Blood clotting is accomplished by a cascade of zymogen activations

Enzymatic cascades are often employed in biochemical systems to achieve a rapid response. In a cascade, an initial signal institutes a series of steps, each of which is catalyzed by an enzyme. At each step, the signal is amplified. For instance, if a signal molecule activates an enzyme that in turn activates 10 enzymes and each of the 10 enzymes in turn activates 10 additional enzymes, after four steps the original signal will have been amplified 10,000-fold. *Hemostasis*, the process of blood clot formation and dissolution, requires a *cascade of zymogen activations:* the activated form of one clotting factor catalyzes the activation of the next (Figure 10.25). Thus, very small amounts of the initial factors suffice to trigger the cascade, ensuring a rapid response to trauma.

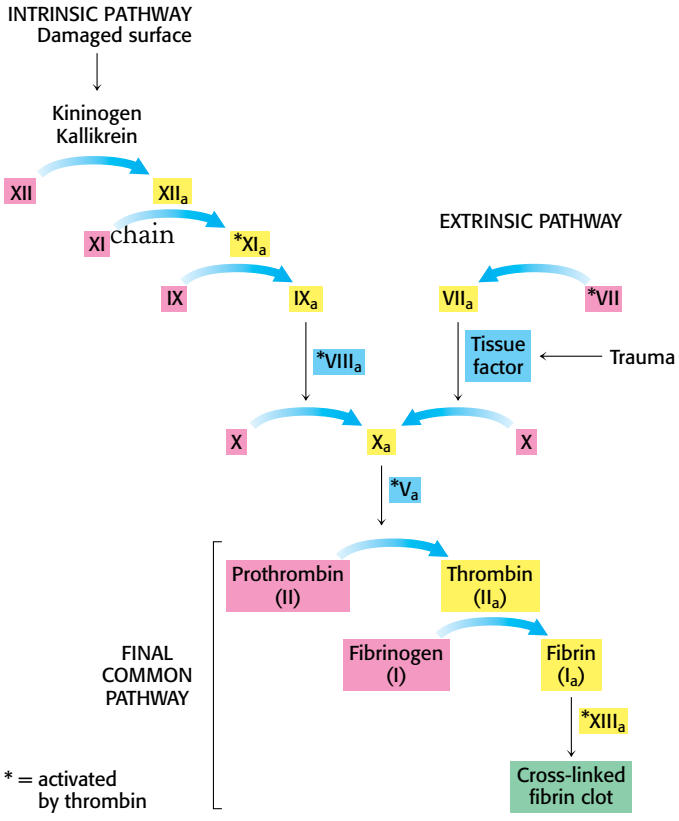
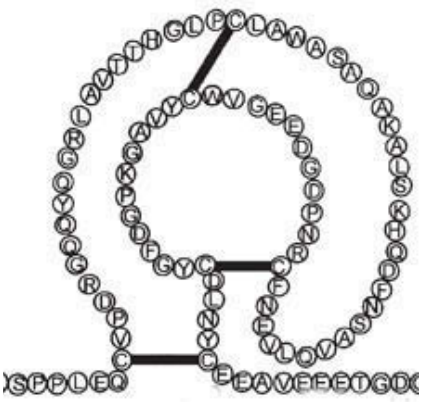


FIGURE 10.25 Blood-clotting cascade. A fibrin clot is formed by the interplay of the intrinsic, extrinsic, and final common pathways. The intrinsic pathway begins with the activation of factor XII (Hageman factor) by contact with abnormal surfaces produced by injury. The extrinsic pathway is triggered by trauma, which releases tissue factor (TF). TF forms a complex with VII, which initiates a cascade-activating thrombin. Inactive forms of clotting factors are shown in red; their activated counterparts (indicated by the subscript "a") are in yellow. Stimulatory proteins that are not themselves enzymes are shown in blue boxes. A striking feature of this process is that the activated form of one clotting factor catalyzes the activation of the next factor.



