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Coagulation Factor Xa

**DATABANKS**

*MEROPS name:* coagulation factor Xa  
*MEROPS classification:* clan PA, subclan PA(S), family S1, subfamily S1A, peptidase S01.216

*IUBMB:* EC 3.4.21.6 (BRENDA)  
*Tertiary structure:* Available  
*Species distribution:* superclass Tetrapoda  
*Reference sequence from:* Homo sapiens (UniProt: P00742)
Name and History

In the mid-1950s it was becoming evident that a coagulation factor different from factors VII and IX was required for the activation of prothrombin to thrombin [1]. In 1957 it was found that the coagulation properties of blood samples from patients thought to lack factor VII (then called proconvertin) could be normalized by mixing the samples. This indicated that the disease was not a homogeneous entity. The new factor, \textit{factor X}, was also called \textit{Stuart's factor} or \textit{Prower's factor}. Factor X was found to require vitamin K for normal biosynthesis, and treatment of patients with vitamin K antagonists such as dicoumarol or warfarin resulted in a decrease in its biological activity [2,3]. It was first purified to homogeneity from bovine plasma and later from human plasma [2,3].

Activity and Specificity

Factor X is the zymogen of a serine protease. The activated enzyme (factor Xa) is an Arg-specific serine protease related to trypsin, but has much narrower substrate specificity. The N-terminal \(\gamma\)-carboxyglutamic acid (Gla) domain binds \(\approx 8 \sim 10\) calcium ions in a cooperative manner (average \(K_d \sim 0.5\) mM) and mediates the interaction of factor Xa with phospholipid membranes [4,5,6]. In the presence of calcium ions, factor Xa forms a phospholipid-bound complex with a cofactor, factor Va. This 'prothrombinase' complex activates prothrombin (Chapter 643) \(\sim 300,000\)-fold more rapidly than does free factor Xa. Studies of this reaction have demonstrated that phospholipid reduces the \(K_d\) for prothrombin, whereas factor Va increases the \(V_{max}\) [5]. \textit{In vivo}, the surface of activated platelets is the main site at which the prothrombinase complex is assembled. Factor Va is derived from factor V by specific proteolytic cleavages mediated by thrombin or factor Xa. Membrane-bound factor Va binds factor Xa with high affinity (\(K_d \approx 1\) nM), due at least in part to interactions between the catalytic domain (residues 163 - 170 [7]) and residues 499 - 505 of factor Va [8], but has no measurable affinity for the zymogen [5]. In the absence of factor Va, factor Xa binds to phospholipid membranes with a \(K_d\) of \(\approx 30 - 100\) nM. In these reactions, the phospholipid can be regarded as a means of reducing the 'dimensionality' of the reaction, from three, to two dimensions, thereby increasing the likelihood of a productive collision between factor Xa and membrane-bound factor Va. Effective binding of factor Xa to membranes requires that phosphatidyserine is exposed on the outer surface, as occurs in vesicles released from activated platelets: phosphatidyserine is translocated to the exterior by phospholipid scramblase when the platelet is activated [9]. This regulatory mechanism restricts binding of factor Xa to discrete types of cells, according to their functional state.

