably of important significance for fIXa function, is the (head-to-tail) arrangement of both EGF domains, which are roughly oppositely oriented along their long axes and inclined to about 110°. The inter-domain interface, which comprises about 80 atom-atom contacts below 4.0 Å, looks like a ball-and-socket joint, where EGE1 (the ball) revolves via its exposed s3 $\rightarrow$ s4 loop (around Gly76) in the concave EGF2 "socket" made by the (strictly conserved) residues Val107, Cys109, Ala86 and the hydrophobic side chain parts of Lys122 and Arg94 (**Fig. 1**). Any inter-domain rotation is, however, restricted by three additional, directing contacts, provided by i) the (covalent) connecting segment Glu83-Leu84-Asp85-Val186, ii) a polar hydrogen bond formed between the side chain of the (always basic residue) Lys122 and Gln74O, and in particular iii) a lateral 2N-2O salt bridge between the carboxylate group of Glu78 and the guanidinium group of Arg94. Both latter residues are strictly conserved in fIX species. Thus, this inter-domain interaction and the relative spatial orientation of both domains observed in these PPACK-fIXa β crystals do not seem to be caused by crystal packing, but to represent an intrinsic structural property of fIXa. It might be noteworthy that in fXa the connecting peptide is longer, that an

Fig. 1 Ribbon plot of the bent fIXa β molecule¹²⁾. The view is onto the "concave" surface, and the phospholipid membrane is presumed to run through the Gla domain (bottom) approximately along a horizontal plane. The active-site groove opens at the upper front of the catalytic domain (top), with the PPack inhibitor (yellow) binding from left to right. The light chain is folded into the (mainly modeled) N-terminal Gla domain/hydrophobic stack (green/yellow color) with the Gla residues (red ball-and-stick) explicitly shown, EGF1 (magenta), EGF2 (light green), and the linker peptide (yellow), whereas the heavy chain forms the catalytic domain (red).

equivalent salt bridge is not possible, and that the residue equivalent to Val75 is polar. This might suggest a less tight, more flexible inter-domainal interaction in fIXa.

At the N-terminal pole of the EGF1 domain, the entering strand 1 and the s2 $\rightarrow$ s3 hairpin loop (Asp64-Ser68) enclose a site, which can accommodate a calcium ion¹⁰⁾. Three of the presumed calcium ligands of fIXa, namely the carbonyl group of Asp65 and the carboxamide/carboxylate side chains of Gln50 and of Asp64, are in our fIXa β structure oriented towards this central site, and the Gly48 carbonyl group of the connecting segment could be easily modeled to com-

plete a favourable calcium liganding sphere. This calcium site is not occupied, however, and the connecting segment from the Gla domain is only defined by appropriate density from Gln50 onwards, due to the absence of calcium in our fIXa β crystals. Addition of calcium does not immediately cause disruption of the crystals, but affects the reflection quality and considerably enhances crystal decay; preliminary attempts to calculate (difference) Fourier maps with such low-resolution data obtained from crystals soaked at calcium or neodymium concentrations up to 10mM did not show density that would unequivocally account for the missing struc-

tures. According to the results obtained with the fXa EGF1 domain in solution¹⁰, the calcium environment would presumably rearrange and rigidify upon unconstrained calcium binding, including fixation of the entering loop segment.

Several lines of evidence^{8,9} suggest that the interaction between EGF1 and the C-terminal helix of the Gla-domain (i.e. its hydrophobic stack) is weakened upon removal of calcium, and that the Gla-domain separates into three organized elements, which are no longer uniquely arranged to one another¹¹, but might still keep their overall spatial relations. The crystal structure of the prothrombin fragment 1 determined in the absence of calcium had revealed electron density for the α -helical hydrophobic stack adjacent to that accounting for the kringle 1 domain⁸. Calcium binding, in contrast, induced a conformational transition towards the ordered Gla-domain (as known from the prothrombin fragment 1 holoform⁸) leading to the exposure of membrane binding determinants¹¹.

In our fIXa β Fourier map, we see a tube-like density beyond the N-terminal pole of EGF1, which could well account for the C-terminal helix of the Gla domain. This feature gave a hint to model the Gla domain of fIXa β in the presence of calcium. The equivalent Gla-domain of calcium-bound prothrombin fragment 1⁸ was therefore, after appropriate allowance for sequence differences, linked to the EGF1 domain by placing its hydrophobic stack-helix in this density and by positioning and orienting it along and about this helix axis in a manner compatible with crystal packing. These constraints approximately limit the Gla arrangement to the geometry shown in **Fig. 1**.

