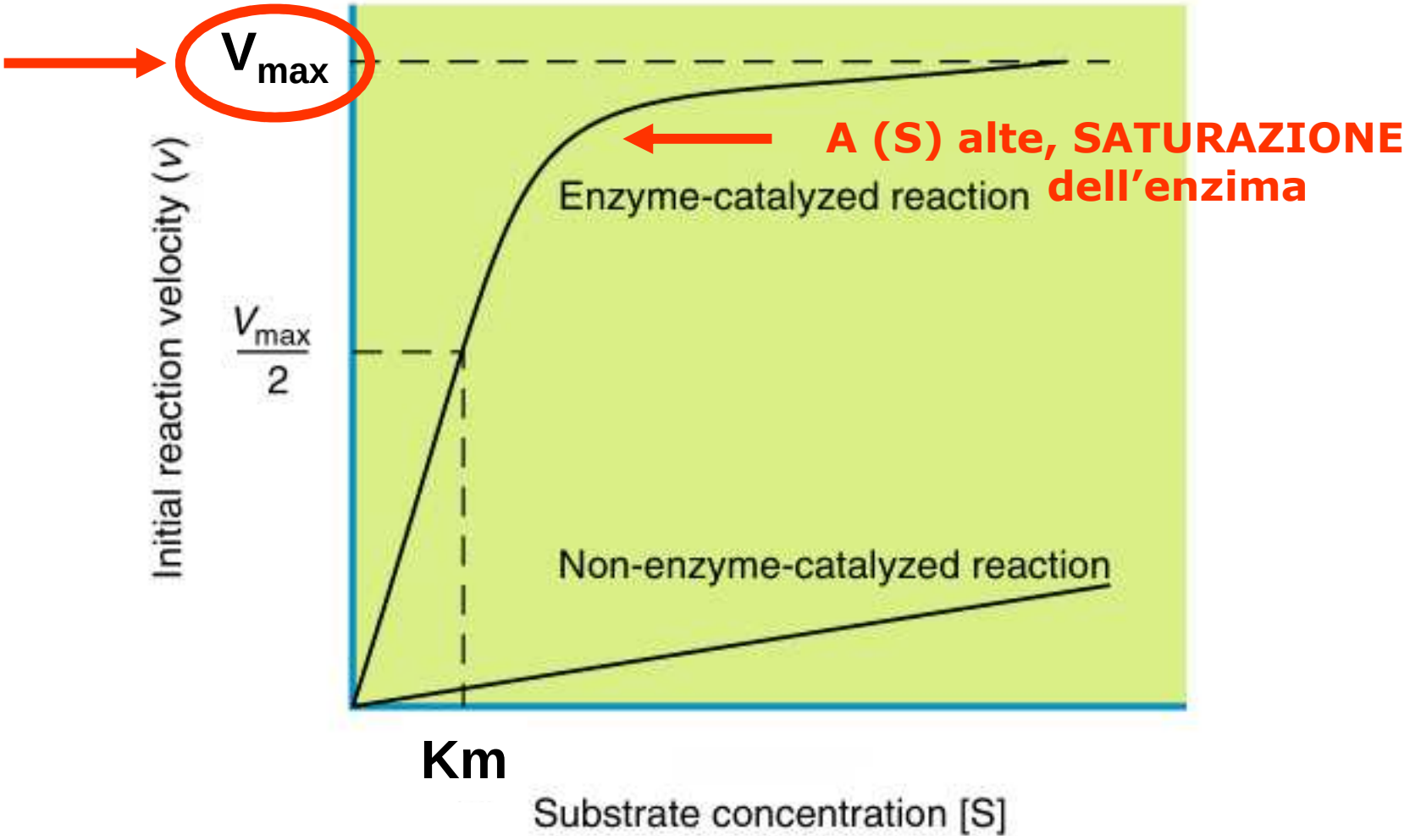
DETERMINANTI DI SPECIFICITA’