Factor Xa activates prothrombin, a single-chain molecule, by hydrolyzing two peptide bonds and the reaction may thus proceed via two pathways [5,10]. In one pathway, cleavage of the Arg320-Ile321 bond yields an intermediate, meizothrombin, which may then be cleaved at the Arg271-Thr272 bond to yield fragment 1-2 and thrombin [11]. During both cleavages, prothrombin and meizothrombin bind to the same exosite [12], and impairing the structural shift that accompanies activation of thrombin, prevents cleavage of meizothrombin [13]. Meizothrombin is inactive against fibrinogen but readily activates protein C (Chapter 644). In a second pathway, the initial cleavage occurs at the Arg271-Thr272 bond and the intermediates fragment 1-2 and prethrombin-2 are formed. Prethrombin-2 is enzymatically inactive and is formed predominantly in the absence of factor Va [14]. Subsequent hydrolysis of the Arg320-Ile321 bond in prethrombin-2 yields thrombin. The Arg284-Thr285 bond is also susceptible to cleavage by factor Xa or thrombin and, in plasma, this site may be prefered over the Arg 271-Ile 272 bond [15]. \textit{In vitro}, factor Va and/or phosphatidyserine modulate the specificity of factor Xa so that the meizothrombin pathway predominates [14,16]. This is generally thought to be the most physiologically relevant activation pathway. Factor Xa can also proteolytically activate factors V and VIII, and factor VII (Chapter 641), thus participating in positive feedback loops that amplify the clotting process. Factor Xa also inactivates factor VIIIa, by cleavage at Lys36 and Arg336 within the A1 subunit [17]. Several synthetic substrates are available for factor Xa, including methoxy-carbonyl-cyclohexylglycyl-Gly-Arg NHPhNO\(_2\) (Spectrozyme Xa), methanesulfonamide-Leu-Gly-Arg NHPhNO\(_2\) (CBS 31.39), and Bz-Ile-Glu-(piperidino amide)-Gly-Arg NHPhNO\(_2\) (S2337). Full activity of factor Xa towards these substrates requires the presence of both Na\(^+\) and Ca\(^2+\) ions, which modulate the catalytic domain [18]. The subsite specificity has been examined in detail using proteome-derived peptide libraries [19], phage display [20], and fluorescence-quenched substrates [21], revealing a preference for Arg in P1 and Gly or Ala in P2; slight preferences for Pro in P4, a small amino acid in P1', and Asp in P3'; and aromatic or aliphatic residues (especially Phe, Leu or Tyr) in P2'.

Free factor Xa in the bloodstream is rapidly inactivated by serine protease inhibitors (serpins) such as antithrombin and \(\alpha_1\)-protease inhibitor [22]. Cleavage of an exposed loop on the serpin by factor Xa leads to formation of a covalently-linked complex (1:1 stoichiometry) and inhibition of enzymatic activity. The rate of inactivation by antithrombin is increased several thousand-fold by heparin/heparin sulfate, shorter, heparin-based, synthetic oligosaccharides, and a sulfated galactan from the red algae \textit{Botryocladia occidentalis} [23]. Another serpin, protein Z-dependent protease inhibitor (ZPI), can inhibit factor Xa,
although it is far less effective than antithrombin, because it has an unfavorable P1 Tyr. Residues in factor Xa essential for inhibition by ZPI have been identified in the autolysis loop and heparin binding exosite [24]. Protein Z accelerates this inhibition, and its interaction with ZPI requires association with the membrane, interaction between the Gla domains in protein Z and ZPI, and Glu313 in the protein Z pseudoactive site [25]. Factor Xa is also inhibited by non-serpin inhibitors such as tissue factor pathway inhibitor (TFPI) and \( \alpha_2 \)-macroglobulin. TFPI consists of three tandem Kunitz-type domains, the second of which interacts with the active site of factor Xa: the complex then associates with tissue factor—factor VIIa to form an inactive quaternary complex [22,26]. Heterologous protein inhibitors of factor Xa include soy bean and corn trypsin inhibitors, as well as some proteins in snake venom [27]. Certain peptides, both naturally occurring (e.g. tick anticoagulant peptide [28], ecotin [29] and the hookworm NAP5 peptide) and synthetic (e.g. dansyl-Ile-Glu-Gly-Arg-CH₂Cl, or DEGR, and SEL2711) can directly inhibit factor Xa. In addition, many specific organic inhibitors of factor Xa have been identified, including DX-9065a [30], a propanoic acid derivative, and benzamidine-based compounds. Synthetic inhibitors have been developed as antithrombotic agents, including biaryl methoxy isonipecotanilides [31], tiophene-anthranilamides [32], quinoxalinones [33], and Razaxaban, Apixaban, Rivaroxaban and Darexaban, which can be taken orally [34–37]. The anticoagulant NAPc2 from the nematode Ancylostoma caninum binds first to a novel exosite on factor Xa, and inhibits factor VIIa only when it is complexed with tissue factor and factor Xa [38]. Membrane-bound factor X/Xa can be inactivated by plasmin. Cleavage of the Lys435|Ser436 bond generates factor Xaβ and further cleavage at the Lys330|Gly331 peptide bond generates factor Xα33/13, which destroys clotting activity [39]. These cleavages expose plasminogen binding sites that augment the generation of plasmin and thus seem to switch factor X/Xa from a procoagulant to an anticoagulant role by stimulating fibrinolysis [40].