According to this model, the EGF1 domain of fIXa β seems to interact with the Gla domain mainly through the strictly conserved

Ile66 and the preceding s2 $\rightarrow$ s3 loop main chain of EGF1 and the side chains of Asn39 and Lys43 of the hydrophobic stack. Gla36 and Gla40, the two Gla-residues which fIXa possesses in addition to the 10 of prothrombin, point towards the EGF1 domain, the latter possibly making a domain-domain interaction via the strictly conserved Lys63 (**Fig. 1**). Along with prothrombin⁸, also in fIXa Gla residues 15, 17, 20, 21, 26, 27 and 30 form a Gla domain-dissecting carboxylate layer¹¹, which is opposed by the carboxylate groups of Gla7 and Gla8 projecting from the exposed N-terminal Ω -loop (see **Fig. 1**). A number of calcium ions intercalate between both layers or are bound in a more exposed manner to the peripheral carboxylates⁸. The detailed mode of membrane binding is not yet known. There is clear evidence, however, that fIXa (alike the other vitamin K-dependent proteinases) docks to phospholipid membranes essentially via its Gla domain, and that this interaction is mediated through calcium ions and requires the intermediate Gla residues^{8,11,19}. Most probably, therefore, Gla residues of this carboxylate layer form the phospholipid attachment surface, which should therefore run approximately horizontally in **Fig. 1**. This assumption seems to be the more corroborated by the excellent agreement between the measured distance from the D-Phell-phenyl ring of the bound PPack to this membrane, amounting to almost 90Å, and distances of 89Å and 73Å calculated for D-Phell-conjugated fluorescence donors and membrane-bound acceptor dyes from fluorescence energy transfer measurements²⁰.

But how might fIXa β interact with its partner proteins in the fXase complex, i.e. with cofactor VIIIa and with its physiological substrate fX? According to competition experiments done with various enzyme fragments and chimeric molecules both the heavy

and the light chain of fIXa seem to share binding sites with fVIIIa, with a somewhat weaker participation of the EGF1 domain²¹⁾. Particularly important interaction sites should be provided by highly conserved surface located residues, which for specific recognition might be often confined to fIX alone; furthermore, residues involved in such interactions should not be optionally substituted in one or the other fIX species; and last not least, single residue replacements (observed either in naturally occurring variants of high antigenic level or upon site-directed mutagenesis) of such interacting residues should affect coagulation. Exploration of the fIXa β surface according to all three criteria suggests that most of the conspicuous surface located residues, mutation of which caused clinical symptoms (c.f. ref. 14), map to a continuous broad strip extending along the entire factor IXa molecule (its "concave" side facing the observer in **Fig. 2**, and its face towards the "left" side). Mutat-

ed, fIX specific and conserved surface residues, respectively, are emphasized in **Fig. 2** by red, pink and light-blue color. For sake of simplicity, only a few exemplary residues will be addressed in the following.

Noteworthy in the Gla-domain are (besides most Gla residues and the N-terminal loop, both probably involved in phospholipid binding or in maintenance of the structure¹⁹⁾²¹⁾ Phe9 located in the Ω -loop, and in particular also Phe25 and Arg29 harboured in the second helix; all three side chains are exposed and directed towards the lumen left by the bent factor IXa β molecule, and all of them are strictly conserved in fIX, with the latter two also occurring in most of the related other factors, however.

Ile66 and Try69²²⁾, positioned in the Gla-touching s2 $\rightarrow$ s3 loop and in the central part of the EGF1 domain, respectively, likewise face this lumen; both residues are strictly conserved not only in fIX, but also in most of the related factors and might thus fulfill a

Fig. 2 Solid surface model of the bent fIXa β molecule¹²⁾. The surface coloring is made according to surface located residues, which upon mutations lead to bleeding disorders (red color), conserved fIX specific residues (pink), other conserved fIX residues (light-blue) and variable residues (blue). This "concave" side and the surface strip to the "left" of the molecule are dominated by mutated, fIX specific or conserved residues and are therefore implicated in fVIIIa binding, whereas the "right" and the "back" side carry more variable residues.

more general structure-stabilizing function. Also the remarkably exposed indole side chain of Trp72, completely conserved only in fIX, is directed towards this lumen. In contrast, in some but the porcine fIX species²⁾ residues 53 and 61 are O-glycosylated Ser residues²³⁾, in agreement with the arrangement of both residues opposite to the lumen (i.e. towards the more variable blue “back” surface not visible in **Fig. 2**).