Relazione tra Velocità e concentrazione del substrato

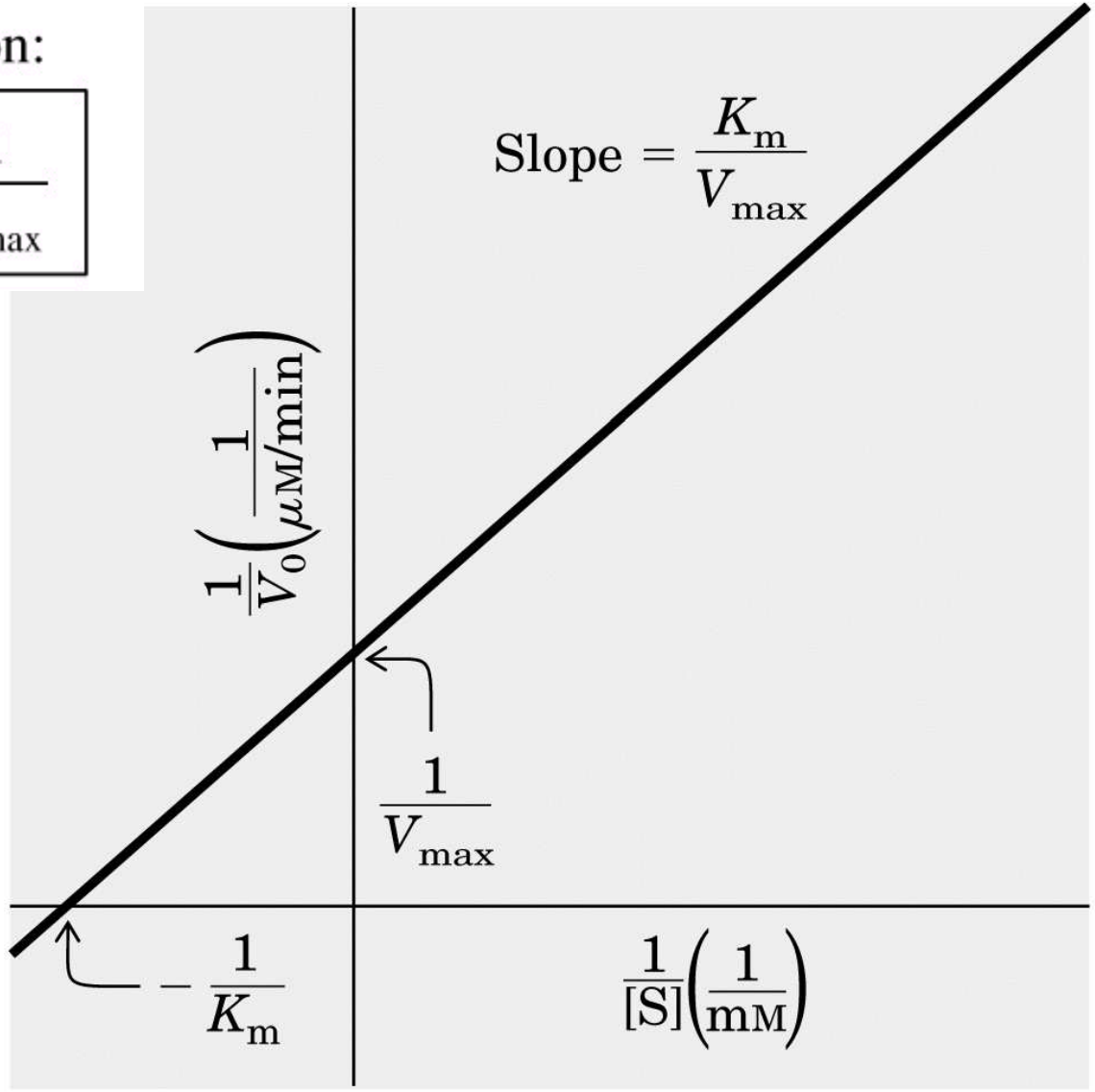


Modo più pratico per descrivere la cinetica:

Grafico degli inversi

Lineweaver-Burk equation:

$$\frac{1}{v_0} = \left(\frac{K_m}{V_{max}}\right)\frac{1}{[S]} + \frac{1}{V_{max}}$$



