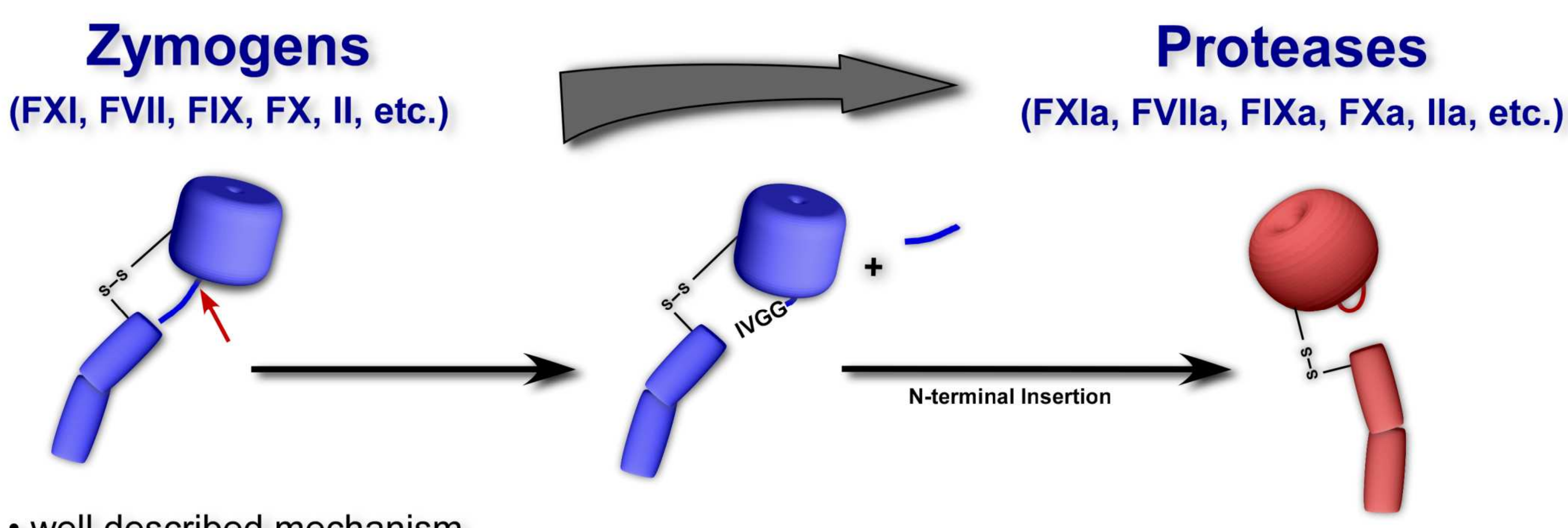
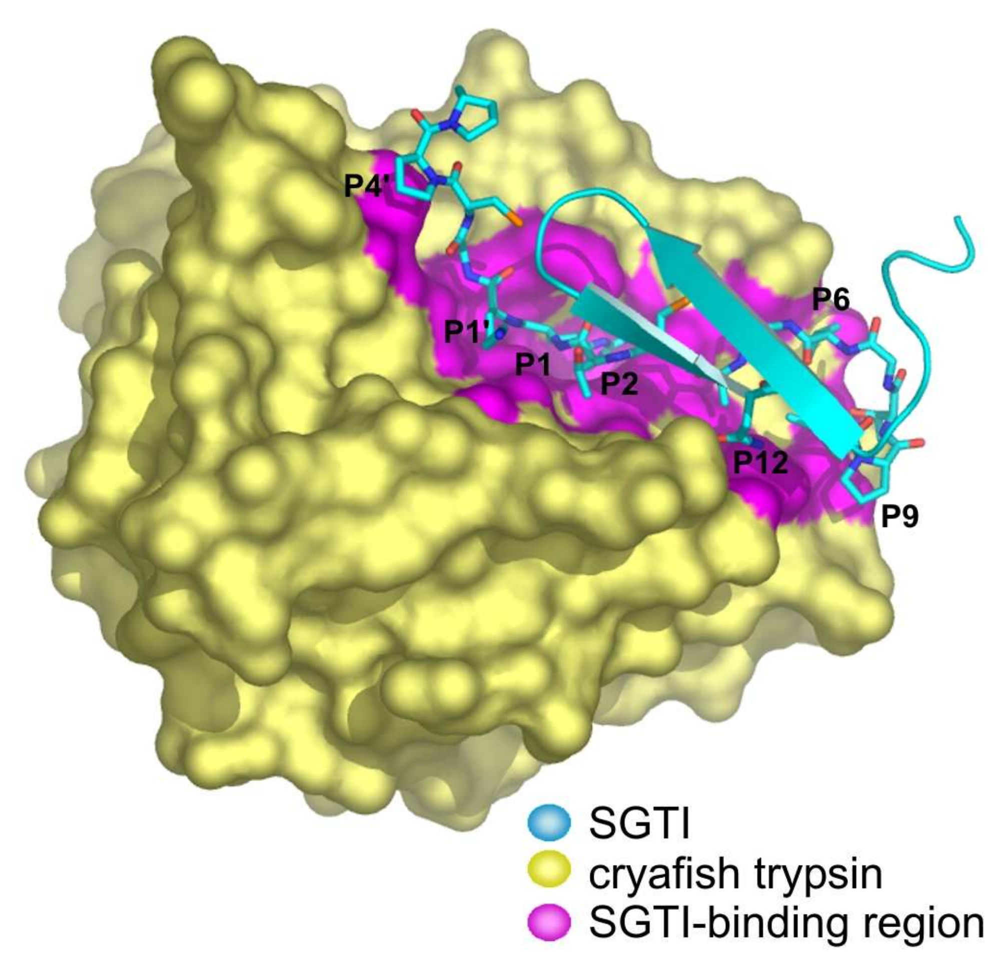
Zymogen Activation as an important way to control enzymatic activity

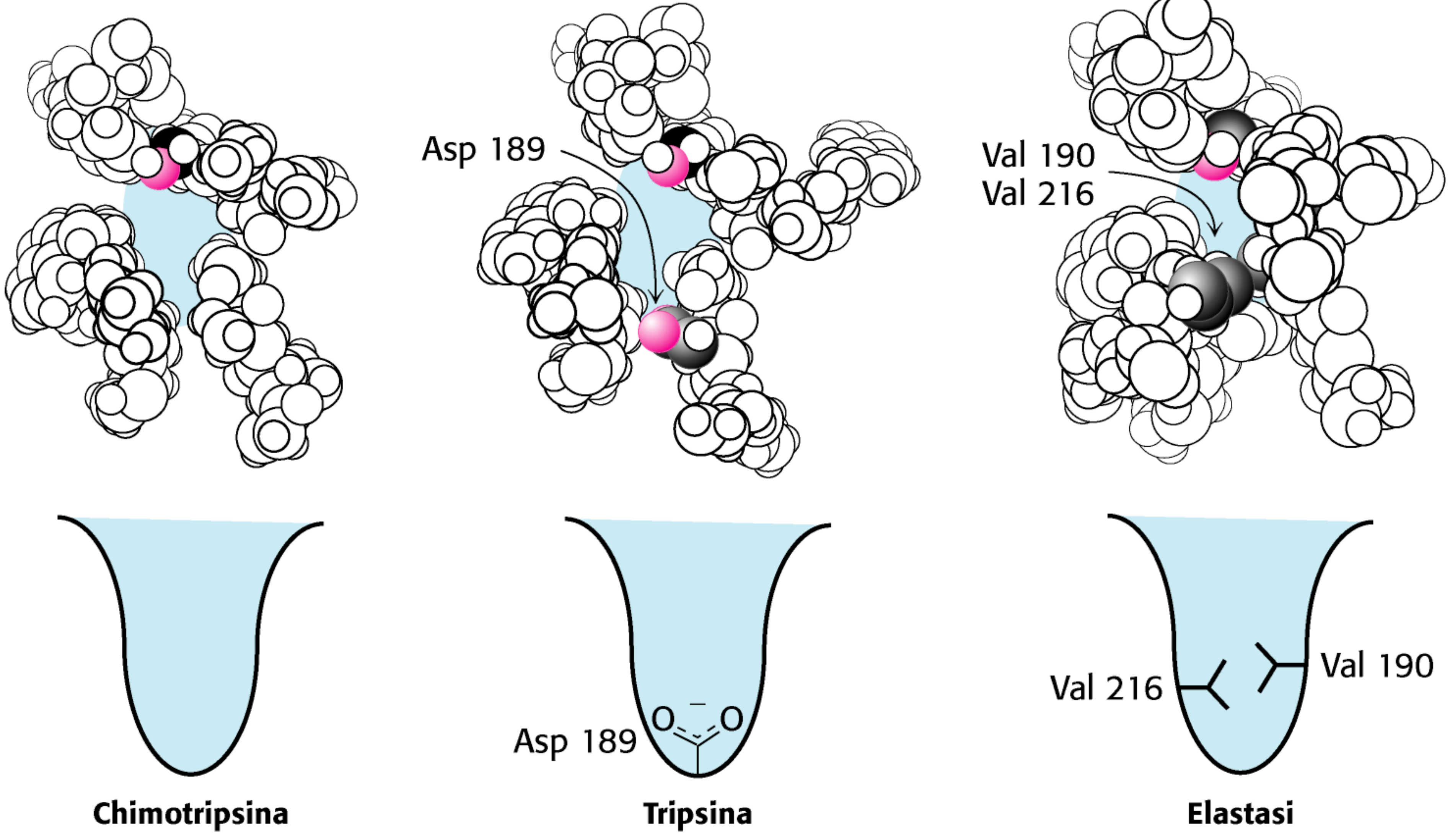


UN ALTRO MODO PER MODULARE L'ATTIVITA' ENZIMATICA

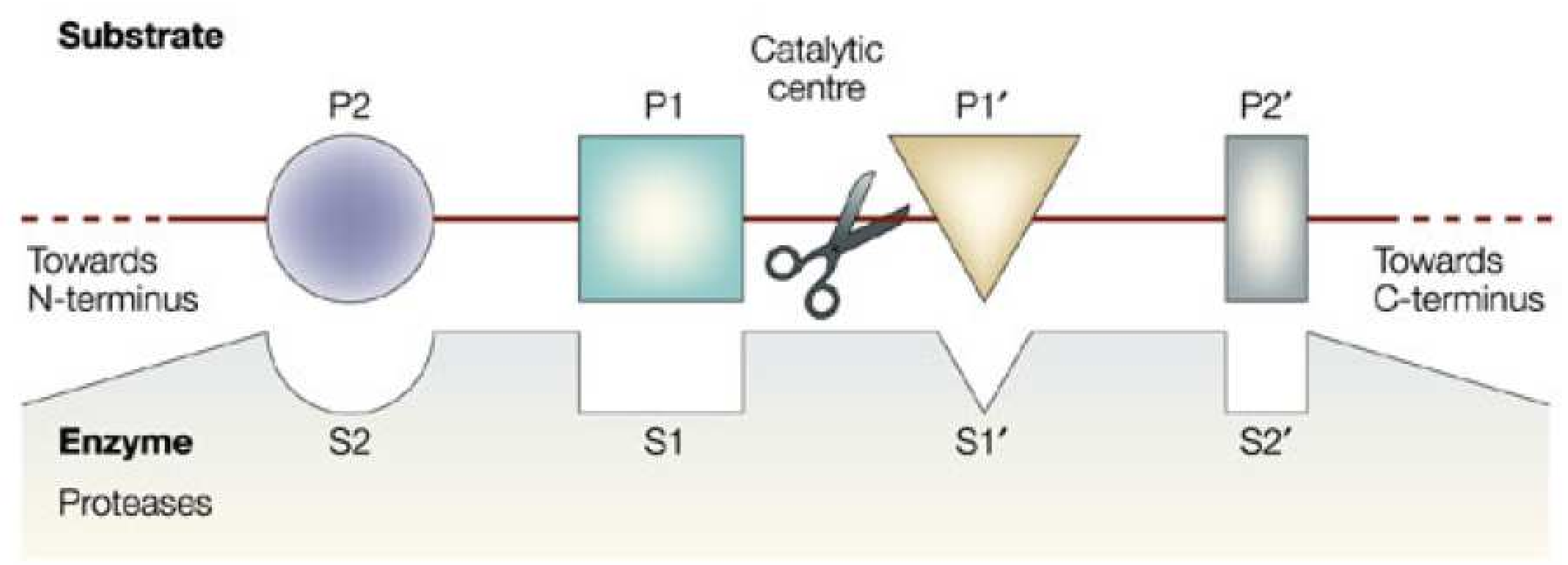
Enzyme Substrate Specificity: Role of Protein Exosites

Digestion Serine proteases are poorly specific



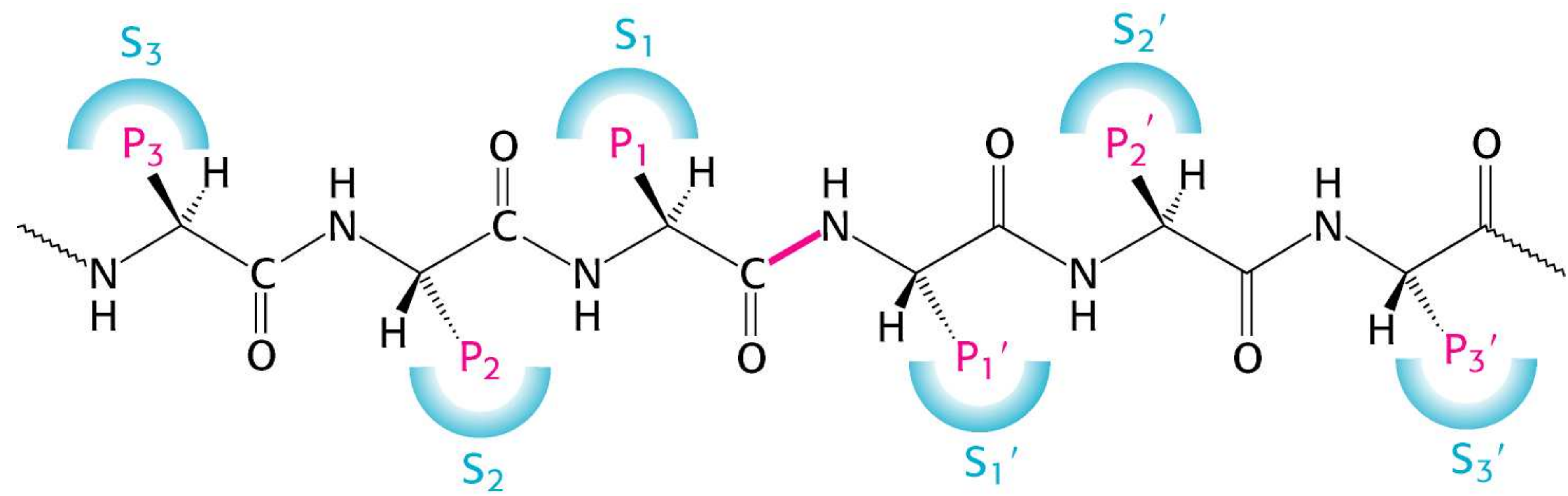

Asp 189
Val 190
Val 216
O
O
Asp 189
Val 216
Val 190
Chimotripsina
Tripsina
Elastasi

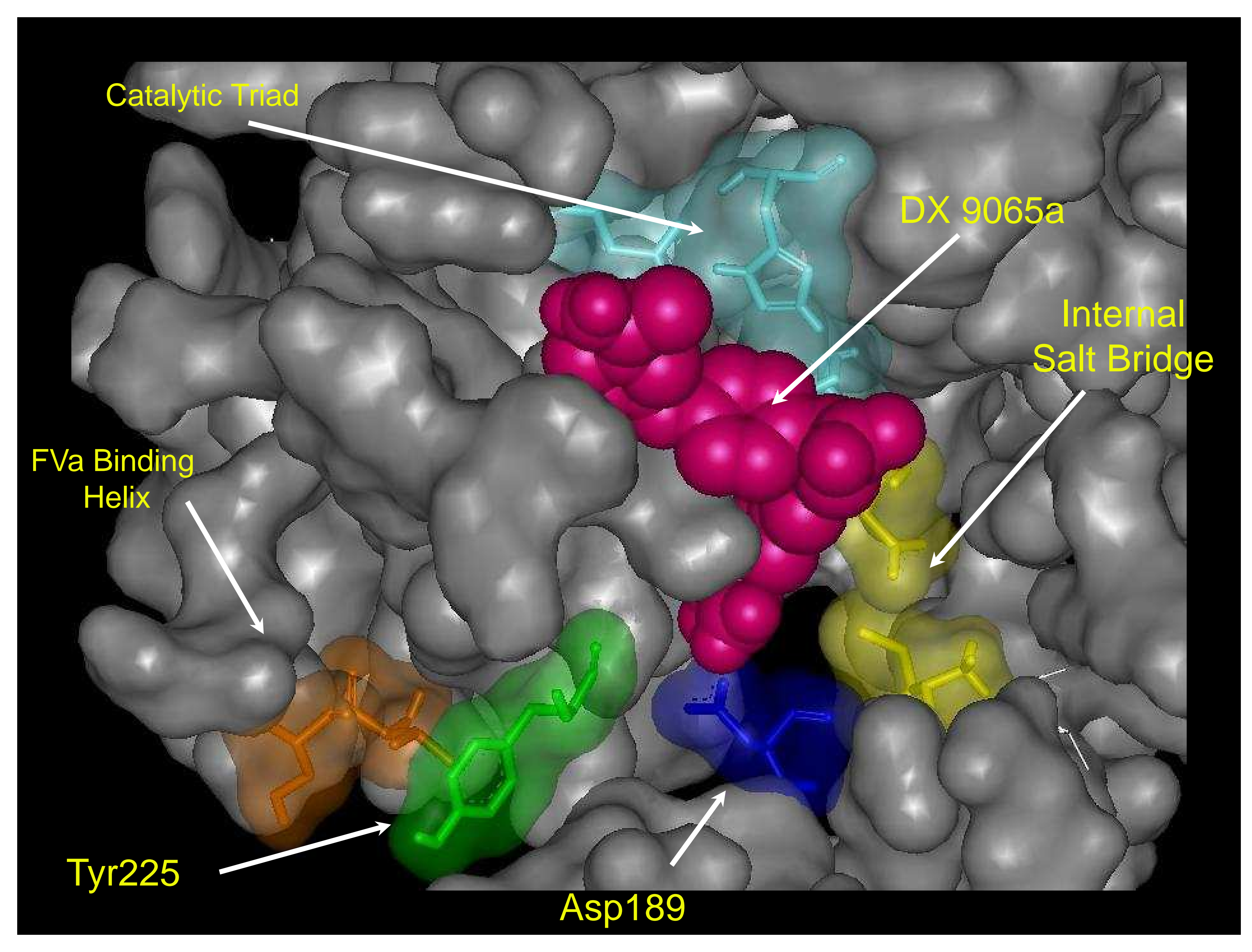
SUBSTRATE RECOGNITION SITES



P1-S1 interaction: S1 = Pocket adjacent to Ser195
Specificity determined by residues 189, 216, 226