**Structural Chemistry**

The primary structure of human factor X has been determined by amino acid and DNA sequencing [41,42]. Factor X sequences from several other vertebrate species are also known. Human factor X is synthesized as a single polypeptide of 488 amino acids, including a 23-residue signal peptide (amino acids −40 to −18) to direct its translocation into the lumen of the endoplasmic reticulum, and a 17-residue propeptide (amino acids −17 to −1), which serves as a recognition site for \( \gamma \)-glutamyl carboxylase [4]; neither of these is found in the mature protein. Prior to secretion, a tripeptide (Arg140-Lys141-Arg142) is excised to yield a heterodimer linked by a disulfide bond between Cys132 and Cys302. The molecular mass of the maturezymogen is 58.9 kDa (sedimentation equilibrium). It consists of a light chain of 139 amino acids and a glycosylated heavy chain of 306 amino acids. When resolved by SDS-PAGE, the chains have apparent masses of ~17 and ~49 kDa. The N-terminus of the heavy chain (residues 143–194) constitutes an activation peptide which is not found in the active enzyme. The N-terminus of the light chain (amino acids 1–45) comprises the Gla domain, including the so-called aromatic amino acid stack. Gla residues reside at positions 6, 7, 14, 16, 19, 20, 25, 26, 29, 32 and 39. The aromatic stack is followed by two EGF-like domains (residues 46–84 and 85–128).

The structure of factor Xa, lacking the Gla domain, has been solved by X-ray crystallography at ~2.2 Å resolution [43,44]. Overall, the folding of the catalytic domain is similar to that of chymotrypsin and thrombin. It contains three disulfide bonds: Cys221-Cys237, Cys350-Cys364 and Cys375-Cys403. His236, Asp282 and Ser379 constitute the catalytic triad involved in hydrolyzing peptide bonds in a substrate. The primary specificity (S1) pocket is formed by two loops (residues 373–379 and 398–403) that are joined by a disulfide bond (Cys375-Cys403). An aspartyl residue (Asp373) sits at the base of the S1 pocket, as is characteristic of trypsin-like proteases. The formation of a salt bridge between Asp373 and Arg residues at the P1 position in a substrate is an important interaction that explains the enzyme’s preference for hydrolyzing substrates on the C-terminal side of Arg residues. The C-terminal EGF-like domain is in close contact with the catalytic domain and, like the N-terminal one, has a fold similar to that of other EGF-like domains. The fold is supported by three disulfide bonds that link the Cys residues in the pattern 1–3, 2–4, 5–6. The three-dimensional structure of the N-terminal EGF-like domain has been determined both in the presence and absence of calcium ions [44–46]. The calcium-bound structures revealed a calcium-binding site that is unique to EGF-like domains, with Gly47, Gln64, \( \beta \)-hydroxylated Asp63, Gly64, and Leu65 identified as ligands for the calcium ion. However, \( \beta \)-hydroxylation of Asp63 is not required for high affinity calcium binding. NMR-based structures showed also that the calcium ion locks the EGF-like domain in the proper position relative to the Gla domain.

The structure of the Gla domain linked to the N-terminal EGF-like domain has been determined in the absence of calcium by NMR spectroscopy [47]. In contrast to a model of the calcium-bound domain, the structure revealed that, in the absence of calcium, Gla residues are exposed to the solvent and three hydrophobic residues near the N-terminus are folded into the interior of the domain. Thus, binding of several calcium ions to the Gla domain pulls the Gla residues to the interior and provides the energy required
to expose the hydrophobic side chains to the solution. Together with site-directed mutagenesis studies, this established that the interaction between a Gla domain and a phospholipid membrane includes associations of a hydrophobic nature.