SIGNIFICATO DELLE COSTANTI CATALITICHE

- K_M
- K_{cat}
- K_{cat}/K_M

Significato della K_m

- Dipende dalle costanti cinetiche:

$$K_m = (k_2 + k_{-1})/ k_1$$

- Se il limiting step è il secondo, allora $k_2 << k_{-1}$ e la K_M si traduce in k_{-1}/ k_1 , che viene detta **Costante di dissociazione K_d.**

- Può quindi essere vista come una costante di dissociazione apparente che misura il grado in cui l'enzima è legato al substrato

- A valori bassi, l'enzima si satura con poco substrato (affinità elevata)

PER MOLTI ENZIMI, I CUI MECCANISMI SONO DIVERSI, QUESTO PRINCIPIO NON VALE

Rapporto k_{cat}/K_m
(costante di specificità)

- Dà una idea della efficienza relativa con cui vengono trasformati substrati diversi (specificità)
- Tiene conto della velocità di catalisi (k_{cat}) e dell'affinità tra E e S (K_m)

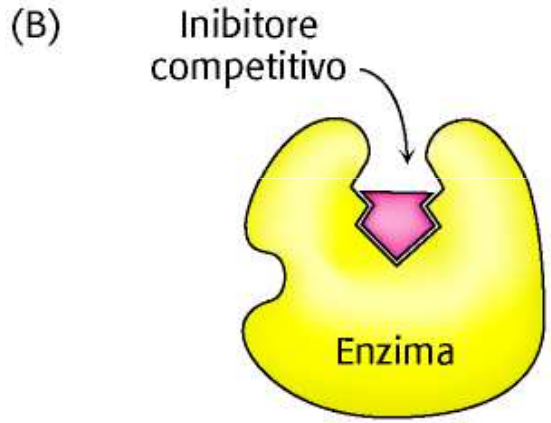
- Dà una idea della efficienza catalitica dell'enzima (enzimi "perfettamente" evoluti hanno costanti cinetiche che si approssimano alla diffusione)

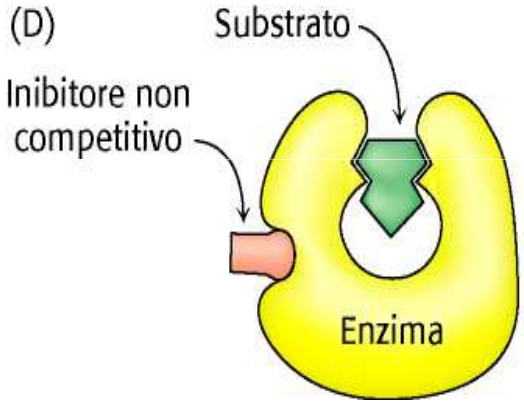
Enzyme	Reaction Catalyzed	K_M (mol/L)	$k_{cat}(s^{-1})$	k_{cat}/K_M $[(mol/L)^{-1}\,s^{-1}]$
Chymotrypsin	Ac–Phe–Ala $\xrightarrow{H_2O}$ Ac–Phe + Ala	1.5×10^{-2}	0.14	9.3
Pepsin	Phe–Gly $\xrightarrow{H_2O}$ Phe + Gly	3×10^{-4}	0.5	1.7×10^{3}
Tyrosyl-tRNA synthetase	Tyrosine + tRNA $\longrightarrow$ tyrosyl-tRNA	9×10^{-4}	7.6	8.4×10^{3}
Ribonuclease	Cytidine 2′, 3′ cyclic phosphate $\xrightarrow{H_2O}$ cytidine 3′-phosphate	7.9×10^{-3}	7.9×10^{2}	1.0×10^{5}
Carbonic anhydrase	$HCO_3^- + H^+ \longrightarrow H_2O + CO_2$	2.6×10^{-2}	4×10^{5}	1.5×10^{7}
Fumarase	Fumarate $\xrightarrow{H_2O}$ malate	5×10^{-6}	8×10^{2}	1.6×10^{8}