The second kringle domain of protrhombin

Kringle pastry

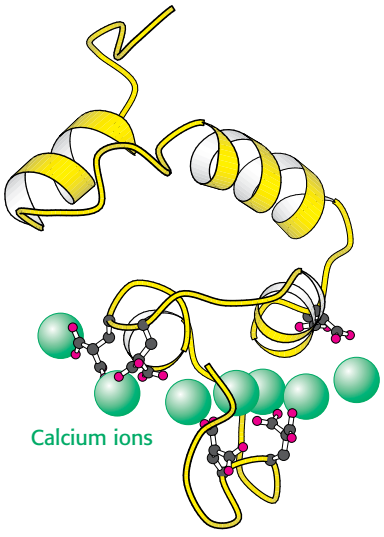


FIGURE 10.27 The calcium-binding region of prothrombin. Prothrombin binds calcium ions with the modified amino acid γ-carboxyglutamate (red). [Drawn from 2PF2.pdb.]

Two means of initiating blood clotting have been described, the *intrinsic pathway* and the *extrinsic pathway*. The intrinsic clotting pathway is activated by exposure of anionic surfaces upon rupture of the endothelial lining of the blood vessels. The extrinsic pathway, which appears to be most crucial in blood clotting, is initiated when trauma exposes *tissue factor* (TF), an integral membrane glycoprotein. Upon exposure to the blood, tissue factor binds to factor VII to activate factor X. Both the intrinsic and extrinsic pathways lead to the activation of factor X (a serine protease), which in turn converts *prothrombin* into *thrombin*, the key protease in clotting. Thrombin then amplifies the clotting process by activating enzymes and factors that lead to the generation of yet more thrombin, an example of positive feedback. Note that the active forms of the clotting factors are designated with a subscript "a," whereas factors that are activated by thrombin are designated with an asterisk.

Prothrombin requires a vitamin K-dependent modification for activation

Thrombin is synthesized as a zymogen called *prothrombin*. The inactive molecule comprises four major domains, with the serine protease domain at its carboxyl terminus (Figure 10.26). The first domain, called the *gla domain*, is rich in γ carboxyglutamate residues (abbreviation gla), and the second and third domains are called *kringle domains* (named after a Danish pastry that they resemble). Vitamin K is required for the synthesis of γ carboxyglutamate, a strong chelator of Ca^{2+}. These three domains work in concert to keep prothrombin in an inactive form. Moreover, because it is rich in γ carboxyglutamate, the gla domain is able to bind Ca^{2+} (Figure 10.27). What is the effect of this binding? The binding of Ca^{2+} by prothrombin anchors the zymogen to phospholipid membranes derived from blood platelets after injury. This binding is crucial because it brings prothrombin into close proximity to two clotting proteins, factor X_a and factor V_a (a stimulatory protein), that catalyze its conversion into thrombin. Activation is begun by proteolytic cleavage of the bond between arginine 274 and threonine 275 to release a fragment containing the first three domains. Cleavage of the bond between arginine 323 and isoleucine 324 yields active thrombin.

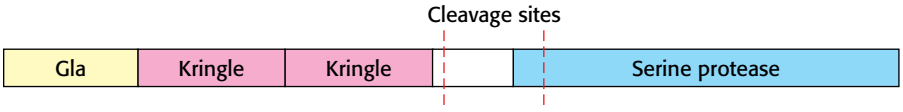


FIGURE 10.26 Modular structure of prothrombin. Cleavage of two peptide bonds yields thrombin. All the γ-carboxyglutamate residues are in the gla domain.

Fibrinogen is converted by thrombin into a fibrin clot

The best-characterized part of the clotting process is the final step in the cascade: the conversion of *fibrinogen* into fibrin by thrombin. Fibrinogen is made up of three globular units connected by two rods (Figure 10.28). This 340-kDa protein consists of six chains: two each of Aα, Bβ, and γ. The rod regions are triple-stranded α-helical coiled coils, a recurring motif in proteins (Section 2.3). Thrombin cleaves four *arginine–glycine peptide bonds* in the central globular region of fibrinogen (p. 217). On cleavage, an A peptide of 18 residues is released from each of the two Aα chains, as is a B peptide of 20 residues from each of the two Bβ chains. These A and B peptides are called *fibrinopeptides*. A fibrinogen molecule