Also the strictly conserved EGF2 residues Ile90 and Arg94 (the latter involved in the unique salt bridge with Glu78) face with their side chains the lumen, as does the intervening Asn92²⁴⁾, which is, however, also conserved in the related proteinases. Leu108 and Gln121, non-conserved and strictly conserved surface residues of the EGF2 domain, respectively, represent the exception rather than the rule in that their mutation affected coagulation function in spite of their location at the outside curvature. In general, however, this “back” surface is covered with more variable residues, not noticeable due to mutations.

The catalytic module of factor fIXa β carries a particularly hot mutational spot in its “intermediate” helix, an exposed substructure in all serine proteinases attached to the main modular body at the “left” side of the inner curvature (in **Fig. 1b** represented by a red bud). Mutations in 6 of the almost strictly conserved 9 residues of the constituting segment Leu330(C162)-Arg338(C170) manifested themselves in bleeding disorders. Particularly noteworthy are three mutations at the central Arg333(C165), which caused severe haemophilia B (c.f. ref.14). The importance of this subsite for fXase function is also underlined by the observation that thrombin cleavage of fIXa β behind Arg327(C159) or ArgC170 leads to a complete loss of procoagulant activity, although fIX cleaved at either site maintains its normal ability to

be activated by factor XIa²⁵⁾. ArgC165 is surrounded by some other surface located basic residues (ArgC170; Lys400(C230), Arg403(C233) and Lys394(C224) arranged along the C-terminal helix of the heavy chain, and Lys293(C126) and Lys301(C132) positioned aside of it), which together form a contiguous positively charged surface patch (extending along the left “back” side, invisible in **Fig. 2**) representing a potential anchoring site for acidic glycosaminoglycans such as heparin. In strong contrast to the heparin binding site in thrombin⁷⁾, this patch does not extend towards the active-site groove in fIXa β , however. Heparin-Sepharose binds more tightly to fIXa β than to fX and fVIIIa, but heparin has been shown to inhibit the activity of intrinsic fXase in a non-competitive manner, without impairing its initial assembly²⁶⁾. A possible structural explanation would be that this “heparin binding site” of fIXa β becomes only involved in tight fVIIIa contacts when reaching the transition state upon fX binding.

Another mutational “hot spot” centers around the N-terminus of the heavy chain¹³⁾, showing up in **Fig. 1b** as a red surface right in the middle of the catalytic domain. In particular the first four residues, Ile(Val)181(C16), ValC17, GlyC18 and GlyC19, are required for the correct zymogen $\rightarrow$ active enzyme rearrangement and for the active state stabilization, similar to the related proteinases¹⁸⁾, and must therefore not necessarily represent specific fVIIIa recognition sites. In the fIX zymogen, this N-terminus is connected with the light chain C-terminus (the exposed appendage to the bottom right of the catalytic domain in **Fig. 1**) via the 35 residue activation peptide, which is presumably accommodated on the surface between both termini (i.e. the surface facing the observer in **Fig. 1**). FIX is not known to complex

fVIIIa¹²¹), what might be due to specific fVIIIa recognition sites around GlyC18 becoming exposed upon activation to fIXa β . FIXa α (a fIXa species, where only the second (180-181) activation site is cleaved, while the activation peptide remains covalently linked to the light chain) requires the additional cleavage at Arg145-Ala146 and activation peptide removal for attaining full cofactor binding activity¹¹), probably indicating some sterical hindrance with the surface located activation peptide. Therefore, the “front” surface of fIXa β as well as its “left” side (with respect to **Fig. 2**) seem to be primarily involved in fVIIIa binding, while its “right” and “back” sides, which carry only a few mutational sites but many non-conserved residues on the surface, appear to be more exposed in the fXase complex.

For fX activation, not only fIXa β residues in, but also around the active site must be in direct contact with the fX cleavage site. This probably holds for a number of conserved surface residues, mutation of which caused hemophilia B (c.f. ref. 14), such as Arg316 (C148) and Gly317 (C149), Ile251 (C86) and Arg252 (C87), or Lys394 (C224) and Tyr395 (C225), which are localized immediately “below”, “above” or to the “left” of the active-site groove. In the absence of a membrane and of fVIIIa, productive collisions between fIXa β and fX will (also due to low mutual affinity) rarely occur, in agreement with the measured very large K_m -value of 33,000 nM, and catalytic events will only slowly happen due to the defective binding site of fIXa β , as reflected by the very small k_{cat} of 0.003 sec⁻¹ ²⁶). A phospholipid membrane enormously increases the collisional frequency for both factors as a result of restricting their diffusion to a two-dimensional surface, but should not favour an opening of the binding site, in agreement with a 150fold

reduced K_m , but a nearly unchanged k_{cat} -value.