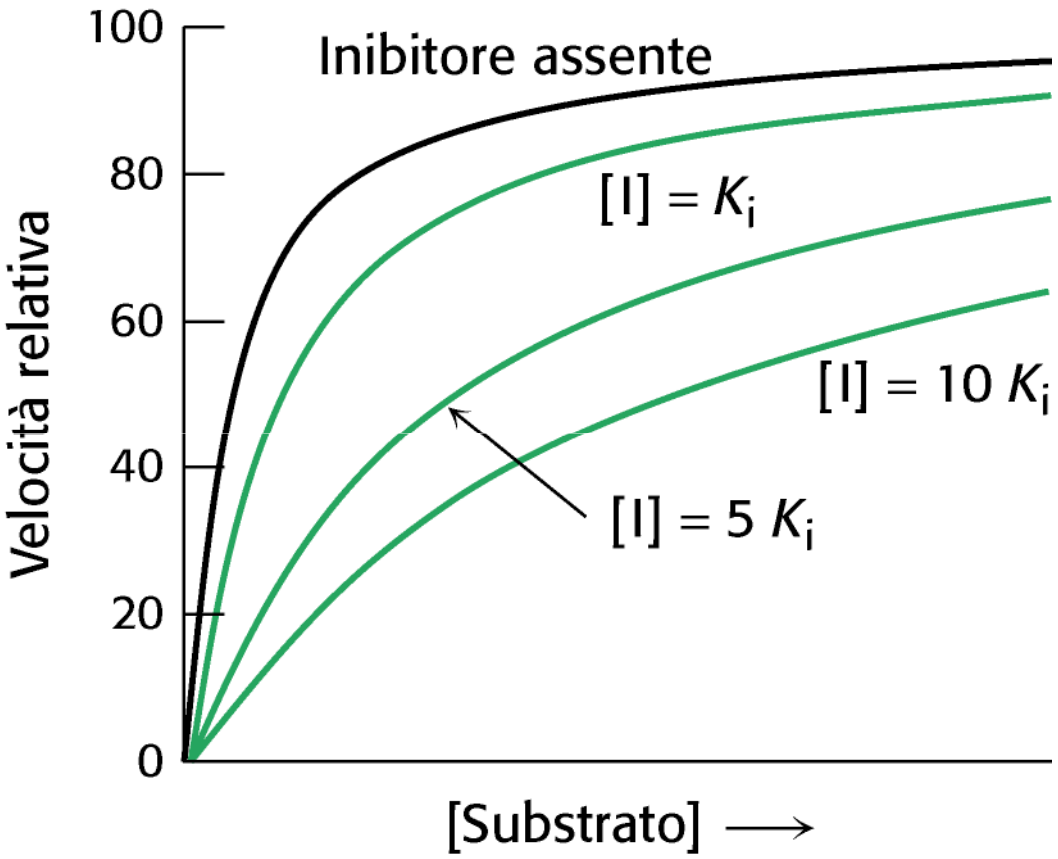
Acetlicolinesterasi	9×10^{-5}	1.4×10^{4}	1.6×10^{8}
Fumarasi	5×10^{-6}	8×10^{2}	1.6×10^{8}