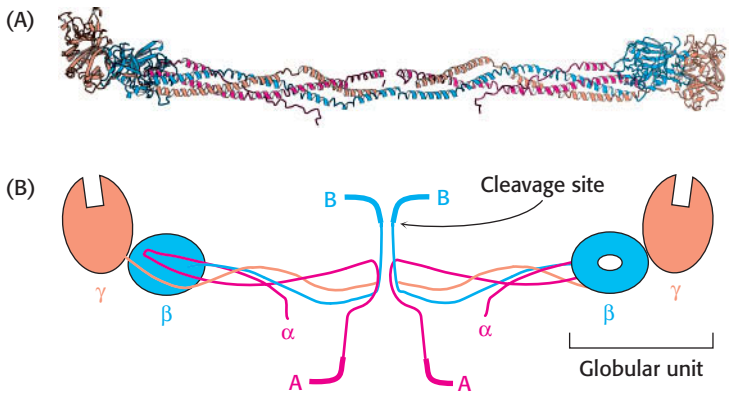


FIGURE 10.28 Structure of a fibrinogen molecule. (A) A ribbon diagram. The two rod regions are α-helical coiled coils, connected to a globular region at each end. The structure of the central globular region has not been determined. (B) A schematic representation showing the positions of the fibrinopeptides A and B. [Part A drawn from 1DEQ.pdb.]

devoid of these fibrinopeptides is called a *fibrin monomer* and has the subunit structure $(\alpha\beta\gamma)_2$.

Fibrin monomers spontaneously assemble into ordered fibrous arrays called *fibrin*. Electron micrographs and low-angle x-ray patterns show that fibrin has a periodic structure that repeats every 23 nm (Figure 10.29). Higher-resolution images reveal how the removal of the fibrinopeptides permits the fibrin monomers to come together to form fibrin. The homologous β and γ chains have globular domains at the carboxyl-terminal ends (Figure 10.30). These domains have binding "holes" that interact with peptides. The β domain is specific for sequences of the form H_3N^+-Gly-His-Arg-, whereas the γ domain binds H_3N^+-Gly-Pro-Arg-. Exactly these sequences (sometimes called "knobs") are exposed at the amino-terminal ends of the β and α chains, respectively, on thrombin cleavage. The knobs of the α subunits fit into the holes on the γ subunits of another monomer to form a protofibril. This protofibril is extended when the knobs of the β subunits fit into the holes of β subunits of other protofibrils. Thus, analogous to the activation of chymotrypsinogen, peptide-bond cleavage exposes new amino termini that can participate in specific interactions. The newly formed "soft clot" is stabilized by the formation of amide bonds between the side chains of lysine and glutamine residues in different monomers.

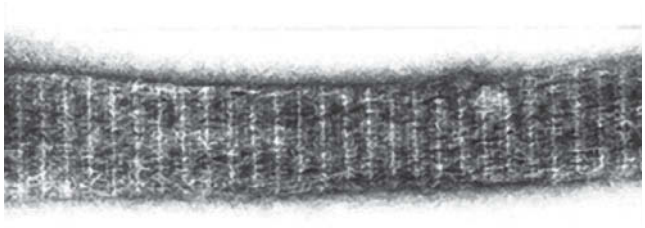


FIGURE 10.29 Electron micrograph of fibrin. The 23-nm period along the fiber axis is half the length of a fibrinogen molecule. [From John L. Woodhead et al., "The Ultrastructure of Fibrinogen Caracas II Molecules, Fibers, and Clots," *J. Biol. Chem.* 271(9):4946–4953, 1996, Mar 1. © American Society for Biochemistry and Molecular Biology.]