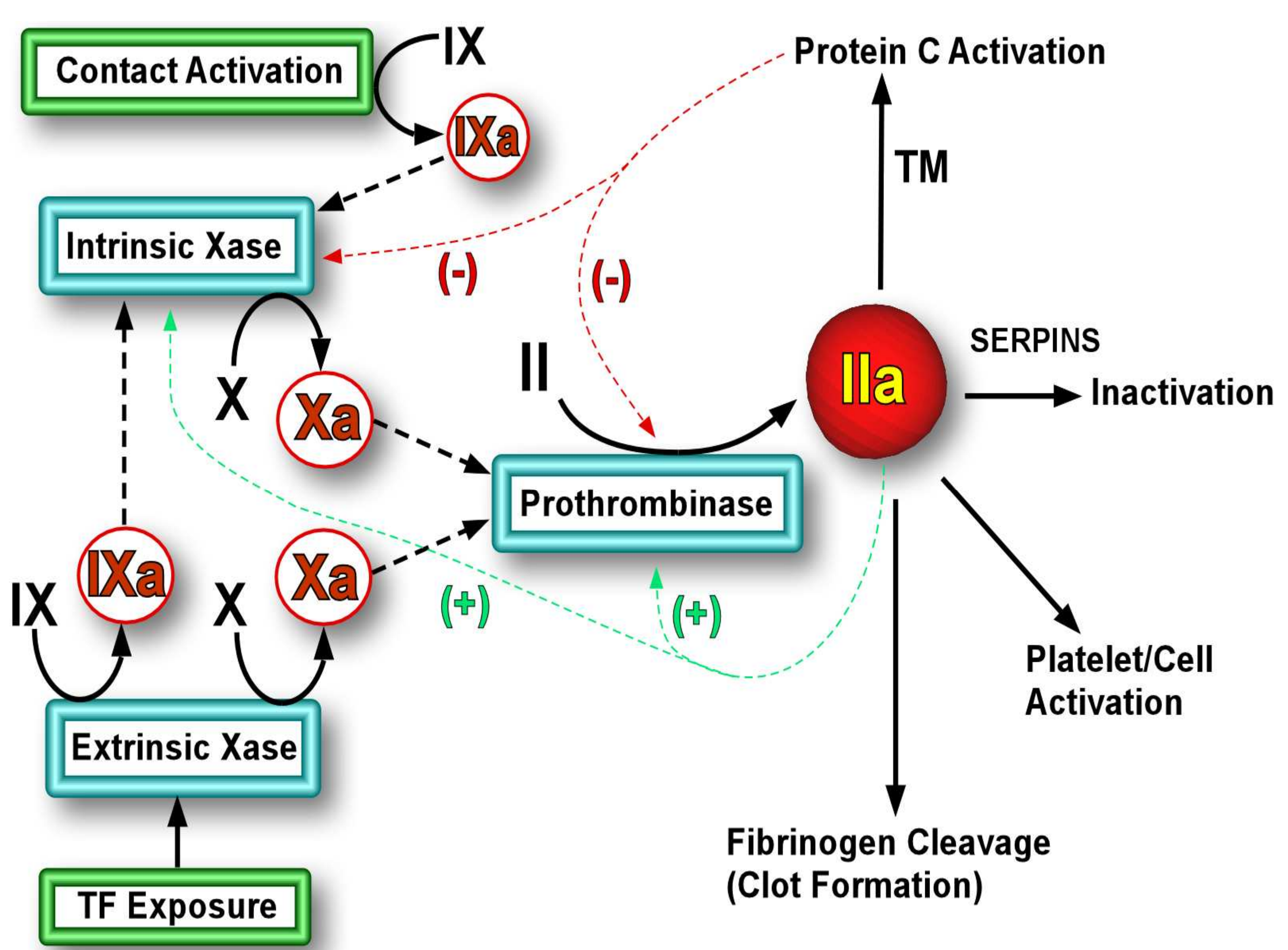
Per molte altre serina proteasi i determinanti di specificità sono molto più complessi




Catalytic Triad
DX 9065a
Internal
Salt Bridge
FVa Binding
Helix
Tyr225
Asp189

Queste interazioni non sono sufficienti a garantire la specificità di taglio di molte serin proteasi !!!!

Blood Coagulation:
A Highly Specific Proteolytic Cascade



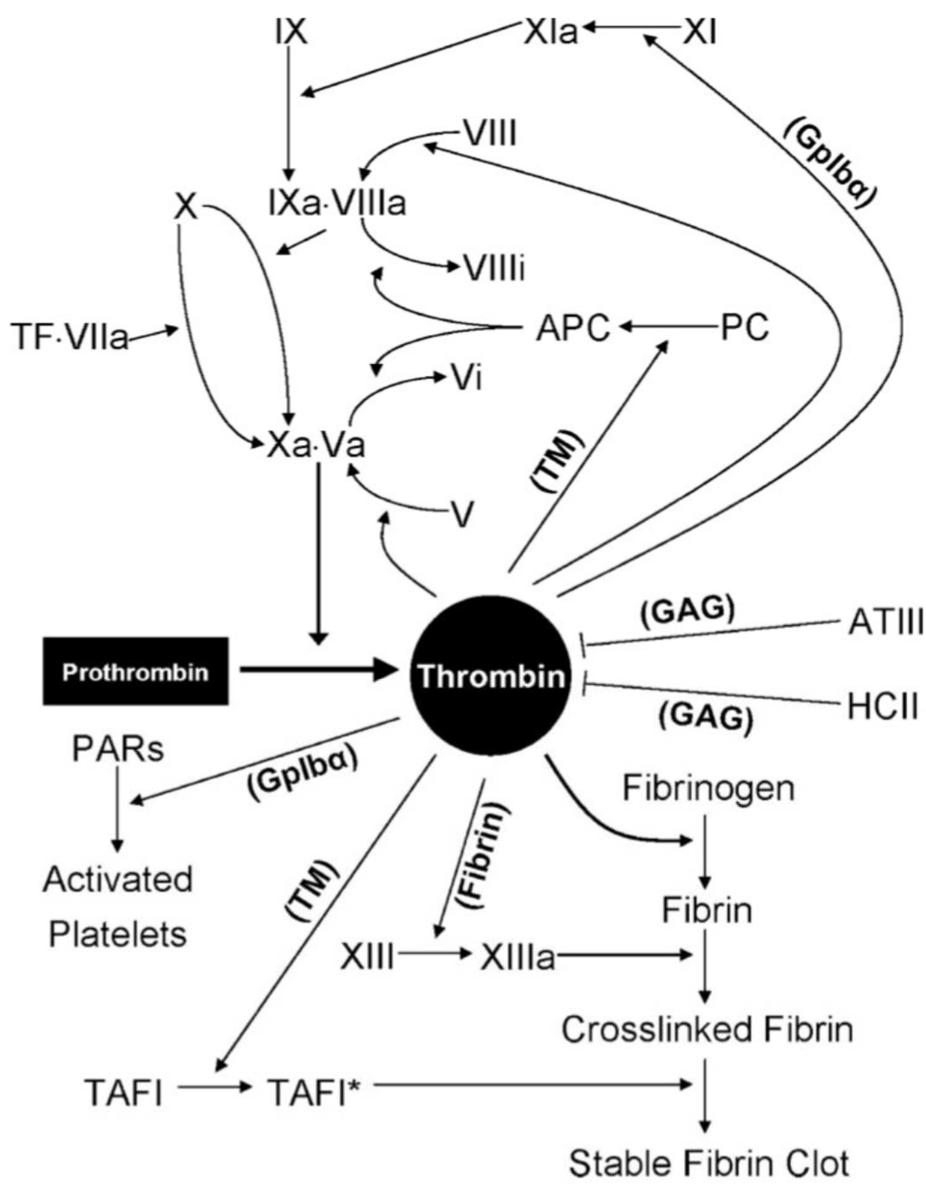


Figure 1. Thrombin activities. Schematic representation of thrombin activities in coagulation, with cofactors indicated in parentheses.

Cleavage Sites for Natural Thrombin Substrates

↓

Fibrinogen A α	GGGVRGP R VVERH
Fibrinogen B β	NEEGFFSA R GHRPLDK
Factor XIII	TVELEGVP R GVNLQQ
Factor VIII	NSPSFIQI R SVAKKH
Factor VIII	LSNNAIGP R SFSNQSR
Factor VIII	QNFVTQSK R ALKQFRL
Factor VIII	DEDENQSP R SFQKKTRH
Factor V	RLAAALGI R SFRNSSLN
Factor V	THHAPLSP R TFHPLRLS
Factor V	DNIAAWYL R SNNGNRRN
Protein C	DQGDQVDP R LIDGKMTR
Thrombin Receptor	ATNATLLDP R FLLRNPNDKY**EPFWEDEE**KNESGLTEY

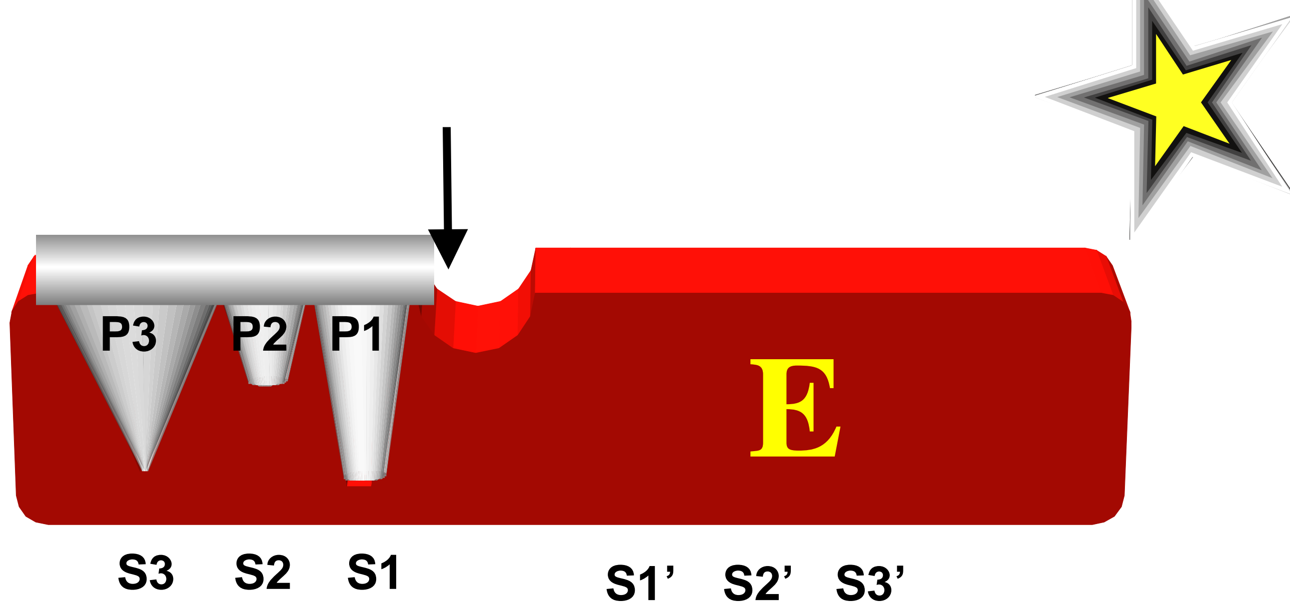
Hirudin

VVYTDCTESGQNLCLCDGSNVCGQGNKCILGSDGEKNQCVTGEGTPKPQSHN**DGDFEEIPEE**YLQ

Table 1 Sites of cleavage in the human vitamin K-dependent zymogens*

Enzyme	Substrate†	P_4	P_3	P_2	P_1	↓	$P_{1'}$	$P_{2'}$	$P_{3'}$	$P_{4'}$
Xa/Va	II	I	E	G	R		T	A	T	S
	$II_{(15–16)}$	I	D	G	R		I	V	E	G
VIIa/TF, IXa/VIIIa	$X_{(15–16)}$	N	L	T	R		I	V	G	G
VIIa/TF, XIa	IX	K	L	T	R		A	E	A	V
	$IX_{(15–16)}$	D	F	T	R		V	V	G	G
VIIa/TF, Xa	$VII_{(15–16)}$	P	Q	G	R		I	V	G	G
IIa/TM	$PC_{(15–16)}$	V	D	P	R		L	I	D	G

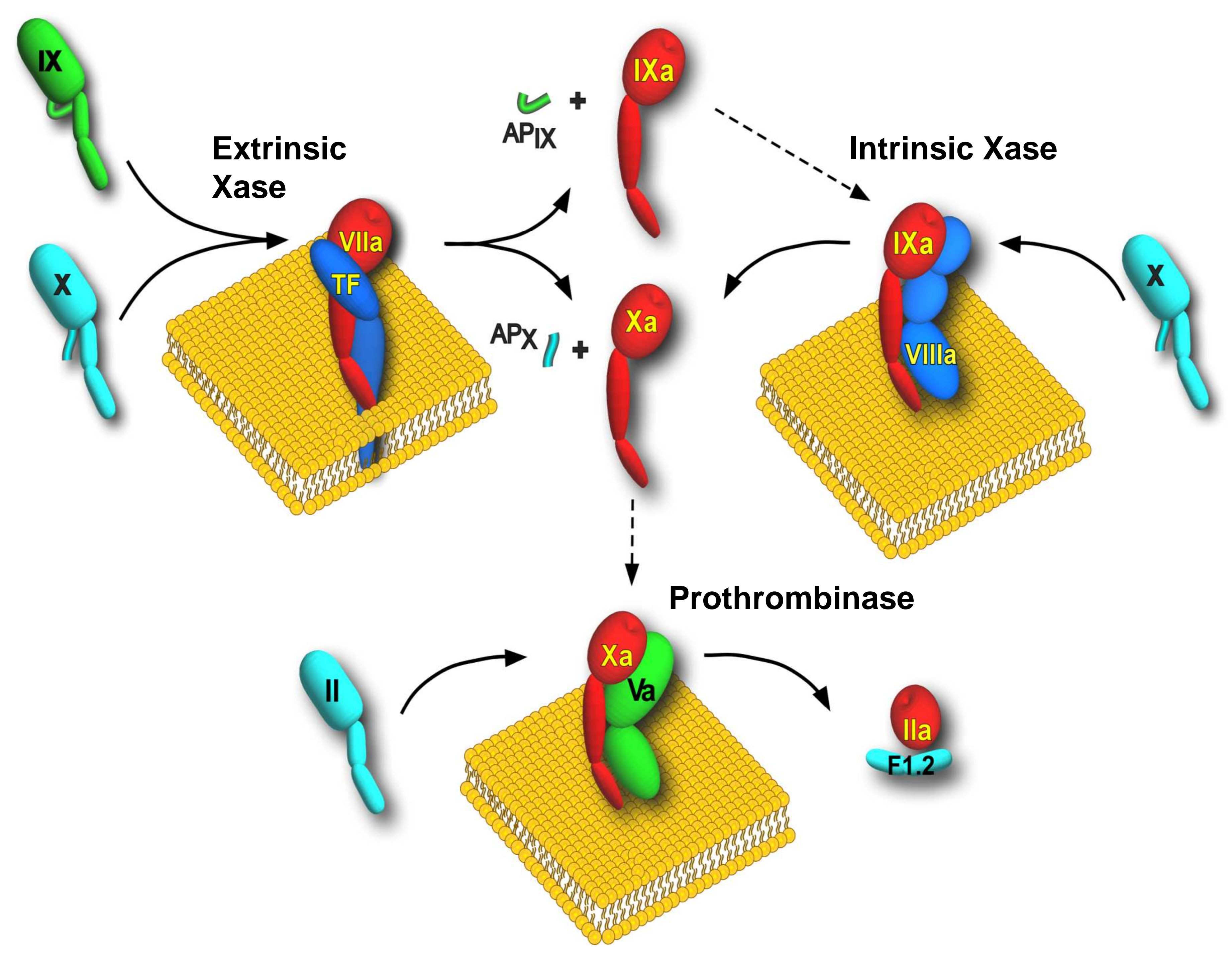
*Sequences flanking cleavage sites relevant to the activation of the vitamin K-dependent zymogens are presented along with the relevant enzymes that catalyze these reactions. The site of bond cleavage is denoted by the arrow. †The site, in each substrate, at which cleavage is required to produce the serine proteinase is indicated as (15–16) corresponding to the homologous residue numbers in chymotrypsinogen [70].



Interazioni macromolecolari estese rendono conto di queste differenti specificità

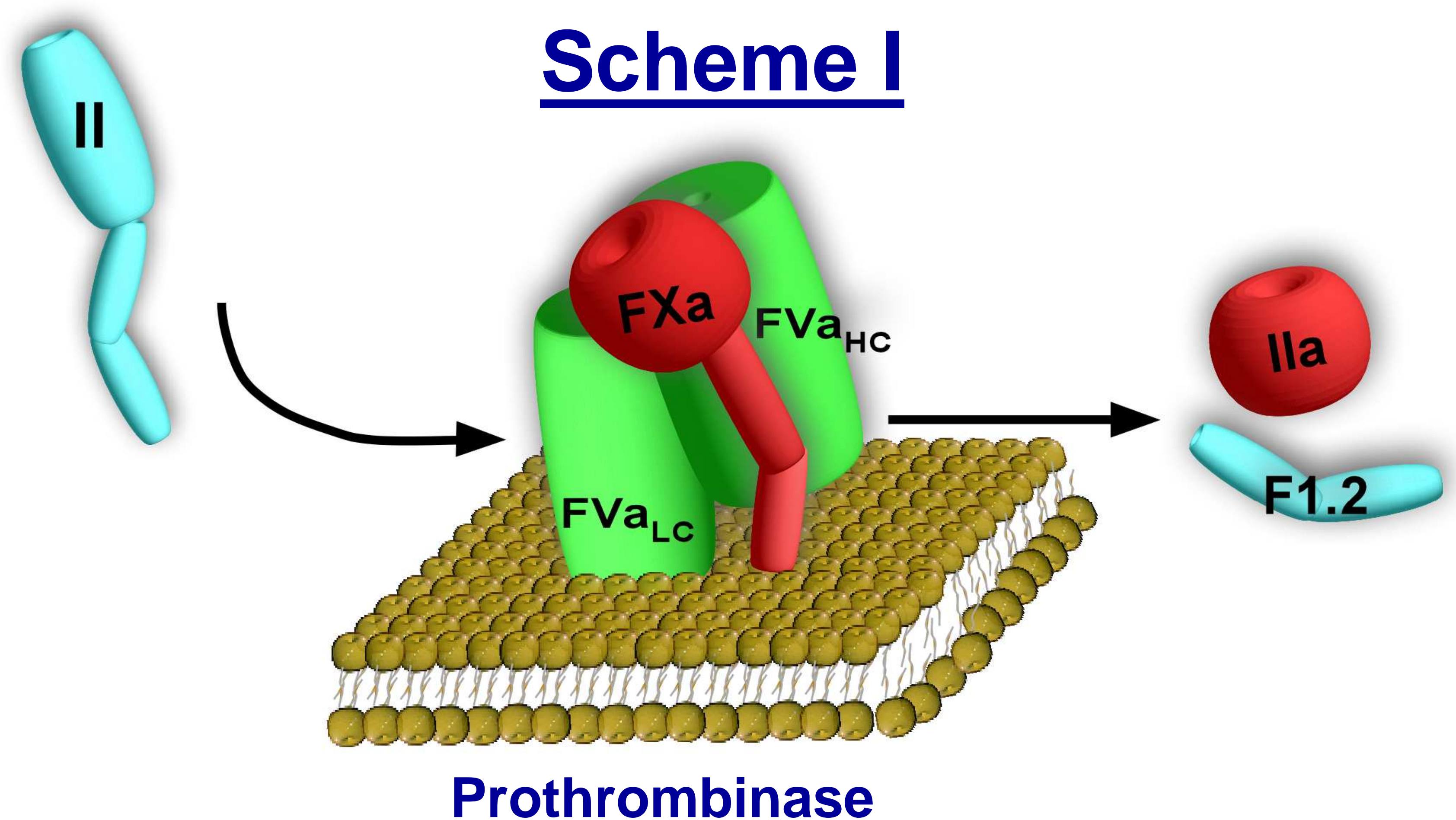
Interazioni macromolecolari estese rendono conto di queste differenti specificità

COMPLESSI MACROMOLECOLARI



IX
X
Extrinsic
Xase
VIIa
TF
APIX
+
IXa
APX
+
Xa
Intrinsic Xase
IXa
VIIIa
X
Prothrombinase
II
Xa
Va
IIa
F1.2

Scheme I



- Coagulation enzyme complexes act on their protein substrates with marked and distinctive specificity.