GLI ENZIMI POSSONO ESSERE INIBITI

Inibizione Reversibile

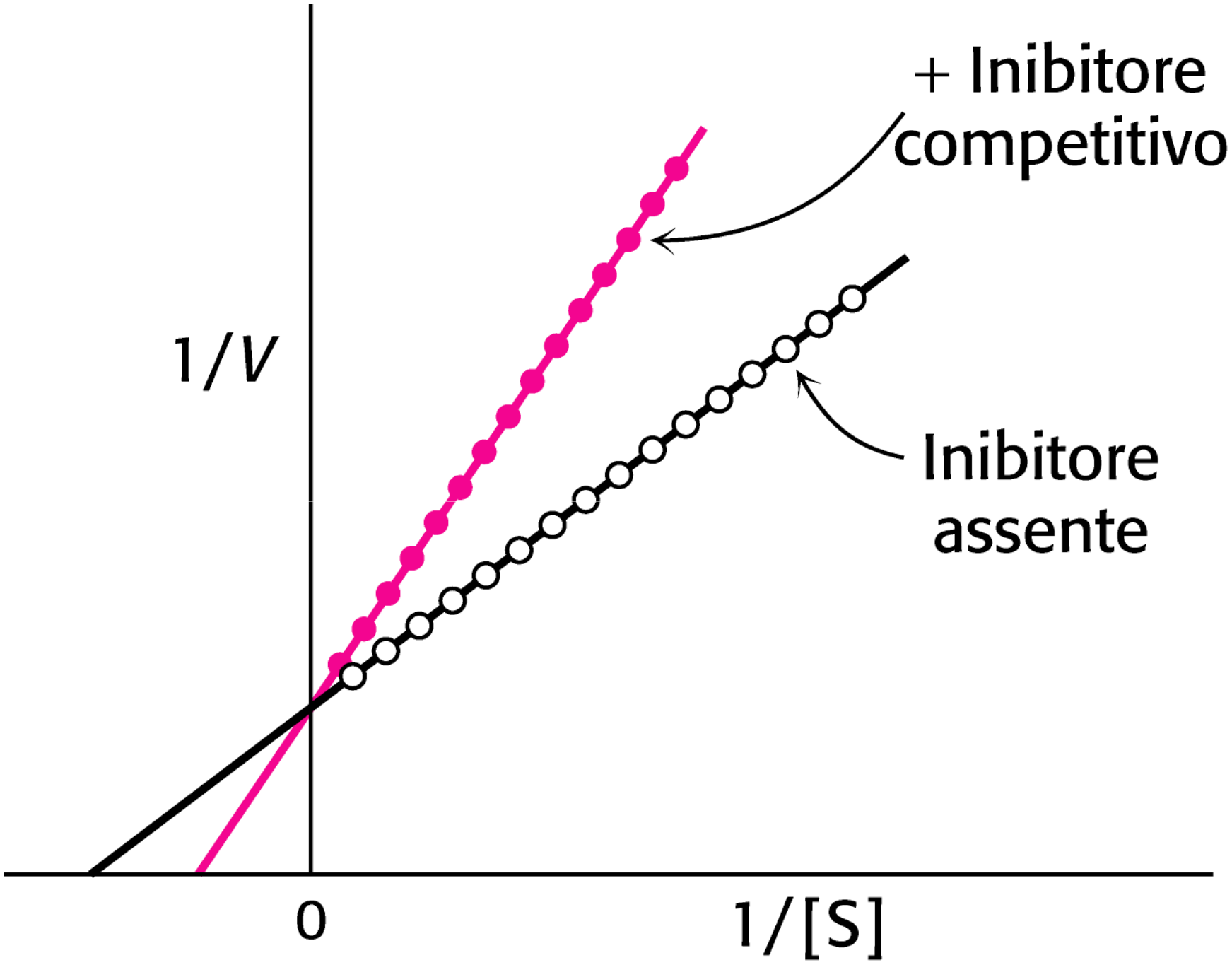


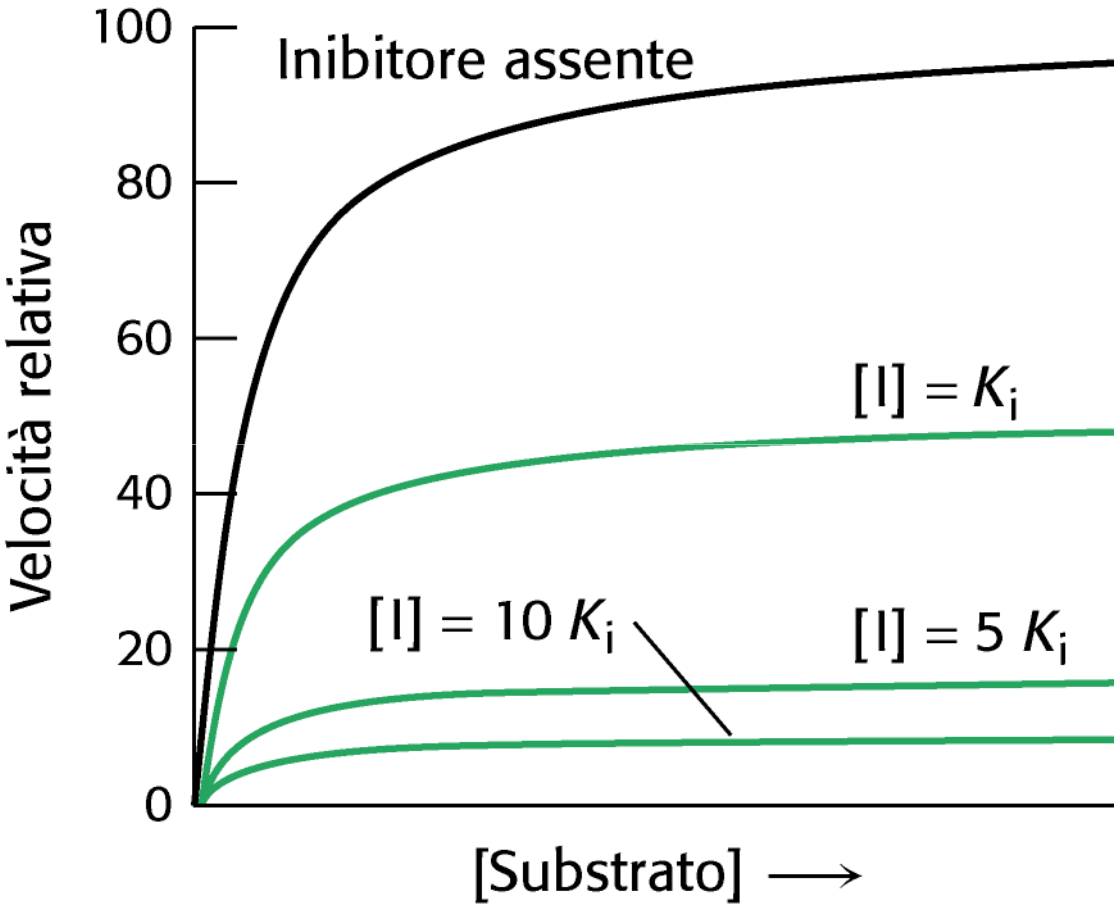