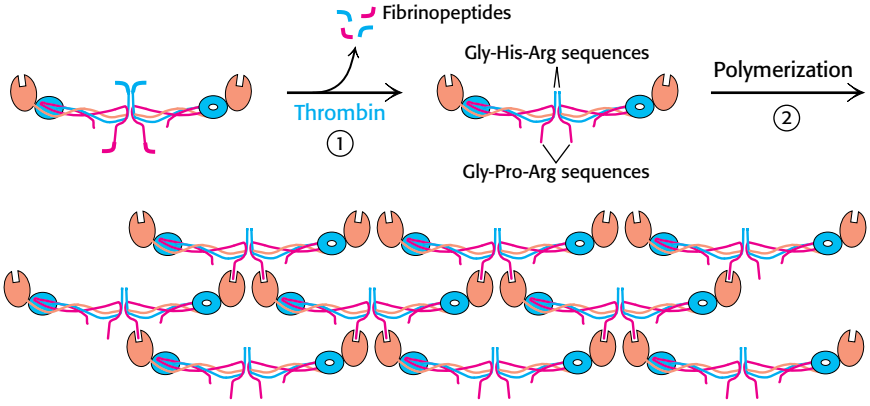
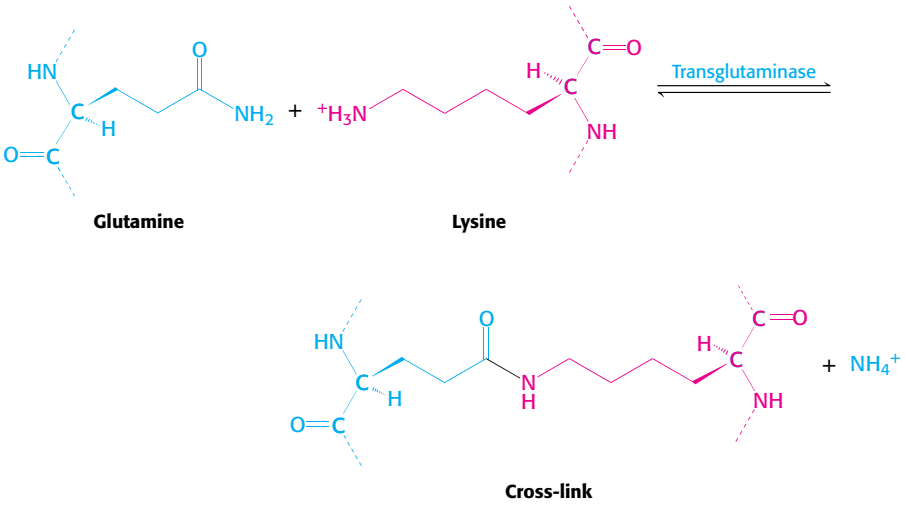


FIGURE 10.30 Formation of a fibrin clot. (1) Thrombin cleaves fibrinopeptides A and B from the central globule of fibrinogen. (2) Globular domains at the carboxyl-terminal ends of the β and γ chains interact with "knobs" exposed at the amino-terminal ends of the β and γ chains to form clots.



This cross-linking reaction is catalyzed by *transglutaminase (factor* $XIII_a$*)*, which itself is activated from the protransglutaminase form by thrombin.

Vitamin K is required for the formation of γ-carboxyglutamate

Vitamin K (Figure 10.31) has been known for many years to be essential for the synthesis of prothrombin and several other clotting factors. Indeed, it is called vitamin K because a deficiency in this vitamin results in defective blood *k*oagulation (Scandinavian spelling). After ingestion, vitamin K is reduced to a dihydro derivative that is required by γ-glutamyl carboxylase to convert the first 10 glutamate residues in the amino-terminal region of prothrombin into γ-carboxyglutamate (Figure 10.32).

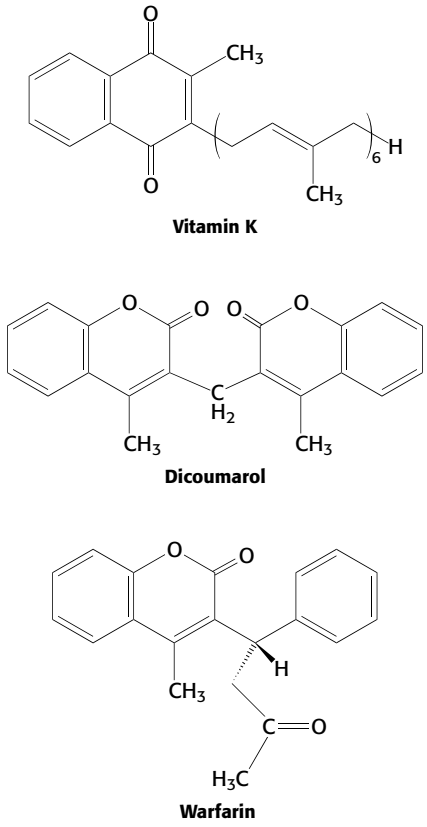


FIGURE 10.31 Structures of vitamin K and two antagonists, dicoumarol and warfarin.