- What is the molecular basis for this specificity?

Cleavage Sites for Natural Thrombin Substrates

↓

Fibrinogen A α	GGGVRGP R VVERH
Fibrinogen B β	NEEGFFSA R GHRPLDK
Factor XIII	TVELEGVP R GVNLQQ
Factor VIII	NSPSFIQI R SVAKKH
Factor VIII	LSNNAIGP R SFSNQSR
Factor VIII	QNFVTQSK R ALKQFRL
Factor VIII	DEDENQSP R SFQKKTRH
Factor V	RLAAALGI R SFRNSSLN
Factor V	THHAPLSP R TFHPLRLS
Factor V	DNIAAWYL R SNNGNRRN
Protein C	DQGDQVDP R LIDGKMTR
Thrombin Receptor	ATNATLLDP R FLLRNPNDKY**EPFWEDEE**KNESGLTEY

Hirudin

VVYTDCTESGQNLCLCDGSNVCGQGNKCILGSDGEKNQCVTGEGTPKPQSHN**DGDFEEIPEE**YLQ

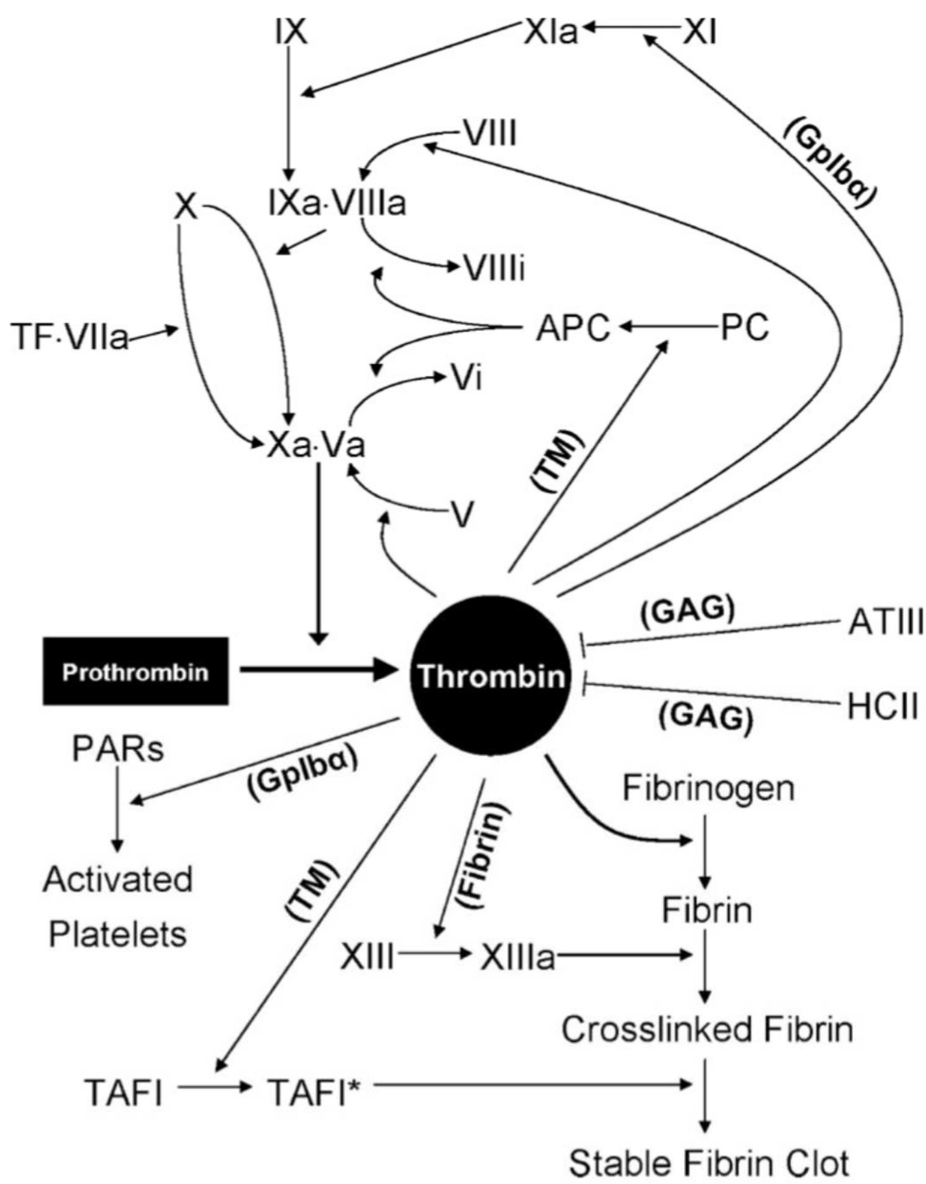
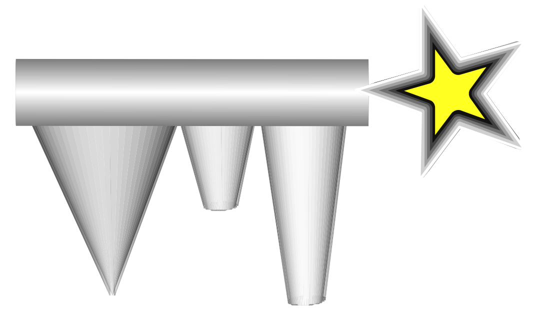
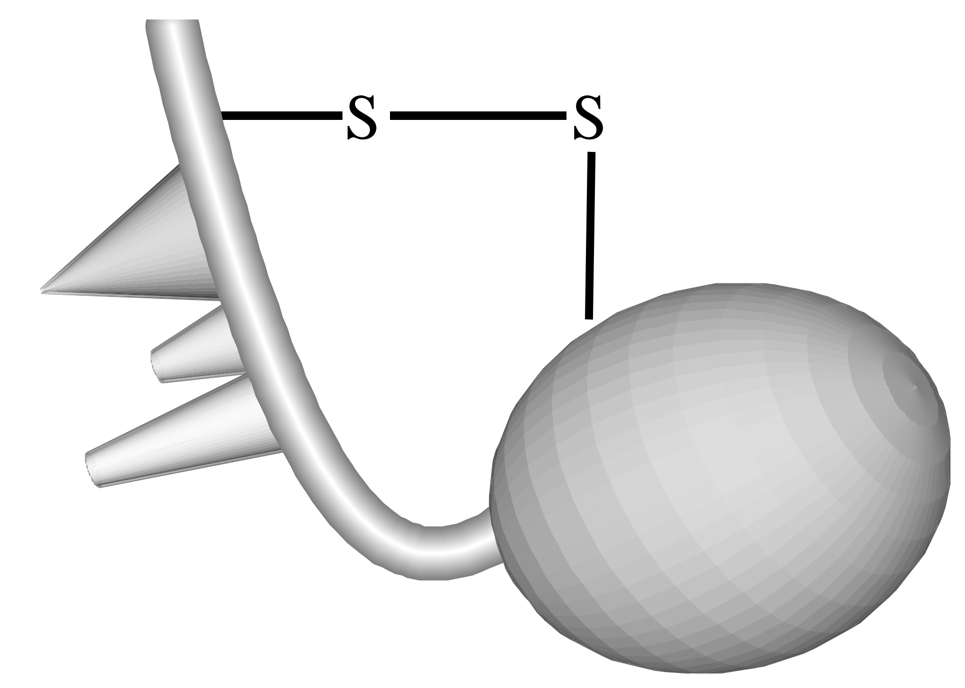


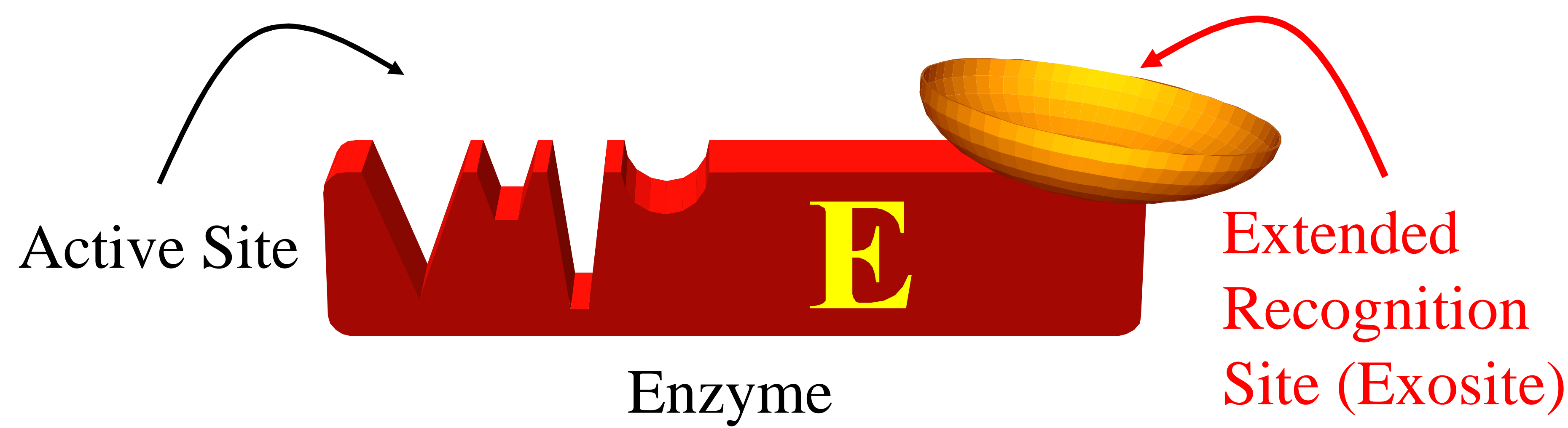
Figure 1. Thrombin activities. Schematic representation of thrombin activities in coagulation, with cofactors indicated in parentheses.



Oligopeptidyl Substrate



Protein Substrate



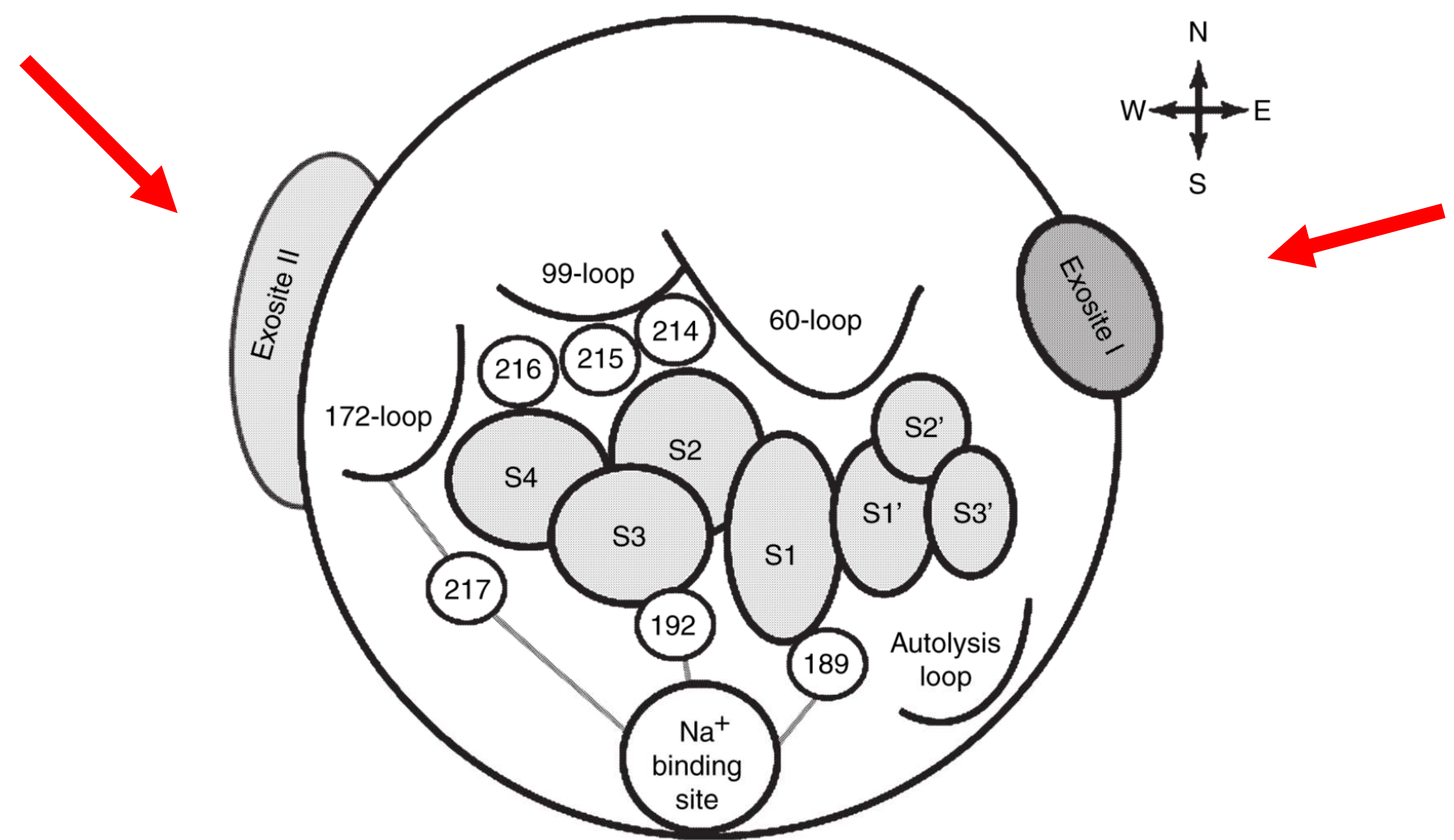


Fig. 1. Schematic representation of the specificity determinants of coagulation proteases. Loops at positions 60, 99, and 172 influence the active site specificity toward small peptide substrates and can impact macromolecular specificity. Exosites I and II, located to the east and west of the active site, are involved in substrate recognition and play fundamental regulatory roles. The Na^+-binding site within the 180 and 220 loops links to the active site and other critical sites throughout the protease domain.

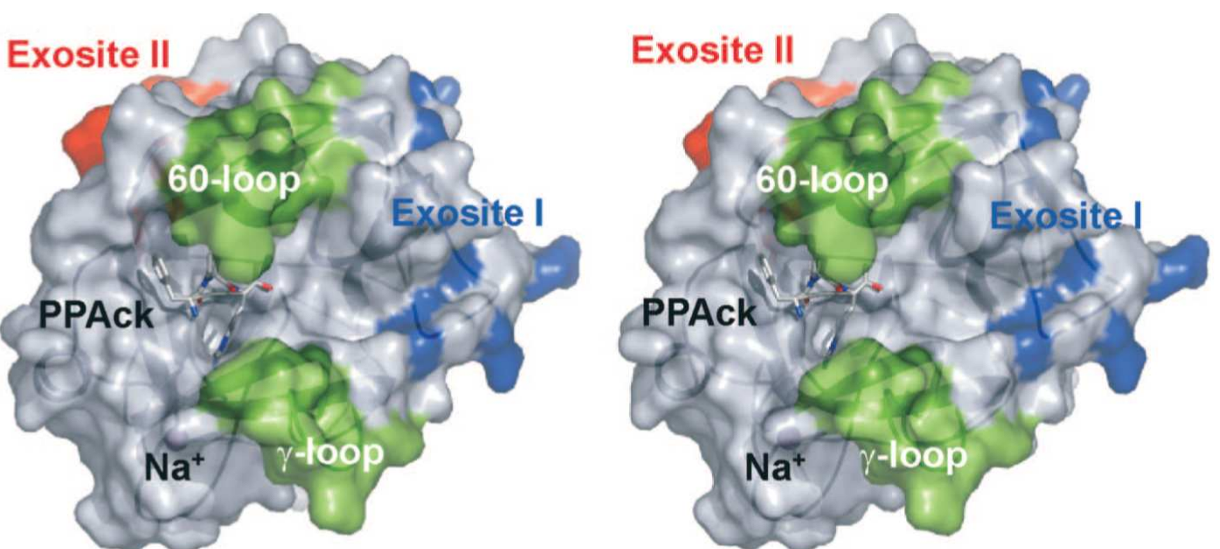


Figure 2. Thrombin topography. Stereo view of surface representations of thrombin, shown in the standard orientation (Protein DataBank entry 1PPB), bound to the active site inhibitor d-Phe-Pro-Arg-ck (PPAck). The figure (displayed with a transparent surface and underlying ribbon structure) shows positions of relevant specificity-determining sites: the 60- and γ-loops (green), residues that make up exosite I (blue), and residues in exosite II (red). The position of coordinated Na^+ is indicated.