INIBIZIONE COMPETITIVA

Stessa Vmax
Aumento Km

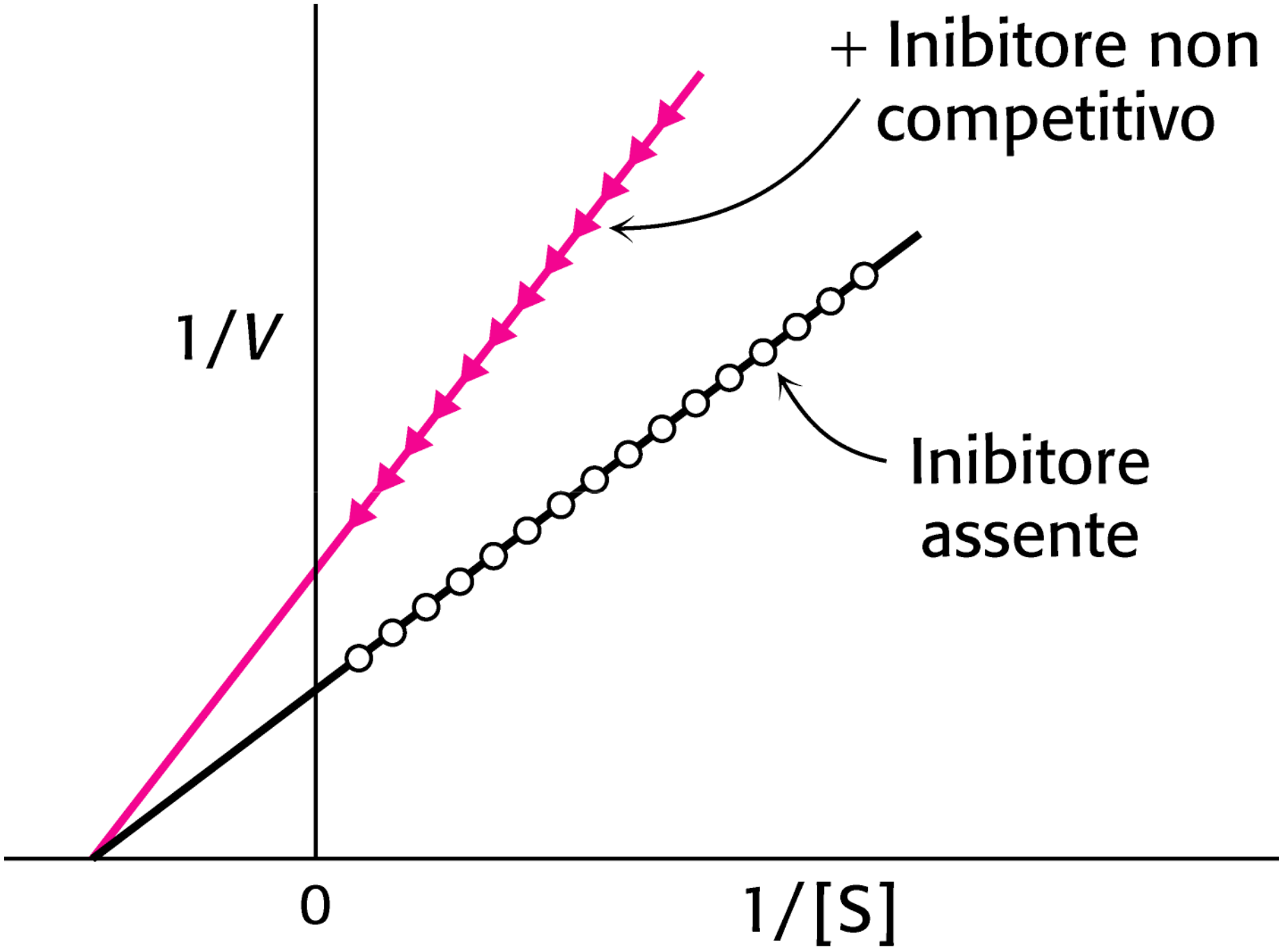

+ Inibitore competitivo
1/V
Inibitore assente
0
1/[S]