The measured high affinity of fIXa β for fVIIIa²⁶) requires an extended interaction site between both factors, provided in particular through the EGF2 domain and the catalytic domain of fIXa β ²¹). Our structural evidences would suggest that the bent fIXa β molecule wraps around (half of) a projecting fVIIIa substructure, with the interacting fIXa β strip defined above providing most of the contacts. Because fVIIIa does not influence the activity of fIXa β towards chromogenic substrates or chloromethylketones, formation of this binary complex is obviously not accompanied by an opening of the cryptic pocket of fIXa β , even in the presence of phospholipid.

Optimal docking of fX (which might possess a less rigid but altogether similar spatial structure as fIXa β) to the opposite side of this binary complex could require extra energy to put the fX cleavage sites very close to fIXa β 's substrate binding site, in this way enforcing opening of its specificity pocket on the cost of binding energy¹²). This enforced assembling should be beneficial for catalysis, in agreement with the observed 300fold enhancement in k_{cat} , but should not necessarily increase the collision frequency, as reflected by the still high K_m -value of 7000 nM²⁶). The number of productive collisions would be tremendously increased by a phospholipid membrane, which would assist in bringing together and ordering all three proteins in a productive manner and reduce their degrees of freedom, as reflected by a 100fold enhancement in K_m (to 60 nM, i.e. far beyond plasma concentration), and a still 10fold enhancement in k_{cat} (8 sec⁻¹) ^{12/26}).

The fXase model¹²) should be considered a starting point to understand the observed tremendous (more than a million fold) catalytic efficiency enhancement of fIXa β upon

formation of the intrinsic fXase complex, and should stimulate future research to localize these interaction sites. Some fundamental geometrical aspects of this model might be transferable to related structures such as the prothrombinase complex. Furthermore, our fIXa β structure displays the exact arrangement of the distinct residues and their embedding in the substructures. This will allow a more reliable assessment of their structural and functional importance and thus hopefully help to better understand the nature of most deleterious mutants¹⁴⁾ and the molecular mechanisms underlying haemophilia B.