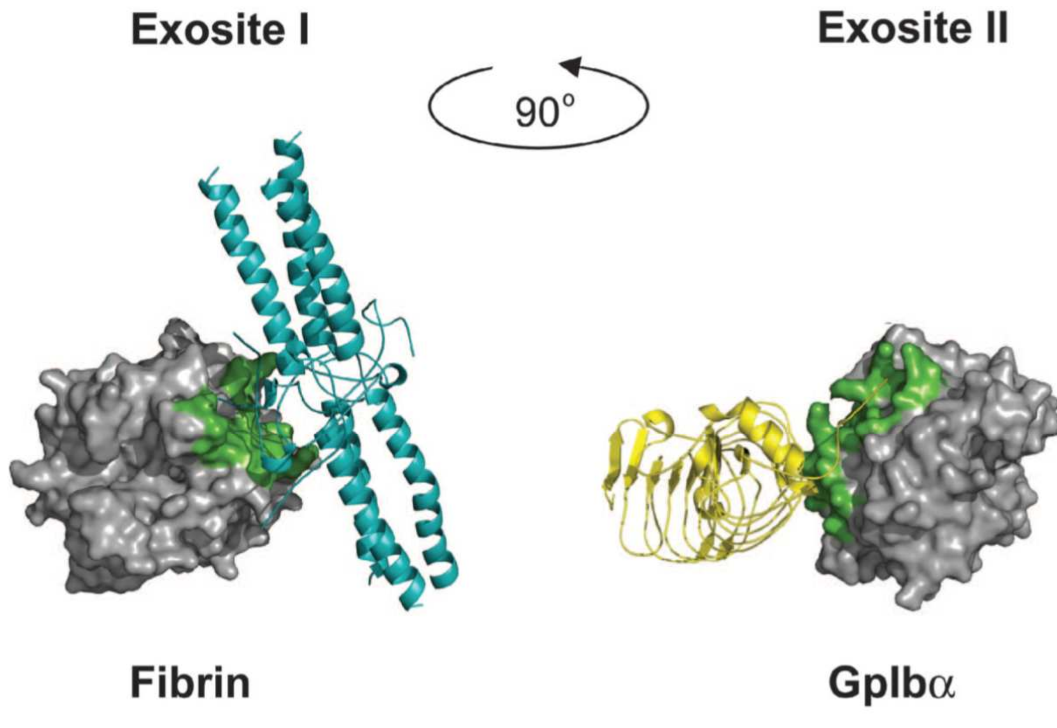
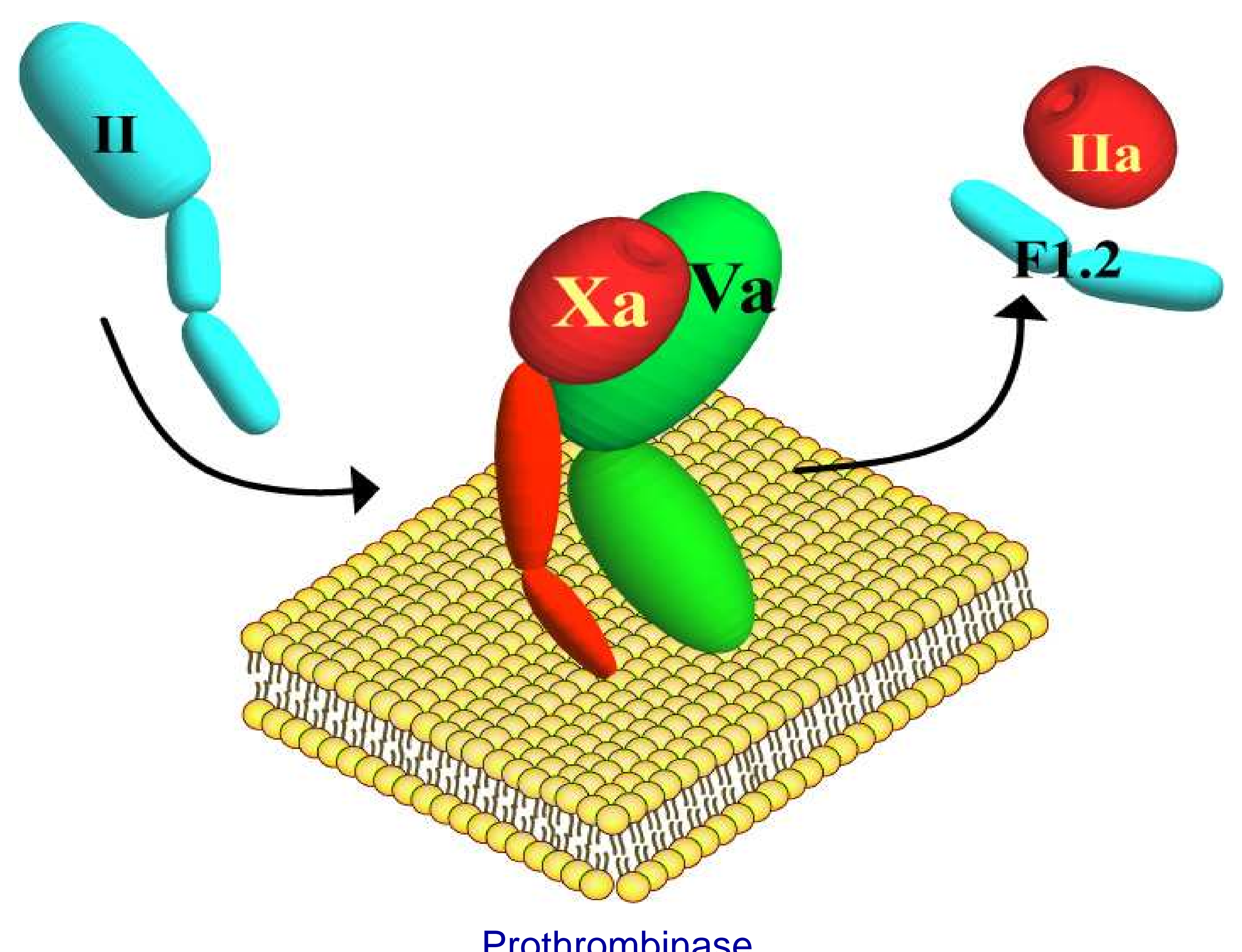


Figure 3. Thrombin-cofactor exosite interactions. The surface representation of thrombin is shown in the standard orientation (left) for exosite I interactions (Protein DataBank entries: fibrin-1QVH, TM-1DX5) and rotated 90° (right) to show exosite II interactions (PDB entries: GPIbα-1P8V, heparin-1XMN). The thrombin residues involved at the cofactor interface (<4 Å distant) are colored as green.

So, the importance of cofactors



Prothrombinase

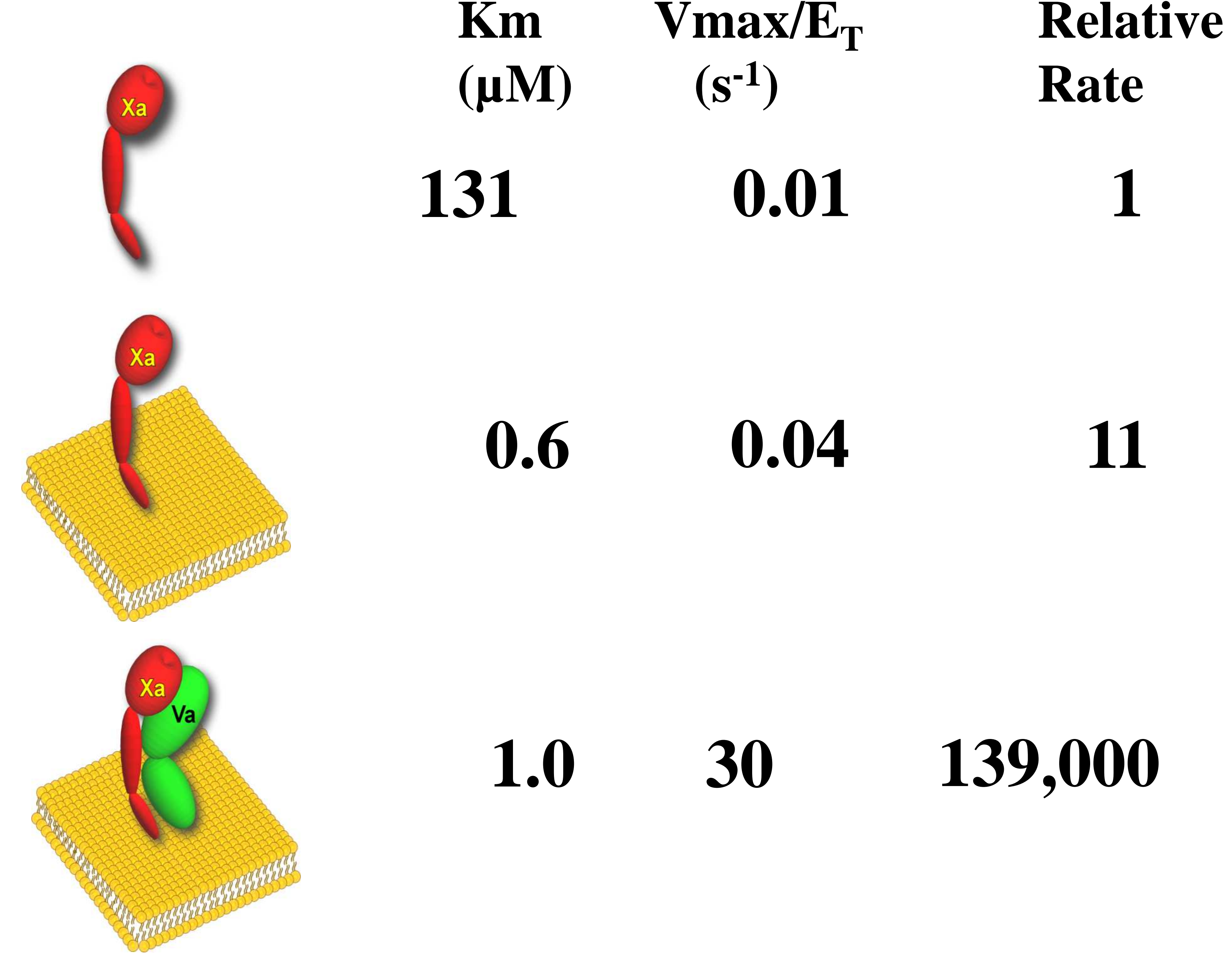

Km
(μM)
Vmax/E$_T$
(s^{-1})
Relative
Rate
Xa
131
0.01
1
Xa
0.6
0.04
11
Xa
Va
1.0
30
139,000

Table 2. Activation of factor X by factor VIIa in the presence of various cofactors of the *extrinsic factor Xase*

Cofactor	Concentration	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, μM^{-1}·min^{-1}
None	NA	>20	$>1.5 \cdot 10^{-4}$	ND
$CaCl_2$	2.5 mM	2.10	$1.0 \cdot 10^{-4}$	$4.8 \cdot 10^{-5}$
Phospholipid (PCPS)[a]	21 μM	0.25	0.016	0.062
Tissue factor[a,b]	9.4 pM	0.23	186	885

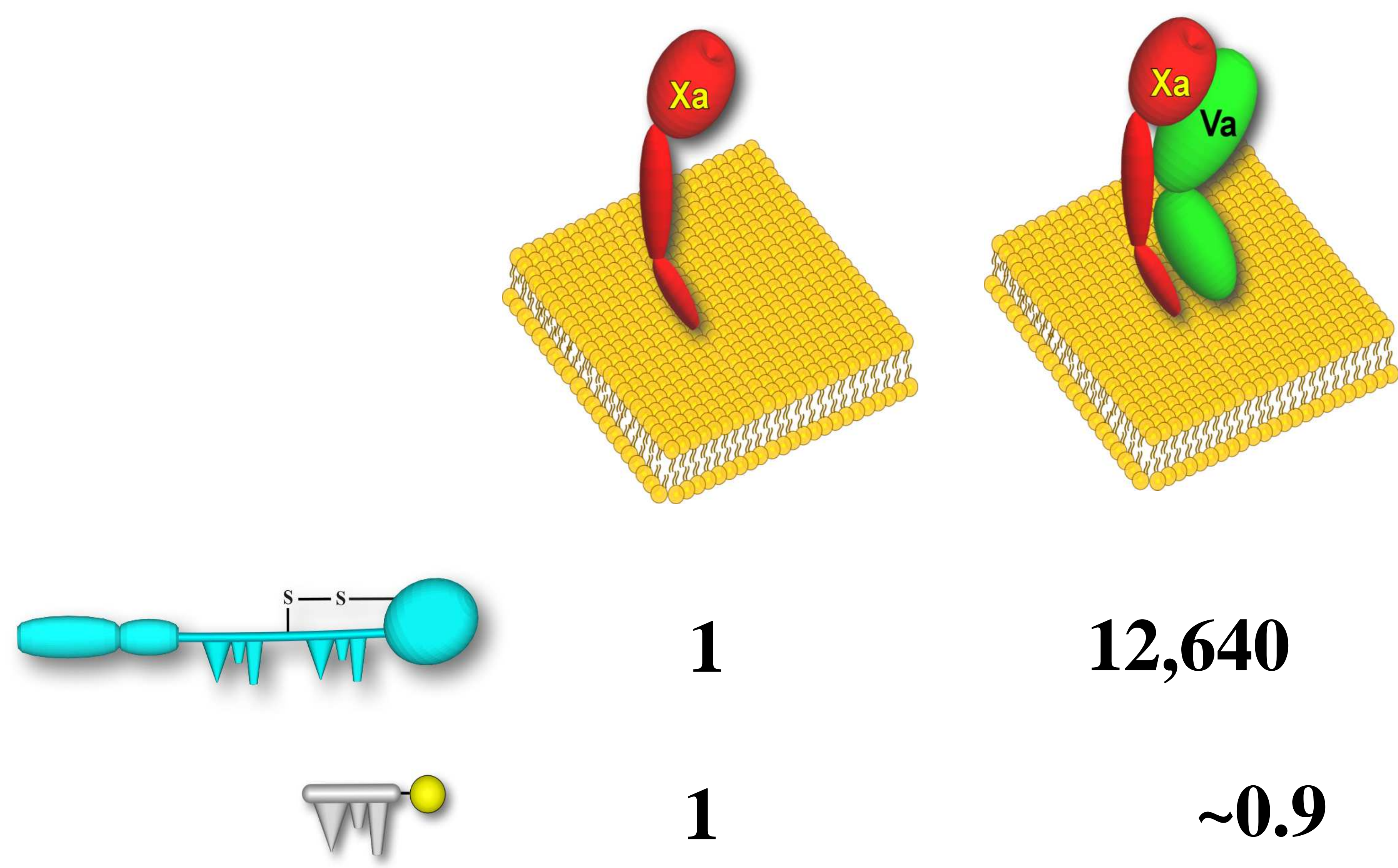
Note: NA, not applicable; ND, not determined; [a] in the presence of 5 mM $CaCl_2$; [b] in the presence of PCPS.

Table 3. Kinetic properties of the vitamin K-dependent enzymes and enzymatic complexes

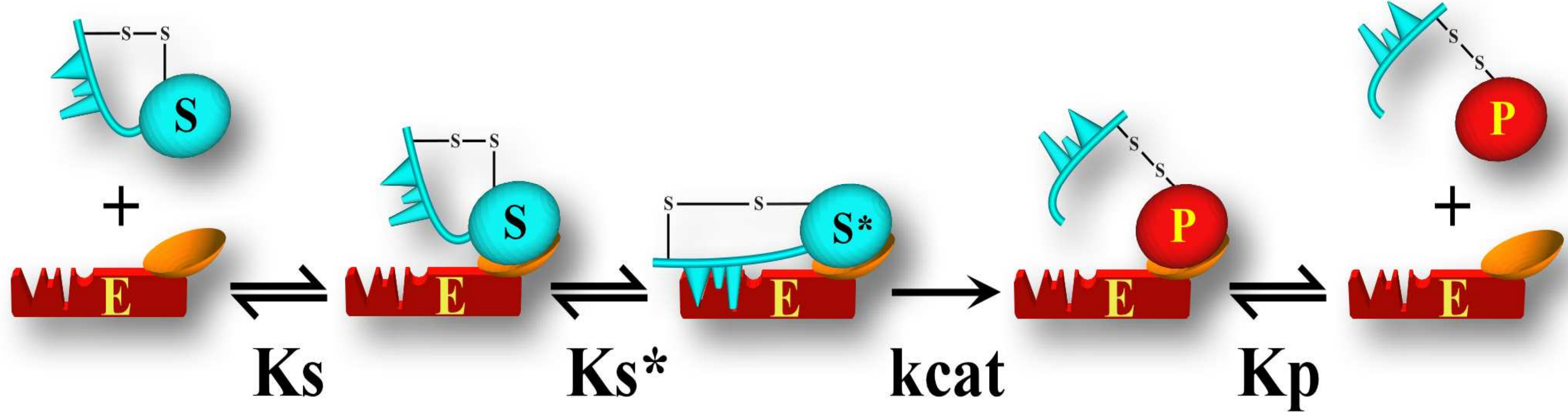
Enzyme	Substrate	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, μM^{-1}·sec^{-1}	Efficiency ratio
Factor VIIa	factor IX	ND	ND	ND	—
Factor VIIa/TF/PCPS/$CaCl_2$	factor IX	0.016	91.9	5560	—
Factor VIIa	factor X	2.1	$1.0 \cdot 10^{-4}$	$4.8 \cdot 10^{-5}$	—
Factor VIIa/TF/PCPS/$CaCl_2$	factor X	0.23	186	885	[illegible]
Factor IXa	factor X	300	0.002	$6.6 \cdot 10^{-6}$	—
Factor IXa/VIIIa/PCPS/$CaCl_2$	factor X	0.063	500	7937	$1.2 \cdot 10^{9}$
Factor Xa	factor II	131	0.6	$4.6 \cdot 10^{-3}$	—
Factor Xa/Va/PCPS/$CaCl_2$	factor II	1.0	5016	5016	$1.1 \cdot 10^{6}$
Factor IIa	protein C	60	1.2	0.02	—
Factor IIa/TM/PCPS/$CaCl_2$	protein C	0.1	214	2140	$1.1 \cdot 10^{5}$

Note: ND, not determined; TM, thrombomodulin.

Complex Assembly Selectively Increases Catalytic Efficiency for Protein Substrate Cleavage



Protein Substrate Recognition by Prothrombinase is a Multi-Step Process



Kinetic studies to understand mechanisms of interaction

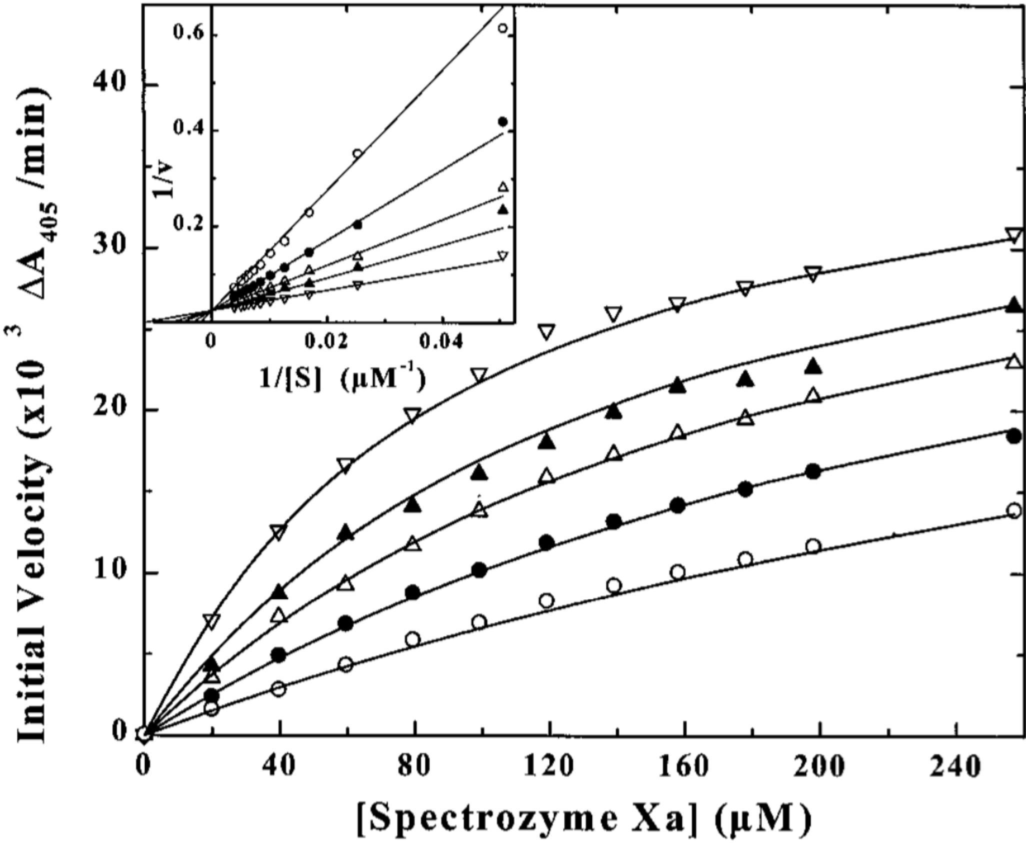
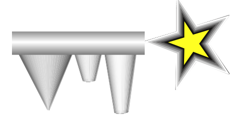
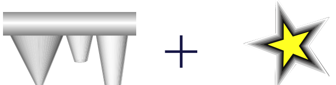


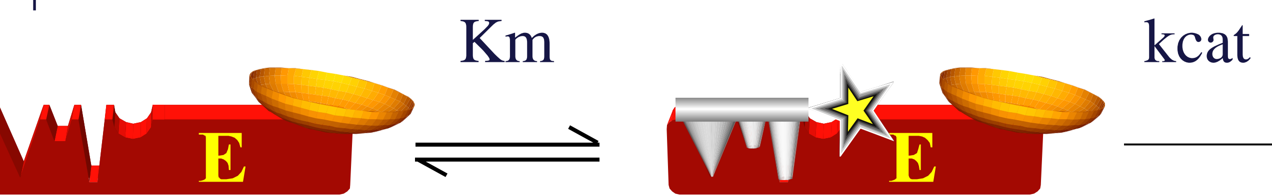
FIGURE 1: Inhibition kinetics of peptidyl substrate hydrolysis by prothrombinase. Initial velocities were measured using 0.5 nM prothrombinase (0.5 nM Xa, 20 nM Va, 50 μM PCPS), increasing concentrations of Spectrozyme Xa with 0 (▽), 15 μM (▲), 30 μM (△), 60 μM (●) and 120 μM (○) PAB. The lines are drawn according to linear competitive inhibition, with the fitted constants listed in Table 1. (Inset) Double reciprocal plot showing effect of PAB on K_m but not on the V_{max} for the reaction.