INIBIZIONE NON COMPETITIVA

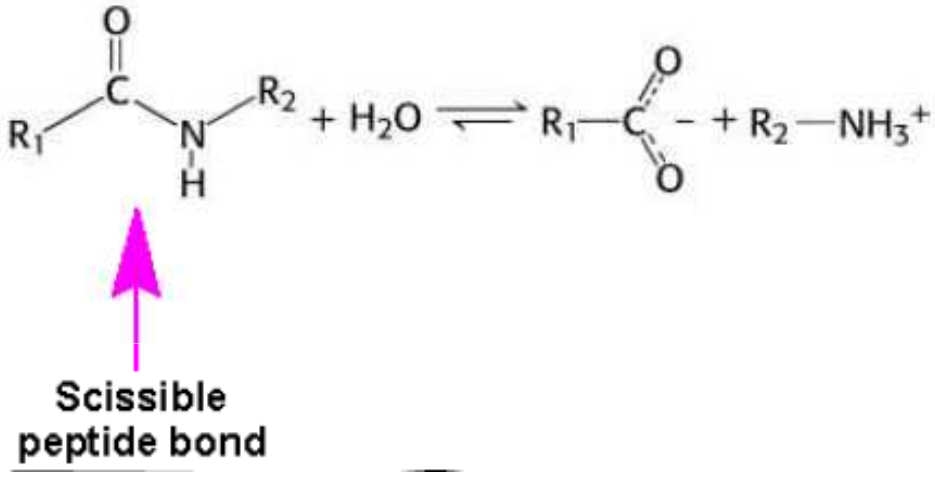
Calo Vmax

Km Invariata


+ Inibitore non competitivo
1/V
Inibitore assente
0
1/[S]