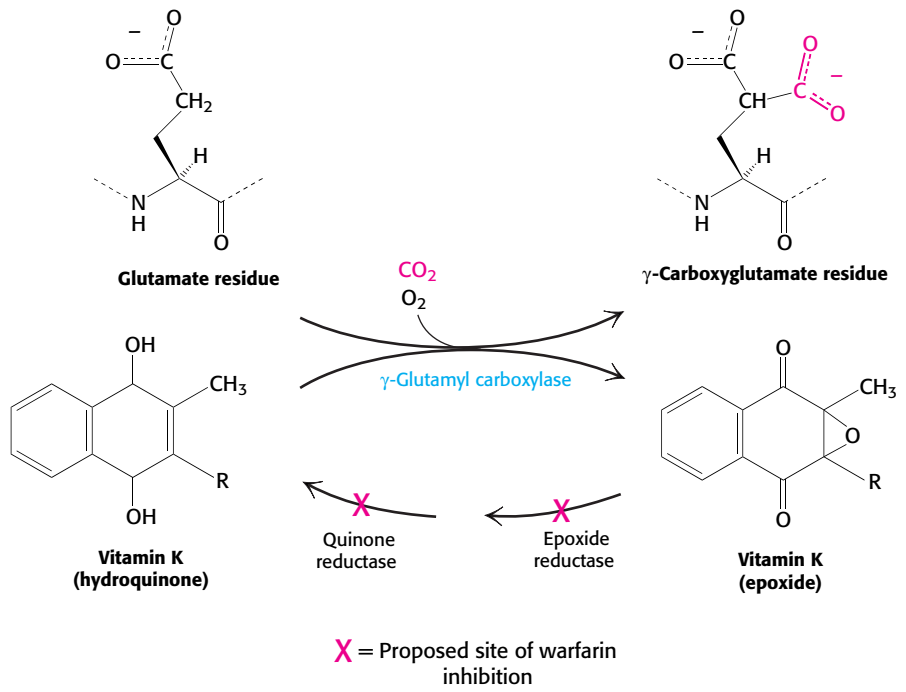


FIGURE 10.32 Synthesis of γ-carboxyglutamate by γ-glutamyl carboxylase. The formation of γ-carboxyglutamate requires the hydroquinone derivative of vitamin K, which is regenerated from the epoxide derivative by the sequential action of epoxide reductase and quinone reductase, both of which are inhibited by warfarin.

Recall that γ-carboxyglutamate, a strong chelator of Ca^{2+}, is required for the activation of prothrombin (p. 304). *Dicoumarol*, which is found in spoiled sweet clover, causes a fatal hemorrhagic disease in cattle fed on this hay. Cows fed dicoumarol synthesize an abnormal prothrombin that does not bind Ca^{2+}, in contrast with normal prothrombin. Dicoumarol was the first *anticoagulant* used to prevent thromboses in patients prone to clot formation. However, it is seldom used now because of poor absorption and gastrointestinal side effects. *Warfarin*, another vitamin K antagonist, is commonly administered as an anticoagulant. Warfarin inhibits the keto reductase and quinone reductase that are required to regenerate the dihydro derivative of vitamin K (Figure 10.32). Dicoumarol, warfarin, and their chemical derivatives serve as effective rat poisons.

An account of a hemorrhagic disposition existing in certain families

"About seventy or eighty years ago, a woman by the name of Smith settled in the vicinity of Plymouth, New Hampshire, and transmitted the following idiosyncrasy to her descendants. It is one, she observed, to which her family is unfortunately subject and has been the source not only of great solicitude, but frequently the cause of death. If the least scratch is made on the skin of some of them, as mortal a hemorrhage will eventually ensue as if the largest wound is inflicted. . . . It is a surprising circumstance that the males only are subject to this strange affection, and that all of them are not liable to it. . . . Although the females are exempt, they are still capable of transmitting it to their male children."