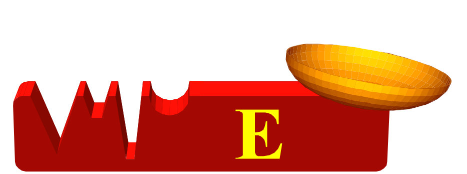
Competitive Inhibition by PAB

<u>Km increases</u>









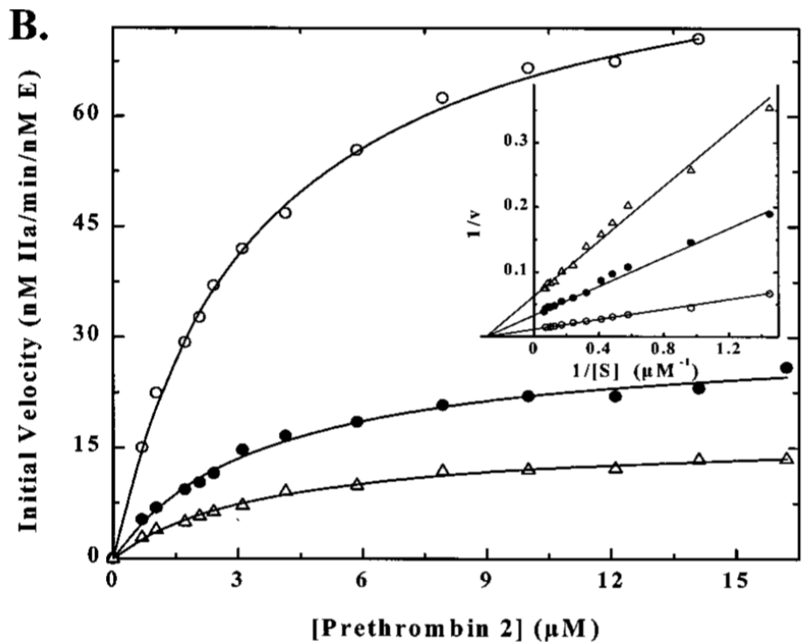
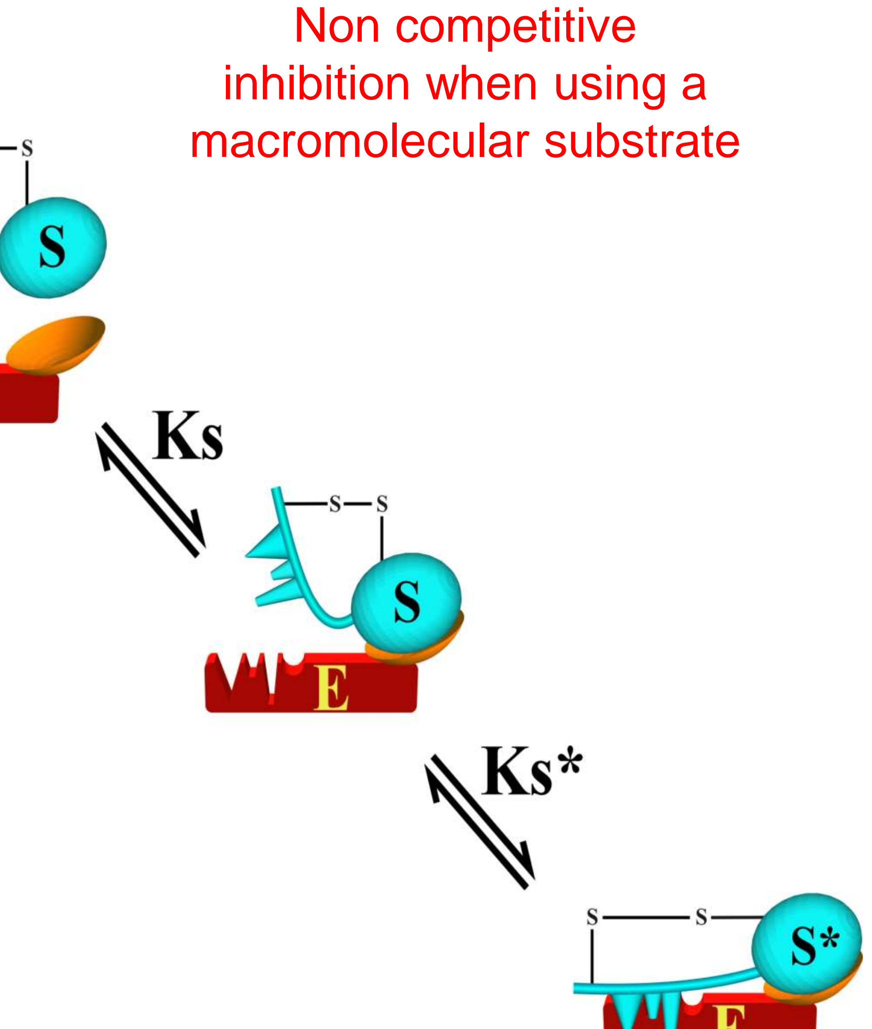
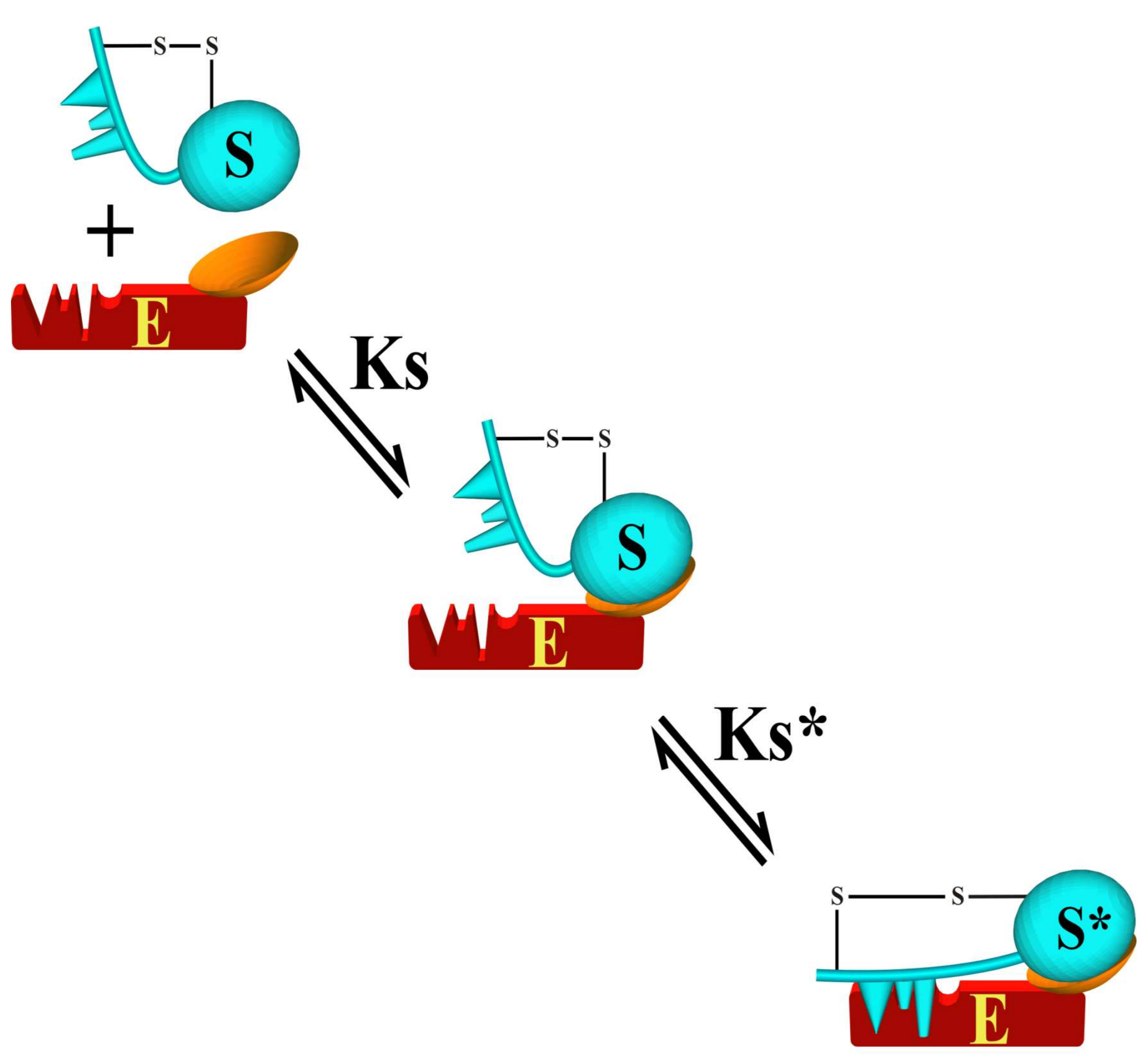


FIGURE 2: Inhibition kinetics of macromolecular substrate cleavage by prothrombinase. The initial velocity for thrombin formation (rate normalized/nanomolar prothrombinase) was determined at increasing concentrations of prethrombin 2 *plus* fragment 1.2 with 0.25 nM prothrombinase (0.25 nM factor Xa, 54 μM PCPS, and 24 nM Va) and 0 (○), 189 μM (●) or 409 μM PAB (△) (Panel A) or increasing concentrations of prethrombin 2 with 5 nM prothrombinase (5 nM Xa, 54 μM PCPS, and 24 nM Va) and 0 (○), 60 μM (●) and 160 μM PAB (△) (panel B). The lines are drawn following analysis according to classical noncompetitive inhibition, with the constants $K_{m_{obs}} = 0.38 \pm 0.02$ μM, $V_{max_{obs}}/E_T = 23 \pm 4$ s^{-1}, and $K_i = 57.3 \pm 4.7$ μM (panel A) or $K_{m_{obs}} = 3.39 \pm 0.1$ μM, $V_{max_{obs}}/E_T = 1.46 \pm 0.02$ s^{-1}, and $K_i = 31.8 \pm 0.64$ μM (panel B). Insets illustrate that PAB changes V_{max} but not K_m.

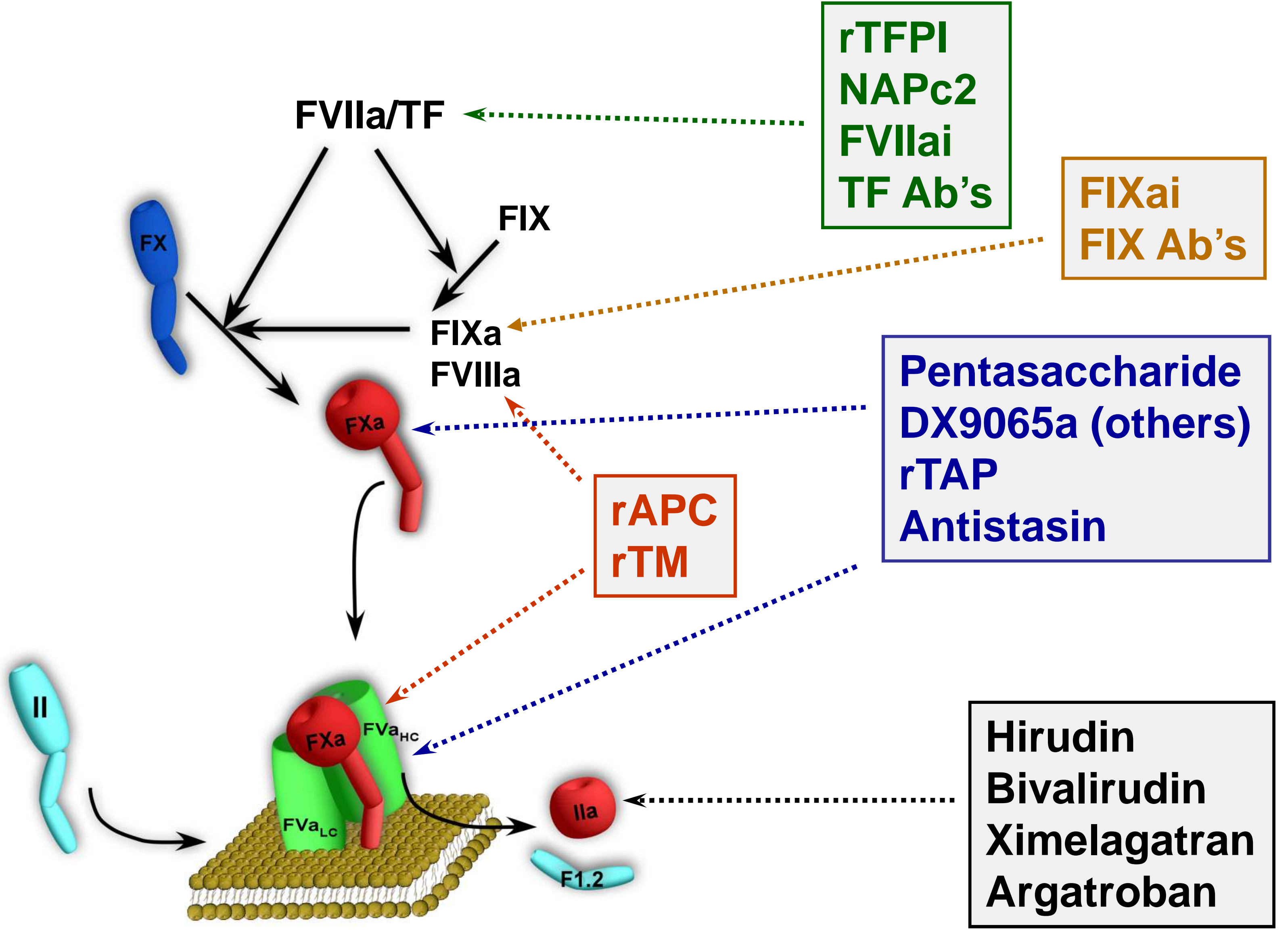



S—S
S
+
E
Ks
S—S
S
E
Ks*
S
S
S*
E

Extended interactions at exosites drive substrate affinity and contribute to substrate specificity.

Active site docking of macromolecular substrates significantly influences the catalytic rate (k_{cat}).

New Anticoagulants Under Development



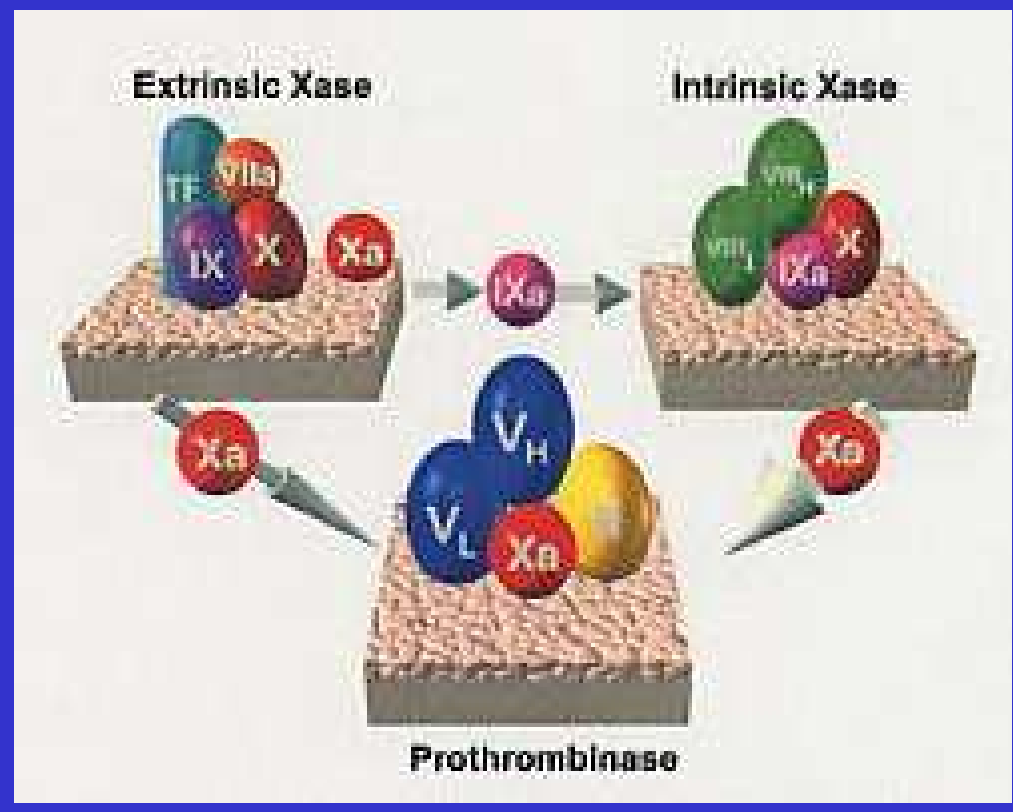
Complessi macromolecolari della coagulazione

- Enzima (serin-proteasi vitamina K-dip.)
- Cofattore non enzimatico
- Substrato (zimogeno)
- Superficie fosfolipidica
- Ioni Ca^{2+}

Interazione con le membrane: dal 3D al 2D

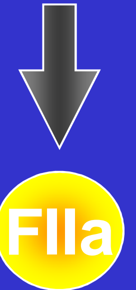
Cascata coagulativa

TF/FVIIa
FX, FIX



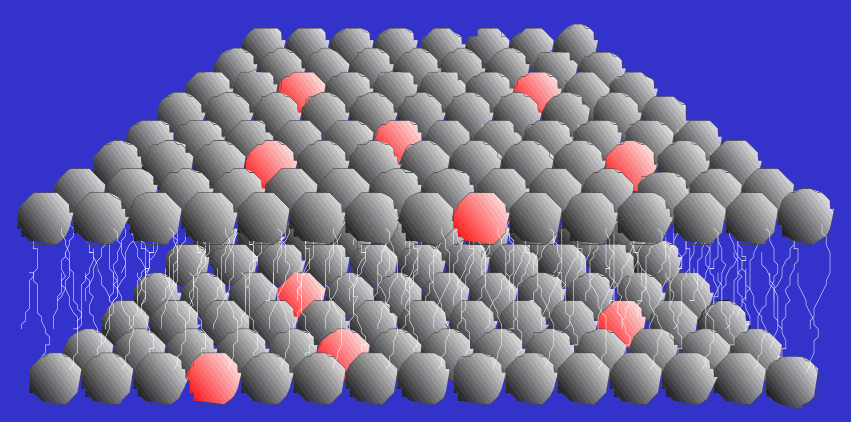
FIXa/FVIIIa
FX

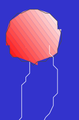
FXa/FVa
PT



FIIa

Superficie fosfolipidica





Fosfolipidi anionici
- **fosfatidilserina**
- **fosfatidilinositolo**

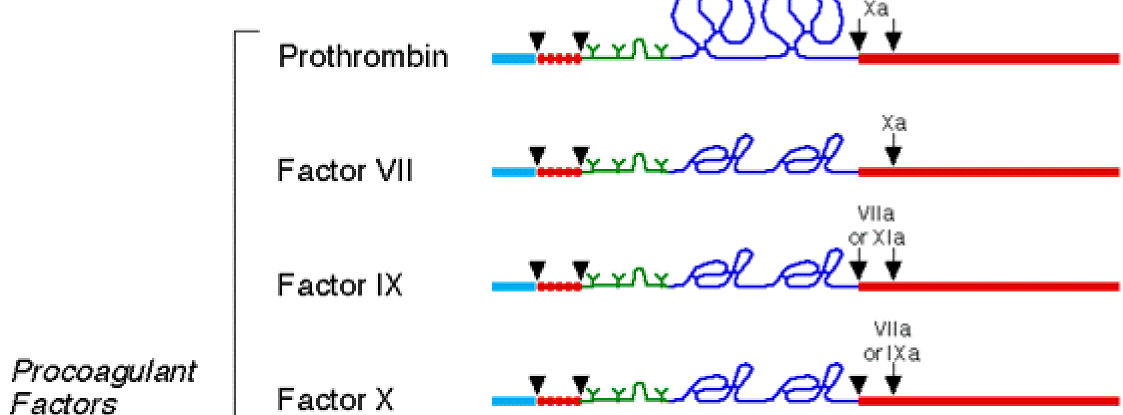
- Supporto fisico dei complessi macromolecolari della coagulazione
- Fornita dalle membrane delle piastrine attivate (*in vivo*) o da vescicole fosfolipidiche sintetiche (*in vitro*)
- Deve contenere fosfolipidi anionici (tipicamente fosfatidilserina)