**Preparation**

Factor X can be purified to homogeneity from plasma using conventional methods. An initial precipitation based on its affinity for insoluble barium salts is usually employed. This is often followed by ammonium sulfate precipitation and anion exchange chromatography. These procedures are sufficient to obtain homogeneous bovine factor X but purification of human factor X may require additional chromatography steps [2,3]. Immunoaffinity chromatography with monoclonal antibodies has also been utilized with success [48–50]. Recently, recombinant factor X has been expressed in several mammalian cell lines. However, at high expression levels, γ-carboxylation and proteolytic processing are inefficient. Substitution of the factor X propeptide with that from prothrombin improves γ-carboxylation [51] and chromatography on hydroxyapatite can effectively isolate fully γ-carboxylated factor X [52]. Site-directed mutagenesis has been employed to enhance cleavage of the propeptide [49], which can also be removed by incubation with furin.

**Biological Aspects**

The factor X gene is located on chromosome 13 at position q34, adjacent to the factor VII gene. It spans ~27 kb and has seven introns and eight exons. Exon I encodes the signal peptide, exon II the propeptide/Gla domain, exon III the C-terminal part of the Gla domain and the aromatic amino acid stack, exons IV and V the EGF-like domains, exon VI the activation peptide region, and exons VII and VIII the catalytic domain [42]. Factor X is synthesized mainly in the liver but its ~1700-nucleotide mRNA and/or protein has been detected in several other tissues. Factor X is secreted into the blood (normal concentration, 5–10 μg ml⁻¹). The protein undergoes extensive post-translational modification [53]. The signal peptide is removed by signal peptidase during translocation into the endoplasmic reticulum, where the 11 Glu residues in the Gla domain are γ-carboxylated by γ-glutamyl carboxylase. This is followed by proteolytic removal of the propeptide by the subtilisin-like enzyme furin. Asp63 in the first EGF-like domain is converted to erythro-β-hydroxyaspartic acid by a dioxygenase [54,55]. In the activation peptide, Thr159 and Thr171 are O-glycosylated and Asn181 and Asn191 are N-glycosylated. The O-linked carbohydrate moieties appear to be important for factor X to be activated efficiently. The activation peptide of bovine factor X contains a sulfate group O-esterified to Tyr160.

In the trans-Golgi apparatus, the factor X polypeptide is cleaved at the Arg142 | Ser143 bond to yield a disulfide-bonded dimer. The three C-terminal residues of the light chain (Arg140-Lys141-Arg142) are somehow removed either before secretion or in the plasma.

Activation of factor X to a serine protease occurs predominantly by hydrolysis of the Arg194 | Ile195 bond in the heavy chain, which releases a 52-residue activation peptide to form factor Xα. The cleavage causes the new N-terminus of the heavy-chain to rearrange so that Ile195 can participate in formation of the substrate binding pocket by forming a salt bridge with Asp378 [56]. This also contributes to the formation of the Na⁺ and factor Va binding sites [57], and appears to cause the transition from zymogen to active protease. A second cleavage, plasmin-mediated or autocatalytic, at the Lys435 | Ser436 bond yields factor Xα3 [39]. The procoagulant activity of both forms of factor Xα is similar.

Activation of factor X occurs via two principal pathways. It is activated by factor VII/VIIa in complex with a non-enzymatic membrane-bound cofactor, tissue factor (TF). This pathway is called the ‘extrinsic pathway’ and is responsible for the initiation of coagulation, proceeding mainly on the surface of damaged endothelial cells and macrophages, but probably also on activated platelets [58,59]. Alternatively, factor X is activated on the platelet surface by a membrane-bound ‘tenase’ complex comprising factor IXa, its cofactor factor VIIIa, and calcium ions, which activates factor X ~ 10⁶-fold more rapidly than factor IXa alone [5]. This ‘intrinsic pathway’ is responsible for amplifying the coagulation process (see also Chapter 640) and its importance is illustrated by the fact that hereditary deficiency of factors IX or VIII causes hemophilia B and A, respectively. Thus, factor X plays a pivotal role in blood clotting at the point of convergence of the two coagulation pathways. Accordingly, several rare mutations in the factor X gene have been identified that give rise to bleeding tendencies of variable severity (e.g. Chafa et al. [60], Bereczky et al. [61]). Theoretically, injection of factor X into patients with hemophilia should bypass the intrinsic pathway and permit generation of thrombin, but this fails because of the short half-life in plasma of factor Xα. However, mutants in which Ile16 or Val17 are replaced have a much longer half-life because they do not form complexes with antithrombin III or tissue factor inhibitor in hemophilic plasma, yet still are able to activate prothrombin and thus may be useful therapeutic agents [62,63].