John Otto (1803)

The clotting process must be precisely regulated

There is a fine line between hemorrhage and thrombosis, the formation of blood clots in blood vessels. Clots must form rapidly yet remain confined to the area of injury. What are the mechanisms that normally limit clot formation to the site of injury? The lability of clotting factors contributes significantly to the control of clotting. Activated factors are short-lived because they are diluted by blood flow, removed by the liver, and degraded by proteases. For example, the stimulatory protein factors V_a and $VIII_a$ are digested by protein C, a protease that is switched on by the action of thrombin. *Thus, thrombin has a dual function: it catalyzes the formation of fibrin and it initiates the deactivation of the clotting cascade.*

Specific inhibitors of clotting factors are also critical in the termination of clotting. For instance, *tissue factor pathway inhibitor* (TFPI) inhibits the complex of TF–VII_a–X_a that activates thrombin. Another key inhibitor is *antithrombin III*, a member of the serpin family of protease inhibitors (p. 302) that forms an irreversible inhibitory complex with thrombin. Antithrombin III resembles α_1-antitrypsin except that it inhibits thrombin much more strongly than it inhibits elastase (Figure 10.23). Antithrombin III also blocks other serine proteases in the clotting cascade—namely, factors XII_a, XI_a, IX_a, and X_a. The inhibitory action of antithrombin III is enhanced by *heparin*, a negatively charged polysaccharide (Section 11.3) found in mast cells near the walls of blood vessels and on the surfaces of endothelial cells (Figure 10.33). Heparin acts as an *anticoagulant* by increasing the rate of formation of irreversible complexes between antithrombin III and the serine protease clotting factors.

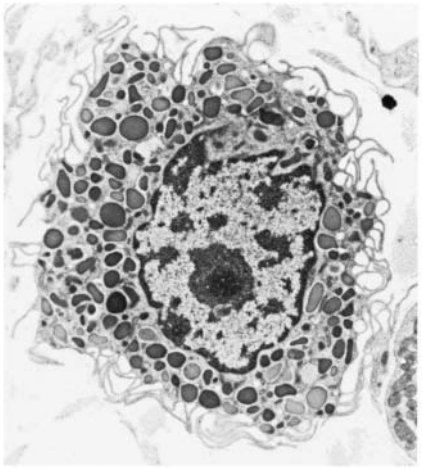


FIGURE 10.33 Electron micrograph of a mast cell. Heparin and other molecules in the dense granules are released into the extracellular space when the cell is triggered to secrete. [Courtesy of Lynne Mercer.]

The importance of the ratio of thrombin to antithrombin is illustrated in the case of a 14-year-old boy who died of a bleeding disorder because of a mutation in his α_1-antitrypsin, which normally inhibits elastase. Methionine 358 in α_1-antitrypsin's binding pocket for elastase was replaced by arginine, resulting in a change in specificity from an elastase inhibitor to a thrombin inhibitor. Activity of α_1-antitrypsin normally increases markedly after injury to counteract excess elastase arising from stimulated neutrophils. The mutant α_1-antitrypsin caused the patient's thrombin activity to drop to such a low level that hemorrhage ensued. We see here a striking example of how a change of a single residue in a protein can dramatically alter specificity and an example of the critical importance of having the right amount of a protease inhibitor.

Antithrombin limits the extent of clot formation, but what happens to the clots themselves? Clots are not permanent structures but are designed to

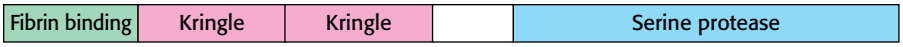


FIGURE 10.34 Modular structure of tissue-type plasminogen activator (TPA).