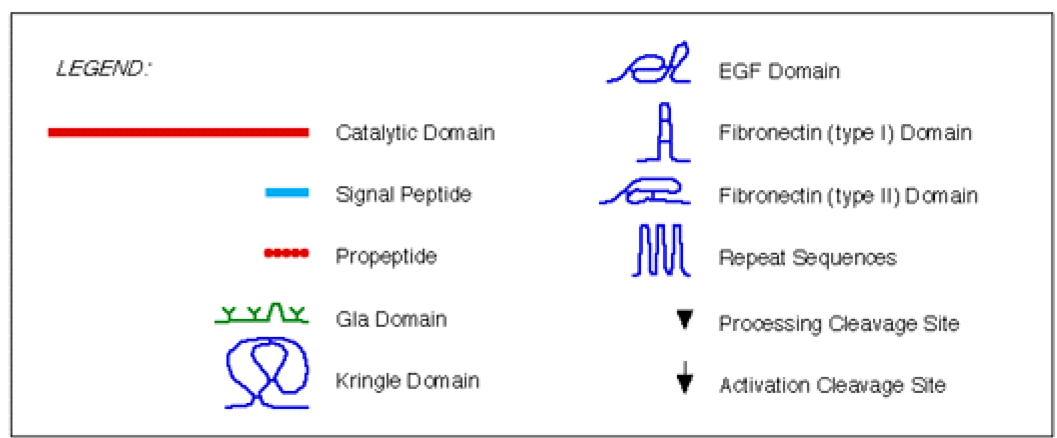
Serin-proteasi della coagulazione

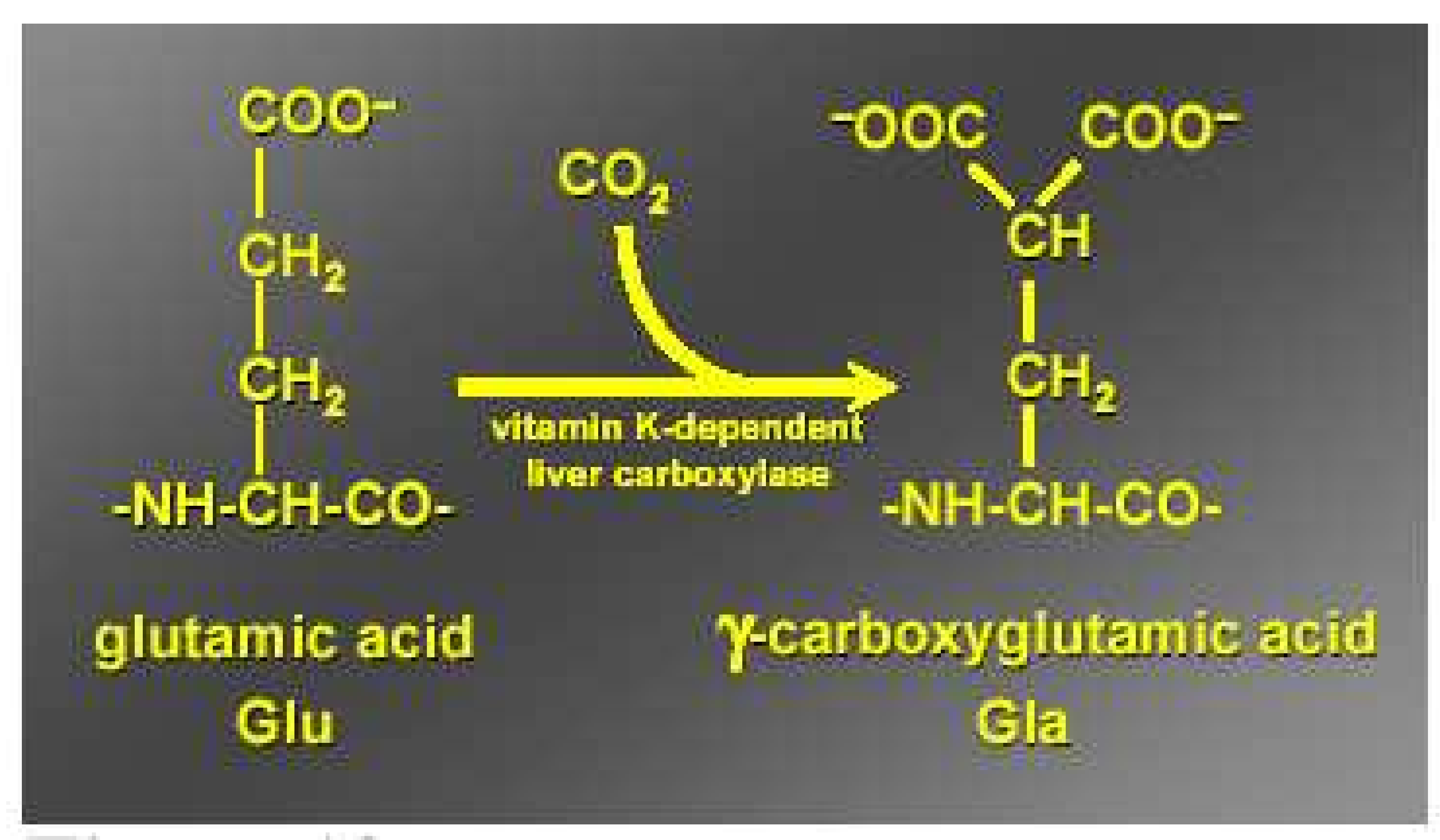
NH_2	Gla	EGF	EGF	AP	Dominio catalitico	COOH

Struttura a domini altamente conservata (→ origine comune):

- Dominio Gla (10-12 residui di acido γ-carbossiglutammico): legame Ca^{2+}-dipendente alle membrane fosfolipidiche
- Domini EGF (o kringle): interazione con altre proteine del complesso
- Dominio di attivazione: contiene il/i sito/i di taglio per l'attivazione dello zimogeno
- Dominio serin-proteasico: attività catalitica

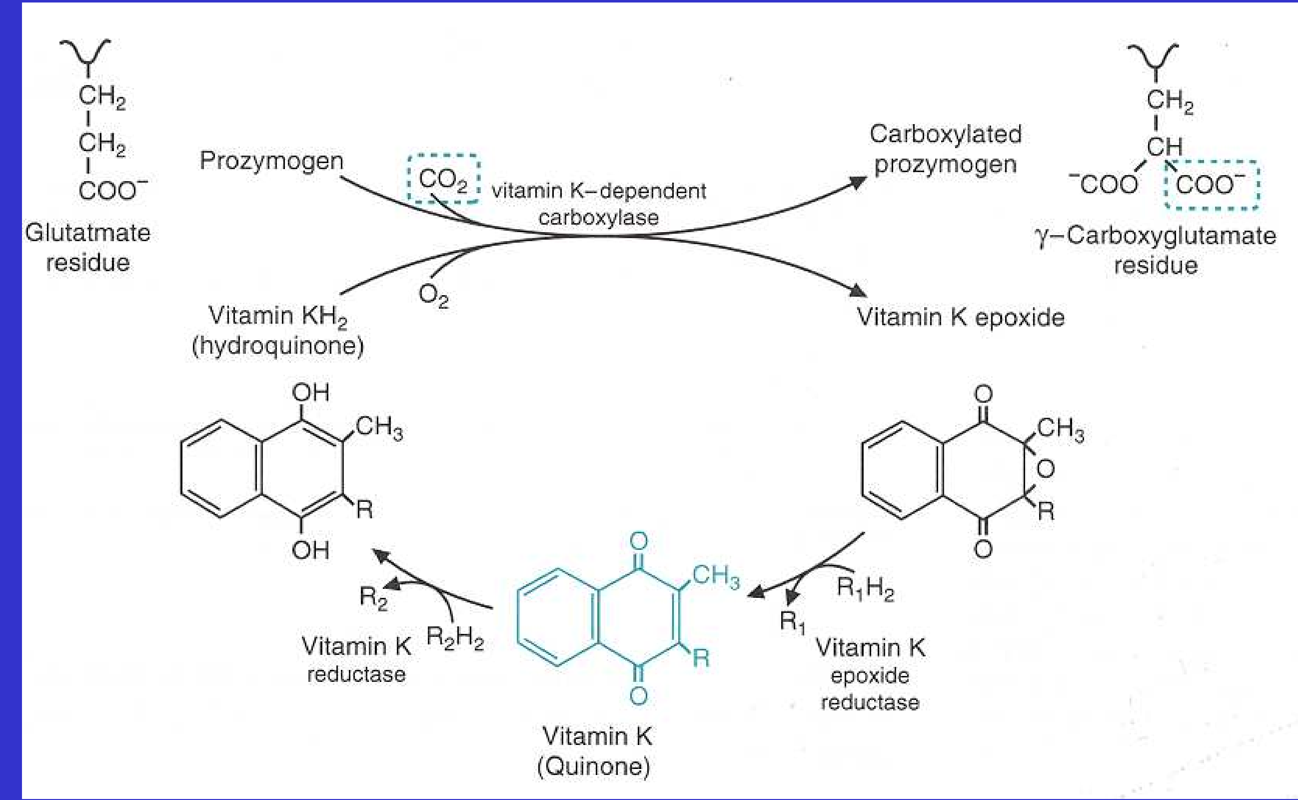

Procoagulant Factors
Prothrombin
Xa
Factor VII
Xa
Factor IX
VIIa
or XIa
Factor X
VIIa
or IXa


LEGEND:
Catalytic Domain
Signal Peptide
Propeptide
Gla Domain
Kringle Domain
EGF Domain
Fibronectin (type I) Domain
Fibronectin (type II) Domain
Repeat Sequences
Processing Cleavage Site
Activation Cleavage Site


COO⁻
CH2
CH2
-NH-CH-CO-
CO2
vitamin K-dependent
liver carboxylase
⁻OOC
COO⁻
CH
CH2
-NH-CH-CO-
glutamic acid
Glu
γ-carboxyglutamic acid
Gla

La γ-carbossilazione

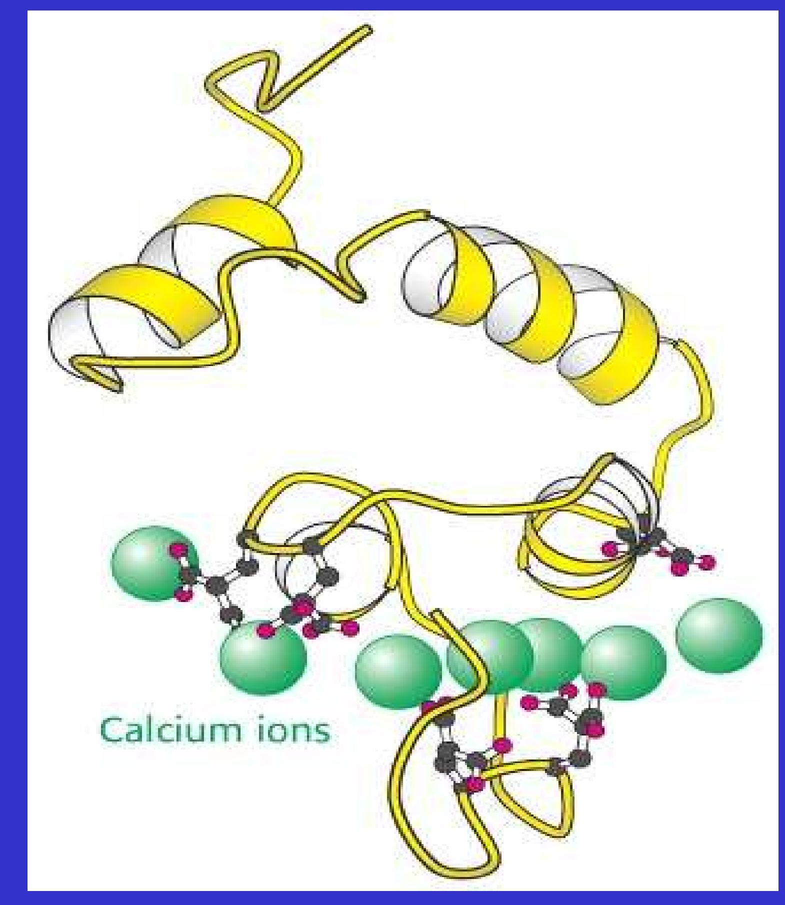
(nel reticolo endoplasmatico)



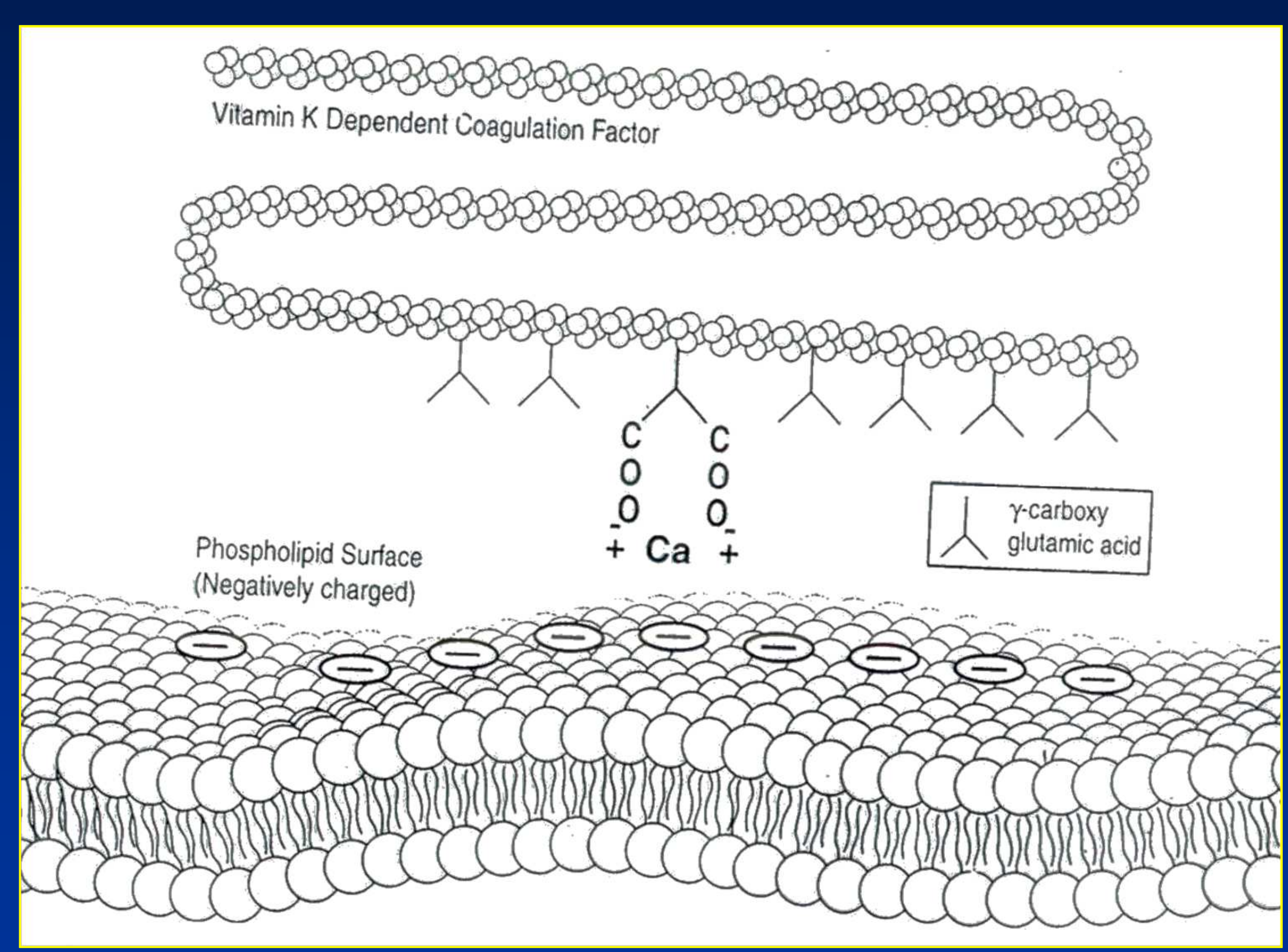
Cumarina: inibitore della KO reduttasi, impedisce il riciclo della vitamina K. Utilizzato come farmaco anti-coagulante.