Factor X can also be activated by an alternative pathway which is initiated on the surface of leukocytes and can trigger clotting. In this case the zymogen is bound by the β₂-integrin Mac-1 (CD11b) and activation occurs through hydrolysis of the Leu177 | Leu178 peptide bond in the activation peptide; a cleavage effected by
cathepsin G, which is secreted by stimulated leukocytes [64,65]. Mac-1 binds factor X with high affinity ($K_d \sim 30 \text{ nM}$) but has no affinity for factor Xa. Enzymes present in venom from snakes (e.g. RVV-X; [66]) (Chapter 235) and other toxic animals can also activate factor X.

In addition to its direct involvement in blood coagulation, factor Xa interacts with signalling receptors on the surface of many types of cells. It can thus elicit a variety of responses, including cell activation, gene expression and mitogenesis. A factor Xa receptor termed effector cell protease receptor-1 (EPR-1), with some structural similarity to the light chain of factor V, has been cloned [67]. EPR-1 does not bind factor X, whereas factor Xa forms a protease–receptor complex that induces cytokine gene expression and the release of platelet-derived growth factor. In endothelial cells, factor Xa appears to exert its effects by docking to EPR-1 and subsequently cleaving and activating protease-activated receptor-2 (PAR-2) [68]. PAR-2 is a member of a family of G protein-coupled receptors that are activated by cleavage of an N-terminal peptide; the new N-terminus (a ‘tethered ligand’) then inserts into the body of the receptor and activates it. There is also evidence that factor Xa can induce cell signalling in vascular wall cells by activating PAR-2 and/or PAR-1 by a mechanism that is independent of EPR-1 (e.g. McLean et al. [69]). Factor Xa activates PAR-1 with the effect that epithelial-derived tumor cells enter apoptosis [70] and breast, colon and lung cancer cell migration is inhibited [71]. In epithelial cells, signaling is via the extracellular-signal regulated kinase (ERK) pathway, leading to upregulation of Bim and caspase-3 activation [70]. In breast cancer cells, the Rho/ROCK and Src/FAK/paxillin pathways are activated leading to myosin light chain phosphorylation, LIMK1 activation, cofillin inactivation and stabilization of actin filaments which are incompatible with cell migration [72].

Factor Xa has other physiological and pathological roles. It is expressed in bronchoalveolar lavage fluid macrophages from mouse models of asthma, where it induces mucin production [73]. Factor Xa mediates the attachment of adenovirus 5 to hepatocytes via the hexon protein, and basic residues in the serine peptidase domain are essential for this interaction [74]. In SARS coronavirus, the spike protein, which binds to host receptors, is cleaved by factor Xa into subunits, facilitating viral infection [75].

**Distinguishing Features**

Factor X is a liver-synthesized zymogen of a serine protease that requires vitamin K for normal biosynthesis. The protein has the same domain structure as coagulation factors VII and IX, protein C (Chapter 644) and protein Z. Although all are synthesized as single polypeptide chains, factor X and protein C are cleaved to form dimers prior to secretion. The proteins are easily distinguished by SDS-PAGE under reducing conditions. Monoclonal and polyclonal antibodies that can distinguish the proteins are commercially available.

**Further Reading**

For a review, see James [76]. Menegatti & Peyvandi [77] and Auerswald [78] have reviewed factor X deficiencies, and Brown & Kouides [79] have reviewed their diagnosis and treatment. Josic et al. [80] have reviewed methods of preparations for all vitamin K-dependent peptidases, including factor X.

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