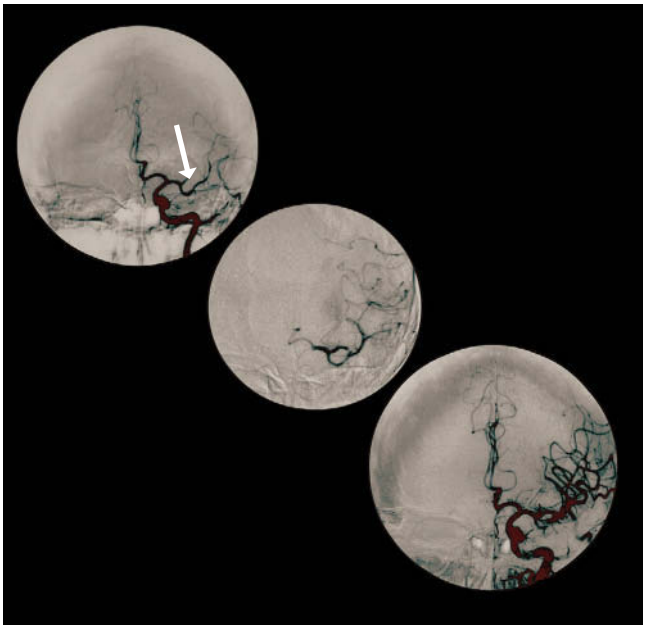


FIGURE 10.35 **The effect of tissue-type plasminogen activator.** Angiographic images demonstrate the effect of TPA administration. The top left image shows an occluded cerebral artery (arrow) prior to TPA injection. The middle image indicates the site of injection. The lower right image, made several hours after injection, reveals the restoration of blood flow to the cerebral artery. [Medical Body Scans/Science Source.]

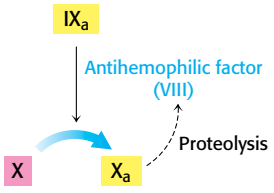


FIGURE 10.36 **Action of antihemophilic factor.** Antihemophilic factor (Factor VIII) stimulates the activation of factor X by factor IX_a. Interestingly, the activity of factor VIII is markedly increased by limited proteolysis by thrombin. This positive feedback amplifies the clotting signal and accelerates clot formation after a threshold has been reached.

dissolve when the structural integrity of damaged areas is restored. Fibrin is degraded by *plasmin*, a serine protease that hydrolyzes peptide bonds in the coiled-coil regions. Plasmin molecules can diffuse through aqueous channels in the porous fibrin clot to cut the accessible connector rods. Plasmin is formed by the proteolytic activation of *plasminogen*, an inactive precursor that has a high affinity for the fibrin clots. This conversion is carried out by *tissue-type plasminogen activator* (TPA), a 72-kDa protein that has a domain structure closely related to that of prothrombin (Figure 10.34). However, a domain that targets TPA to fibrin clots replaces the membrane-targeting gla domain of prothrombin. The TPA bound to fibrin clots swiftly activates adhering plasminogen. In contrast, TPA activates free plasminogen very slowly. The gene for TPA has been cloned and expressed in cultured mammalian cells. TPA administered at the onset of a heart attack or a stroke caused by a blood clot increases the likelihood of survival without physical or cognitive disabilities (Figure 10.35).

Hemophilia revealed an early step in clotting

Some important breakthroughs in the elucidation of clotting pathways have come from studies of patients with bleeding disorders. *Classic hemophilia*, or *hemophilia A*, is the best-known clotting defect. This disorder is genetically transmitted as a sex-linked recessive characteristic. *In classic hemophilia, factor VIII (antihemophilic factor) of the intrinsic pathway is missing or has markedly reduced activity.* Although factor VIII is not itself a protease, it markedly stimulates the activation of factor X, the final protease of the intrinsic pathway, by factor IX_a, a serine protease (Figure 10.36). Thus, activation of the intrinsic pathway is severely impaired in hemophilia.

In the past, hemophiliacs were treated with transfusions of a concentrated plasma fraction containing factor VIII. This therapy carried the risk of infection. Indeed, many hemophiliacs contracted hepatitis and, more recently, AIDS. A safer source of factor VIII was urgently needed. With the use of biochemical purification and recombinant DNA techniques, the gene for factor VIII was isolated and expressed in cells grown in culture. Recombinant factor VIII purified from these cells has largely replaced plasma concentrates in treating hemophilia.