Legame Ca^{2+}-dipendente alla superficie fosfolipidica

- Il dominio Gla media il legame Ca^{2+}-dipendente delle serin-proteasi della coagulazione alle membrane fosfolipidiche
- L'interazione ad elevata affinità tra i residui Gla e il Ca^{2+} determina un riarrangiamento conformazionale della proteina che favorisce l'interazione con le membrane



Fattori vitamina K-dipendenti

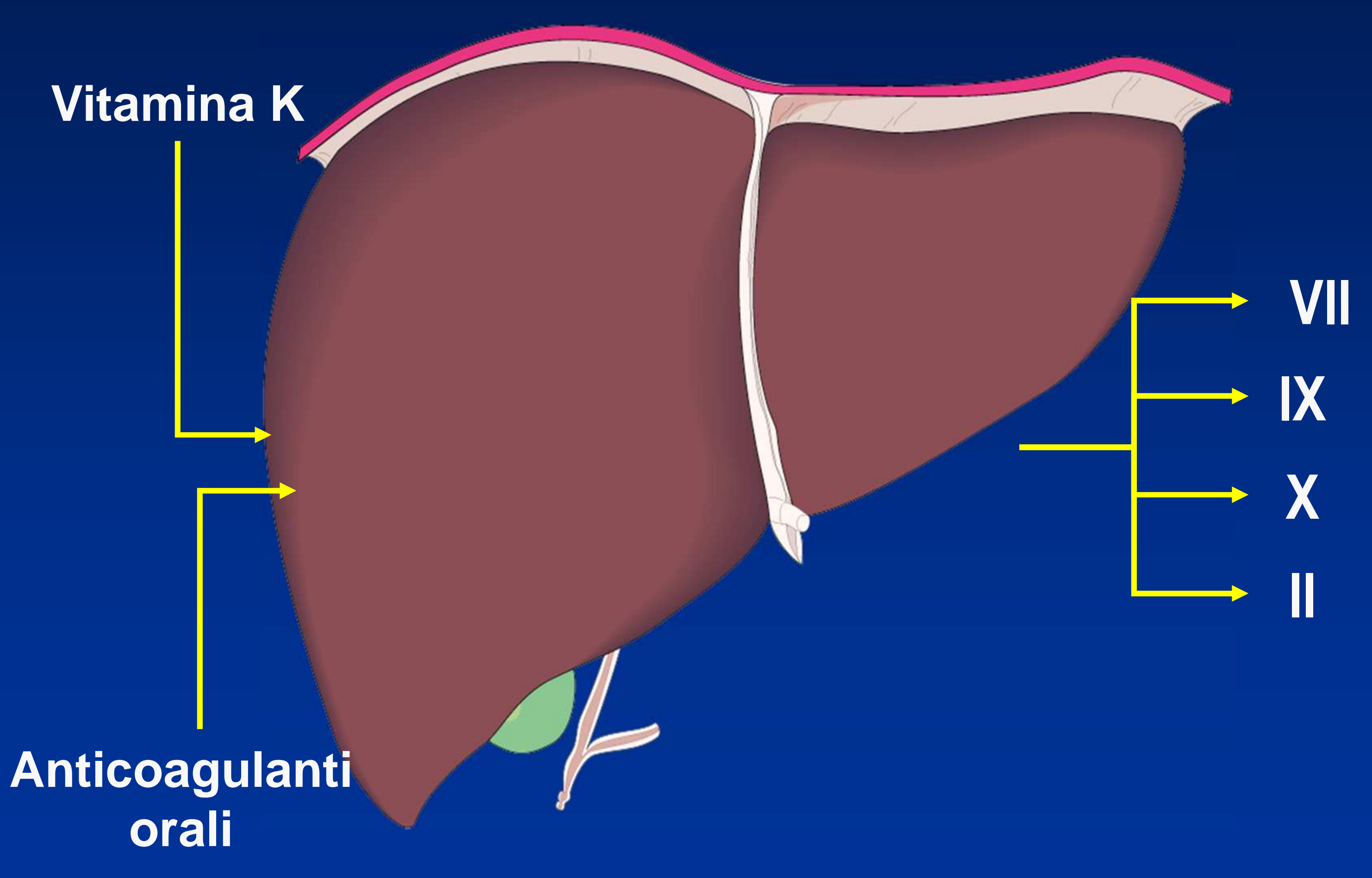


PT-activating mixture	K_m (μM)	V_{max} (mol FIIa/min/mol FXa)
FXa	131 ± 24	0.61 ± 0.08
FXa, Ca^{2+}	84 ± 11	0.68 ± 0.06
FXa, Ca^{2+}, PCPS (7.5μM)	0.058 ± 0.005	2.25 ± 0.05
FXa, Ca^{2+}, PCPS (75μM)	0.35 ± 0.03	3.90 ± 0.10
FXa, Ca^{2+}, FVa	34 ± 5	373 ± 30
FXa, Ca^{2+}, FVa, PCPS (7.5μM)	0.21 ± 0.02	1919 ± 63
FXa, Ca^{2+}, FVa, PCPS (75μM)	1.70 ± 0.60	2748 ± 580

Fosfolipidi → abbassano la K_m di 100 volte

Meccanismo: riduzione della dimensionalita'

Fattori coagulativi vitamina K-dipendenti



Fattori vitamina K-dipendenti e loro tempo di emivita

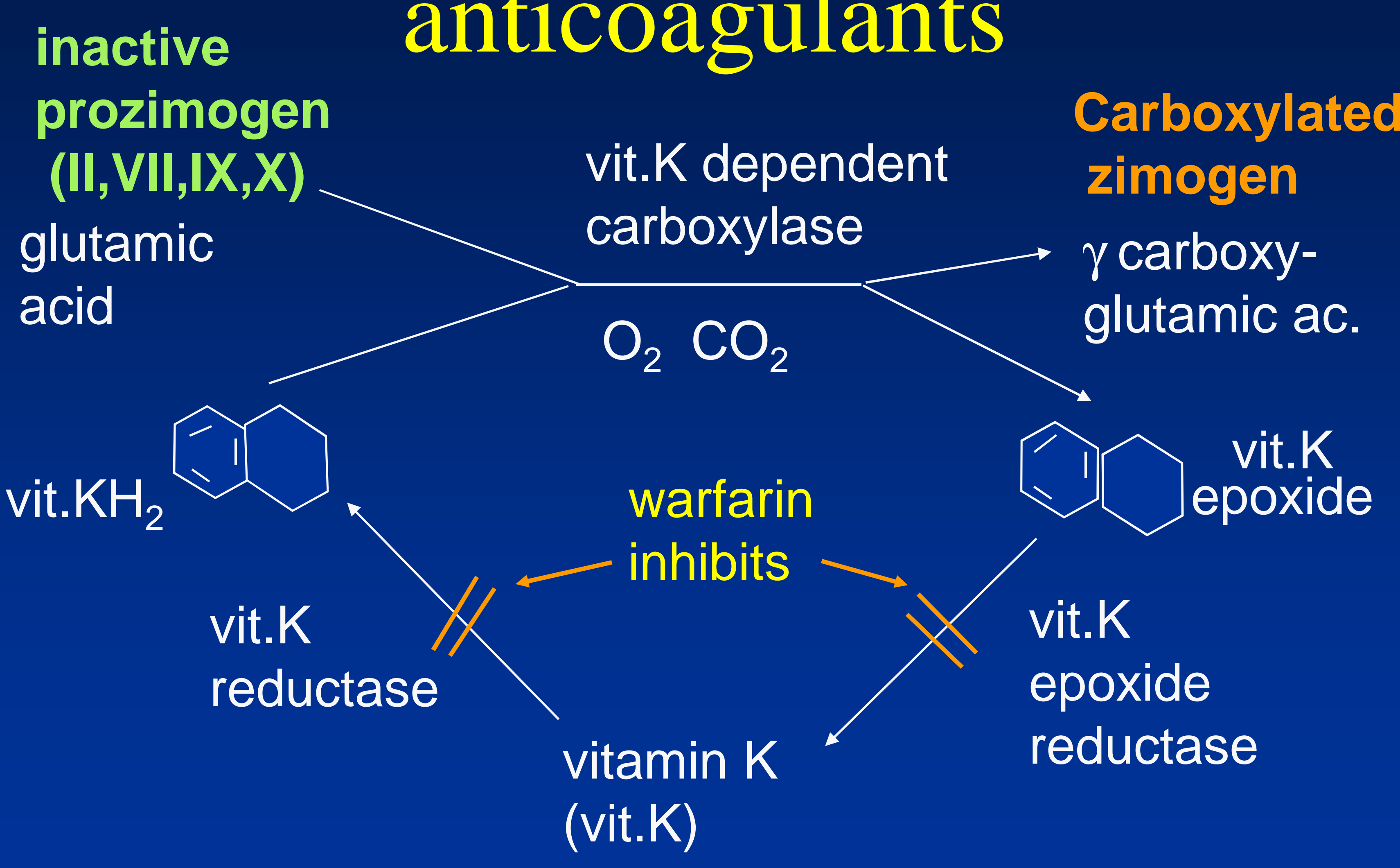
Procoagulanti

• Fattore VII	4-7 h
• Fattore IX	18-30 h
• Fattore X	2 d
• Fattore II	3 d

Anticoagulanti

• Proteina C	6-9 h
• Proteina S	40 h

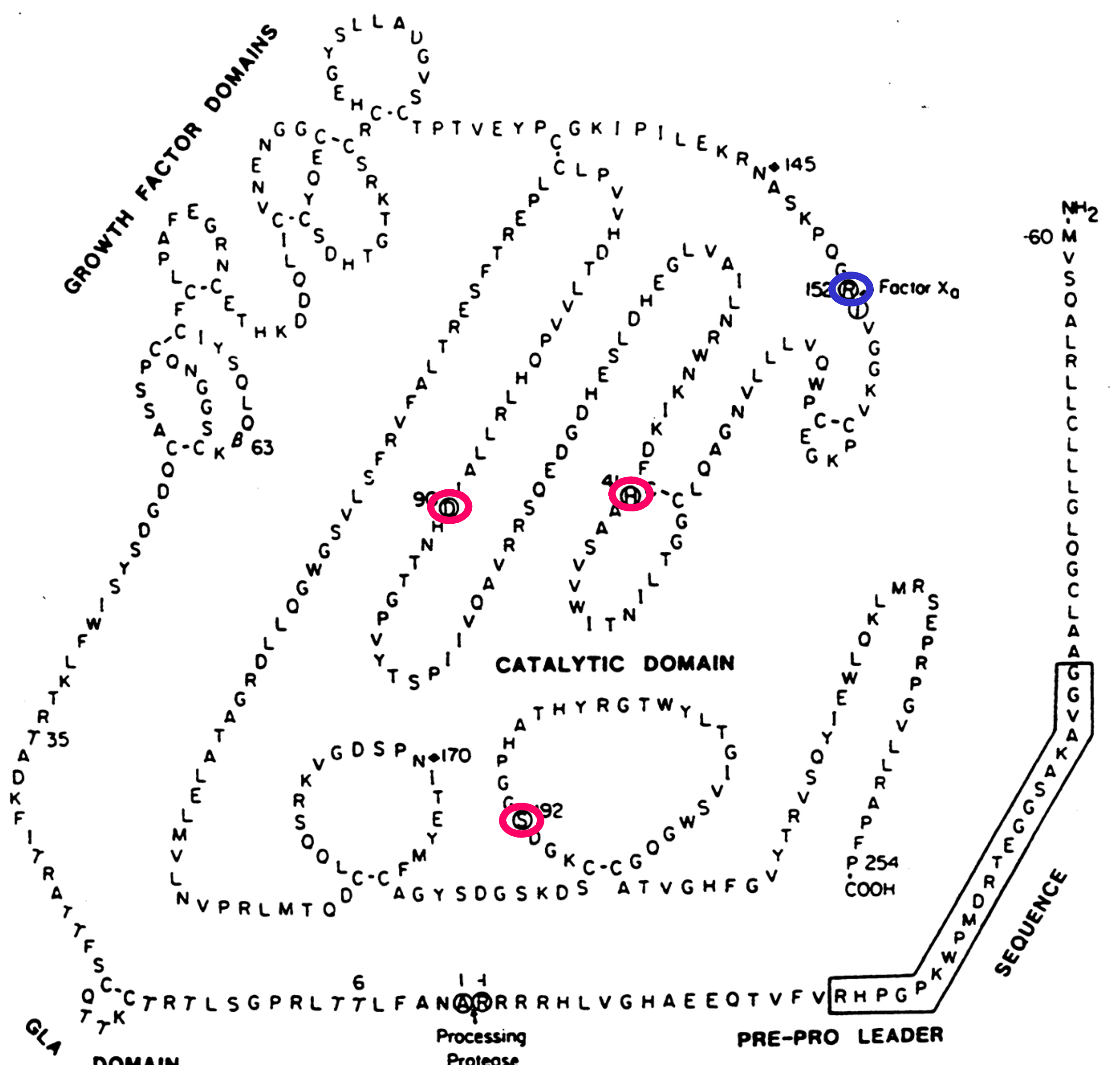
Mechanism of action of oral anticoagulants

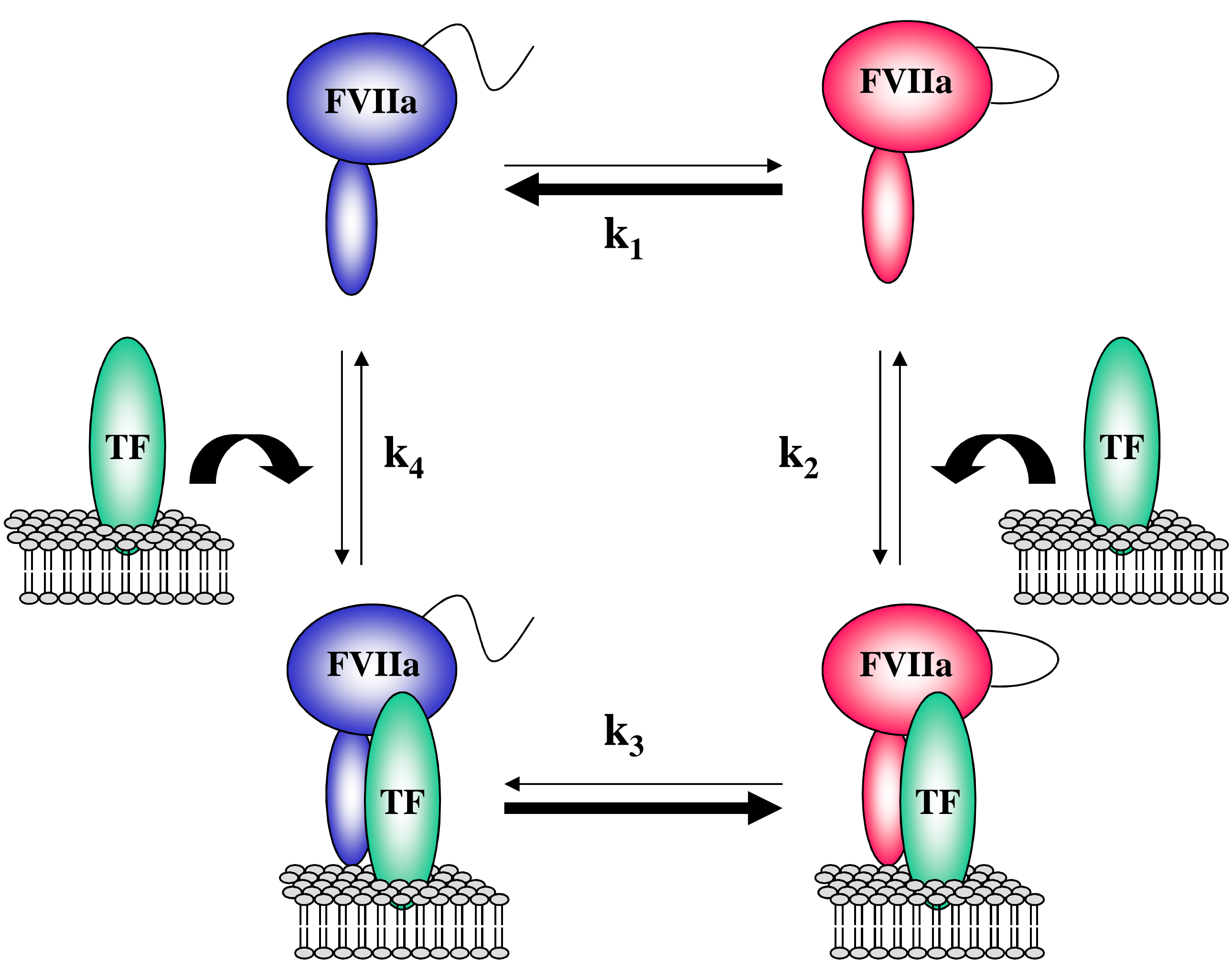


I Cofattori sono essenziali per la regolazione dell'attività delle serin-proteasi della coagulazione.

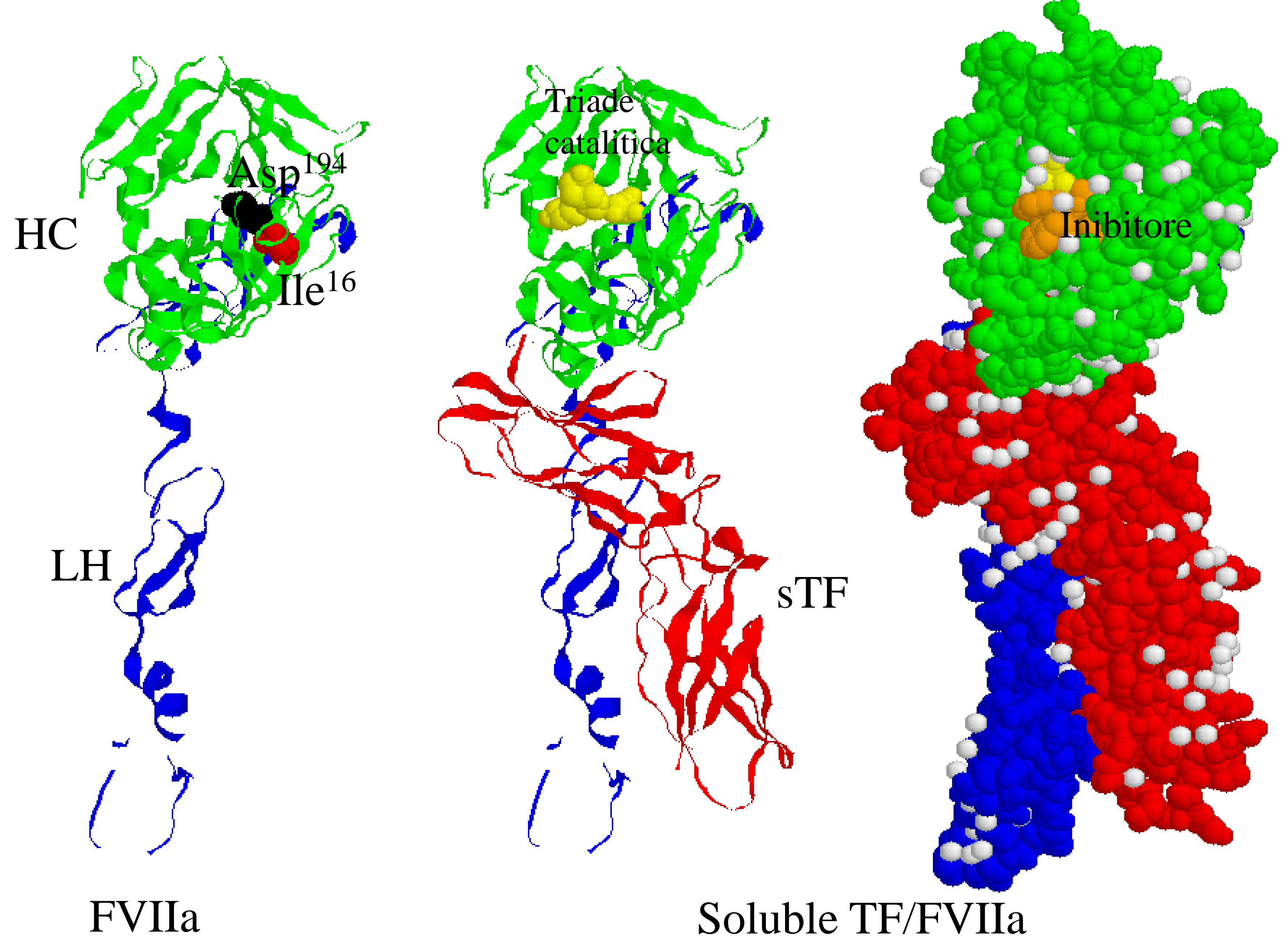
Il Controllo dell'attività del FVIIa da parte del suo cofattore è cruciale