References

- 1) Kurachi K, Kurachi S, Furukawa M, Yao S-N: Blood Coagul Fibrinol 4:953, 1993; Limentani SA, Furie B in Haematology: Basic Principles and Practice (eds Hoffmann R, Bentz EG, Shattil SJ, Furie B, Cohen HG, Silverstein), Churchill Livingstone 1995, pp. 1664; Rainer AP, Davie EW in Haemostasis and Thrombosis, Churchill Livingstone 1994.
- 2) Lollar P, Paker CG, Kajenski PJ, Litwiller RD, Fass DN: Biochemistry 26:7627, 1987; Lollar P, unpublished material; Sarkar G, Koeberl DD, Sommer SS, Genomics 6:133, 1990; Sarkar G, Turner RT, Bolander ME, Genomics 6:133, 1993.
- 3) Fujikawa K, Coan MH, Legaz ME, Davie EW: Biochemistry 13:5290, 1974; Kurachi K, Davie EW: Proc Natl Acad Sci USA 79:6461, 1982.
- 4) Yoshitake S, Schach BG, Foster DC, Davie EW, Kurachi K: Biochemistry 24:3736, 1985.
- 5) Byrne R, Link RP, Castillo FJ: J Biol Chem 255:5336, 1980; McRae et al.: Biochemistry 20:7196, 1981; Castillo MJ, Kurachi K, Nishino N, Ohkubo F, Powers JC: Biochemistry 22:1021; Butenas S, Orfeo T, Lawson JH, Mann KG, Biochemistry 31:5399, 1992; Kam C-M, et al.: J Med Chem 37:1298, 1994.
- 6) vanDieijlen G, Tans G, Rosing J, Hemker HC: J Biol Chem 256:3433, 1981.
- 7) Bode W, et al.: EMBO J 8:3467, 1989; Bode W, Turk D, Karshikov A: Protein Sci 1:426, 1992; Rydel TJ, et al. 249:277, 1990; Arni PK, Padmanabhan K, Padmanabhan KP, Wu TP, Tulinsky A: Biochemistry 32:4727, 1993.
- 8) Seshadri TP, Tulinsky A, Skrzypczak-Jankun E, Park CH: J Mol Biol 220:481, 1991; Soriano-Garcia M, Padmanabhan K, de Vos AM, Tulinsky A: Biochemistry 31:2554, 1992.
- 9) Padmanabhan K, et al.: J Mol Biol 232:947, 1993.
- 10) Baron M, et al.: Protein Sci 1:81, 1992; Selander-Sunnerhagen M, et al.: J Biol Chem 267:19642, 1992.
- 11) Freedman SJ, Jacobs M, Furie BC, Baleja JD, Furie B: Blood 84, Suppl 1:531, 1994; Freedman SJ, Furie BC, Furie B, Baleja JD: Biol J Chem 270:7980-7989, 1995.
- 12) Brandstetter H, Bauer M, Huber R, Lollar P, Bode W: Proc Natl Acad Sci USA 92:9796-9800, 1995.
- 13) Hamaguchi N, Roberts H, Stafford DW: Biochemistry 32:6324, 1993; Bajaj SP, Sabharwal AK, Gorka J, Birktoft JJ: Proc Natl Acad Sci USA 89:152, 1992; Hamaguchi N, Stafford DW: Thromb Haemostas 72:856, 1994.
- 14) Bottema CD, et al.: Am J Hum Genet 49:820, 1991; Roberts HR: Thromb Haemostas 70:1, 1993; Gainelli F, et al.: Nucl Acid Res 22:3534, 1994; Wacey AI, Krawczak M, Kakkar VV, Cooper DN: Human Gen 94:594-608, 1994.
- 15) Sommer SS: private communication.
- 16) Lollar P: unpublished material.
- 17) Huber R, Bode W: Acc Chem Res 11:114, 1978; Bode W, Schwager P, Huber R: J Mol Biol 118:99, 1978, Bode W: J Mol Biol 127:357, 1979; Wang D, Bode W, Huber R: J Mol Biol 185:595, 1985; Vijayalakshmi J, Padmanabhan KP, Mann KG, Tulinsky A: Protein Sci 3:2254, 1994.
- 18) Astermark J, Björk I, Öhlin A-K, Stenflo J: J Biol Chem 266:2430, 1991; Wolberg AS, Cheung WF, Stafford DW, Hamaguchi N: Thromb Haemostas 69:614, 1993; Jacobs M, Freedman SJ, Furie BC, Furie B: J Biol Chem 269:25494, 1994; Medved LV, Vysotchin A, Ingham KC:

- Biochemistry **33** : 478, 1994 ; Cheung WF, Hamaguchi N, Smith KJ, Stafford DW : J Biol Chem **267** : 20529, 1992.
- 19) Mutucumarana VP, Duffy EJ, Lollar P, Johnson AE : J Biol Chem **267** : 17012, 1992.
- 20) Astermark J, Hogg PJ, Björk I, Stenflo J : J Mol Biol Chem **267** : 3249, 1992 ; Ahmad SS, Rawala-Sheikh R, Cheung W-F, Stafford DW, Walsh PN : J Biol Chem **267** : 8571, 1992 ; Bajaj SJ, Rapaport SI, Maki SL : J Biol Chem **260** : 11574, 1985 ; Lin S-W, Smith KJ, Welsh D, Stafford DW : J Biol Chem **265** : 144, 1990 ; Hertzberg MS, Ben-Tal O, Furie B, Furie BC : J Biol Chem **267** : 14759, 1992.
- 21) Hughes PE, Morgan G, Rooney EK, Brownee GG, Handfood P : J Biol Chem **268** : 17727, 1993.
- 22) Hase S, et al. : J Biochem **104** : 867, 1988 ; Nishimura H, Takao T, Hase S, Shimonishi Y, Iwanaga S : J Biol Chem **267** : 17520, 1992.
- 23) Nishimura H, et al. : J Biol Chem **268** : 24042, 1993.
- 24) Kisiel W, Smith KJ, McMullen BA : Blood **66** : 1302, 1985.
- 25) Duffy EJ, Parker ET, Mutucumarana VP, Johnson AE, Lollar P : J Biol Chem **267** : 17006, 1992 ; Duffy EJ, Lollar P : J Biol Chem **267** : 7821, 1992 ; Barrow RT, Parker ET, Krishnaswamy S, Lollar P : J Biol Chem **269** : 26796, 1994 ; vanDieijien G, vanRijn JLML, Govers-Riemslog JWP, Hemker HC, Rosing J : Thromb Haemostas **53** : 396, 1985.
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