FVII

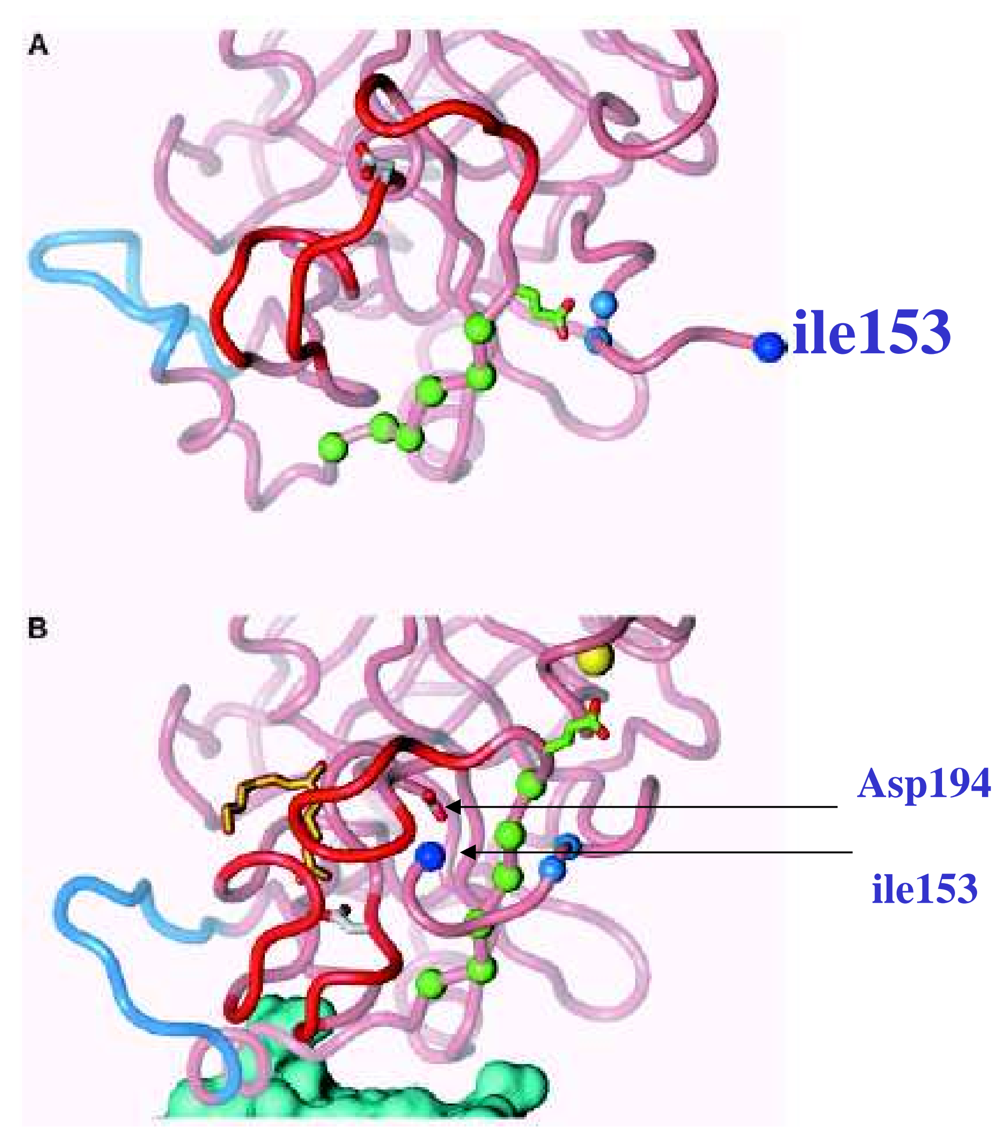




Il complesso Xasico


HC
Asp194
Ile16
LH
Triade
catalitica
sTF
Inibitore
FVIIa
Soluble TF/FVIIa

Incompetent (A) and competent (B) forms of FVIIa



FVIIa/TF complex

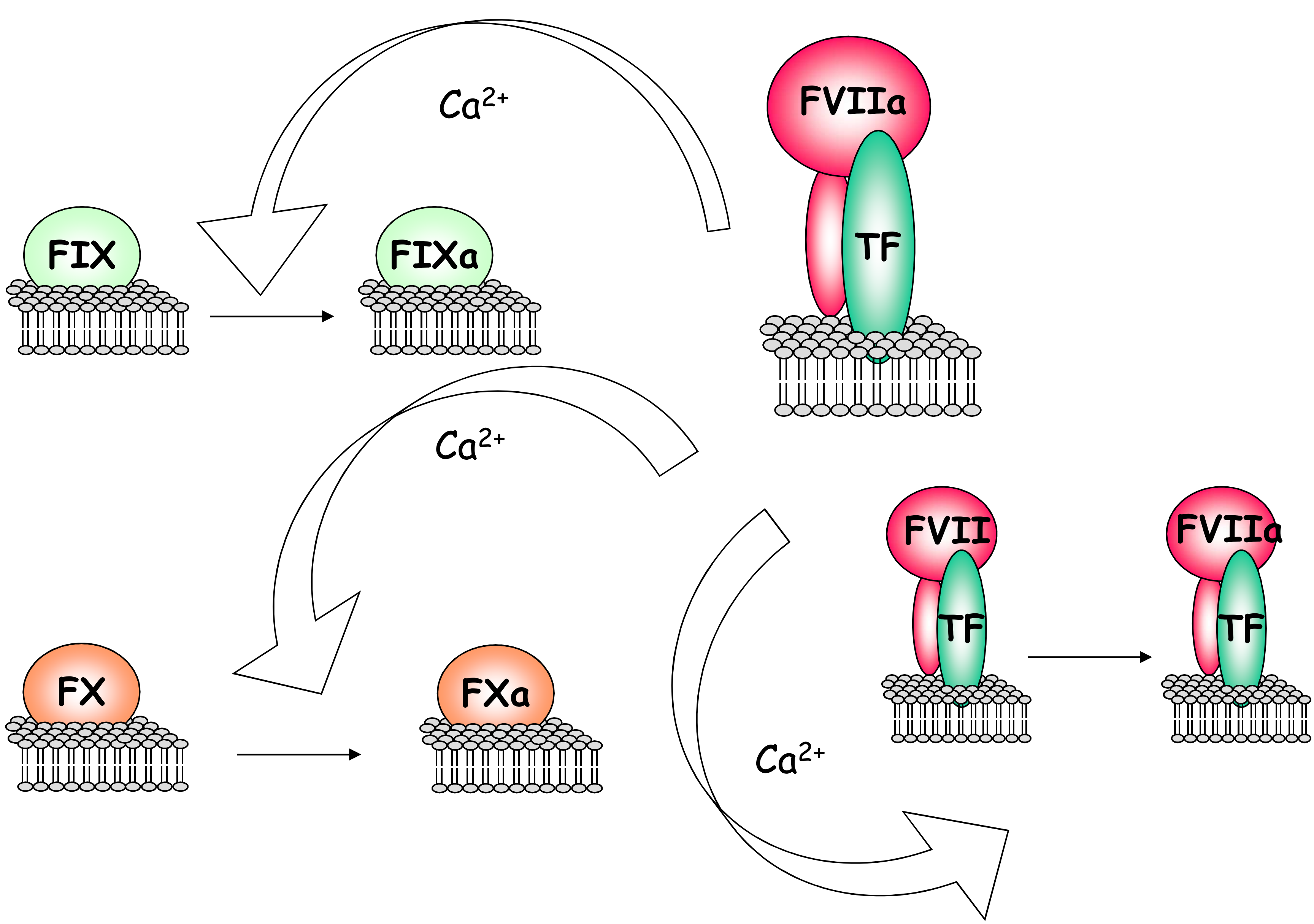
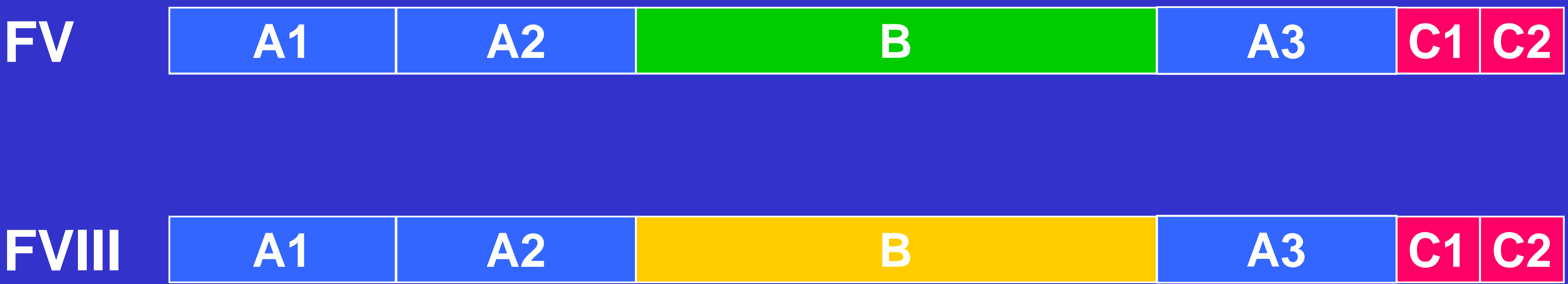


Table 3. Kinetic properties of the vitamin K-dependent enzymes and enzymatic complexes

Enzyme	Substrate	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, $\mu M^{-1} \cdot sec^{-1}$
Factor VIIa	factor IX	ND	ND	ND
Factor VIIa/TF/PCPS/$CaCl_2$	factor IX	0.016	91.9	5560
Factor VIIa	factor X	2.1	$1.0 \cdot 10^{-4}$	$4.8 \cdot 10^{-5}$
Factor VIIa/TF/PCPS/$CaCl_2$	factor X	0.23	186	885

Cofattori non enzimatici



Struttura a domini altamente conservata (→ origine comune):

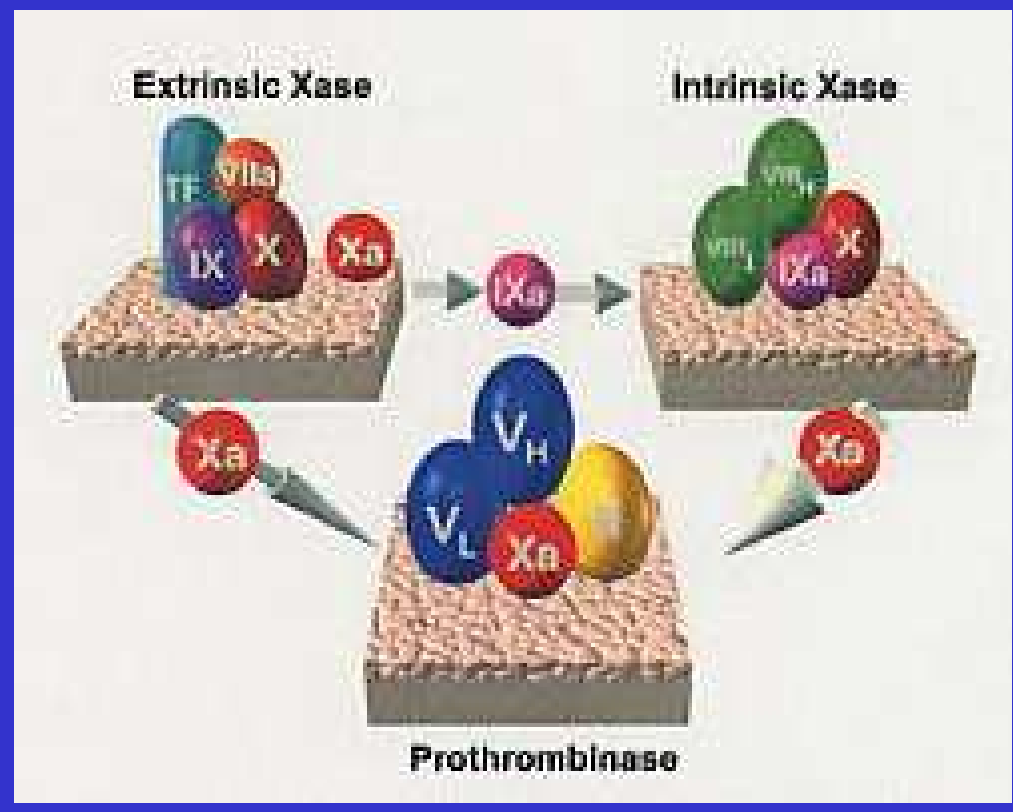
Domini A: forte omologia di sequenza e struttura

Domini B: scarsa omologia

Domini C: forte omologia di sequenza e struttura

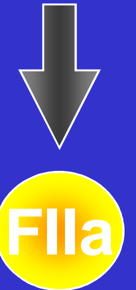
Cascata coagulativa

TF/FVIIa
FX, FIX

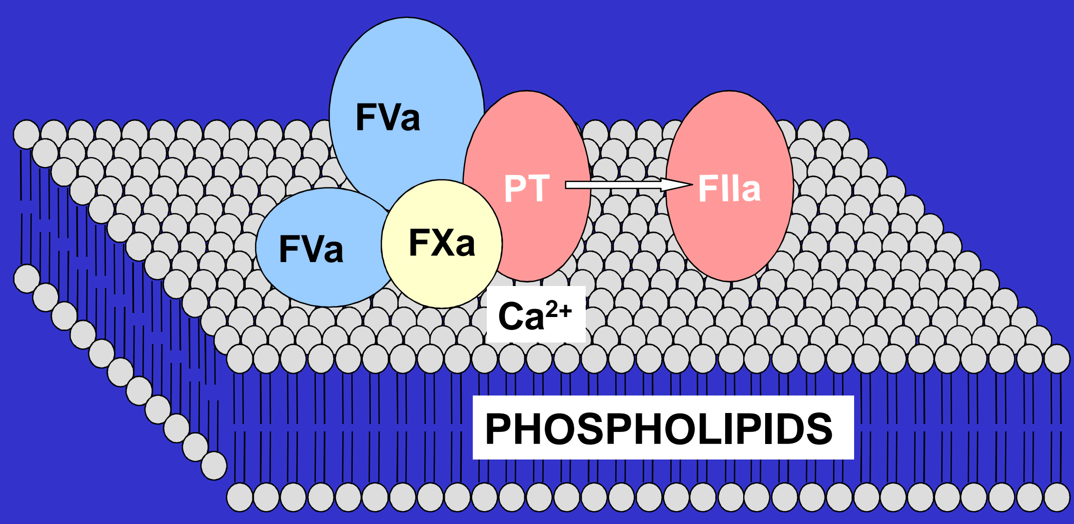


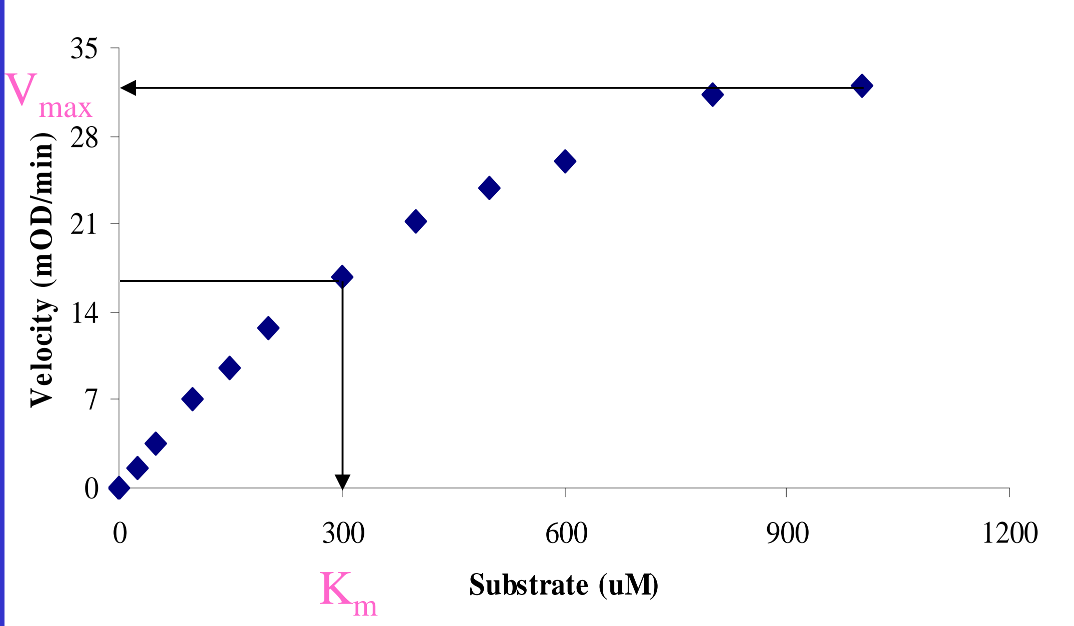
FIXa/FVIIIa
FX

FXa/FVa
PT



Il complesso protrombinasico





$$V = \frac{V_{max}[S]}{K_m + [S]}$$

$K_m = [S] \Rightarrow ½\ V_{max}$

PT-activating mixture	K_m (μM)	V_{max} (mol FIIa/min/mol FXa)
FXa	131 ± 24	0.61 ± 0.08
FXa, Ca^{2+}	84 ± 11	0.68 ± 0.06
FXa, Ca^{2+}, PCPS (7.5μM)	0.058 ± 0.005	2.25 ± 0.05
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FXa, Ca^{2+}, FVa, PCPS (75μM)	1.70 ± 0.60	2748 ± 580

Fosfolipidi ⇨ abbassano la K_m di 100 volte

Meccanismo: riduzione della dimensionalita'

FVa ⇨ aumenta la V_{max} di 3000 volte

Meccanismo:

• il FVa modifica il sito attivo del FXa e/o i siti di riconoscimento della PT sul FXa

Il FVa promuove l’assemblaggio del complesso protrombinasico:

- si lega per primo alle membrane fosfolipidiche
- promuove il legame del FXa della PT alle membrane fosfolipidiche
- media l’interazione fra enzima e substrato

Table 2. Activation of factor X by factor VIIa in the presence of various cofactors of the *extrinsic factor Xase*

Cofactor	Concentration	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, μM^{-1}·min^{-1}
None	NA	>20	$>1.5 \cdot 10^{-4}$	ND
$CaCl_2$	2.5 mM	2.10	$1.0 \cdot 10^{-4}$	$4.8 \cdot 10^{-5}$
Phospholipid (PCPS)[a]	21 μM	0.25	0.016	0.062
Tissue factor[a,b]	9.4 pM	0.23	186	885

Note: NA, not applicable; ND, not determined; [a] in the presence of 5 mM $CaCl_2$; [b] in the presence of PCPS.

Table 3. Kinetic properties of the vitamin K-dependent enzymes and enzymatic complexes

Enzyme	Substrate	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, μM^{-1}·sec^{-1}	Efficiency ratio
Factor VIIa	factor IX	ND	ND	ND	—
Factor VIIa/TF/PCPS/$CaCl_2$	factor IX	0.016	91.9	5560	—
Factor VIIa	factor X	2.1	$1.0 \cdot 10^{-4}$	$4.8 \cdot 10^{-5}$	—
Factor VIIa/TF/PCPS/$CaCl_2$	factor X	0.23	186	885	1[illegible]
Factor IXa	factor X	300	0.002	$6.6 \cdot 10^{-6}$	—
Factor IXa/VIIIa/PCPS/$CaCl_2$	factor X	0.063	500	7937	$1.2 \cdot 10^{9}$
Factor Xa	factor II	131	0.6	$4.6 \cdot 10^{-3}$	—
Factor Xa/Va/PCPS/$CaCl_2$	factor II	1.0	5016	5016	$1.1 \cdot 10^{6}$
Factor IIa	protein C	60	1.2	0.02	—
Factor IIa/TM/PCPS/$CaCl_2$	protein C	0.1	214	2140	$1.1 \cdot 10^{5}$

Note: ND, not determined; TM, thrombomodulin.