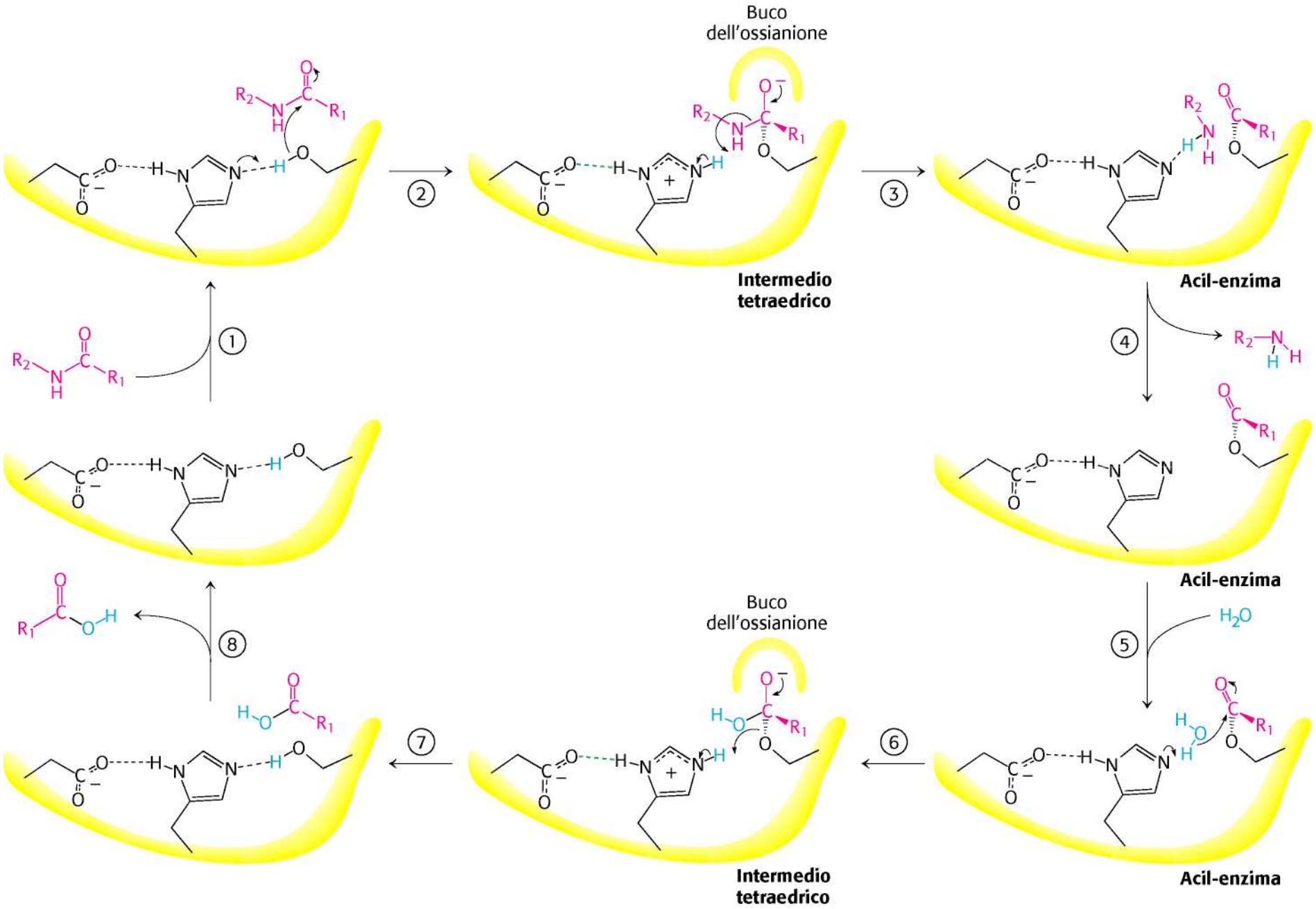
LE SERIN PROTEASI COME MODELLI

la reazione consiste nell'idrolisi di legami peptidici dal lato carbossilico di particolari AA

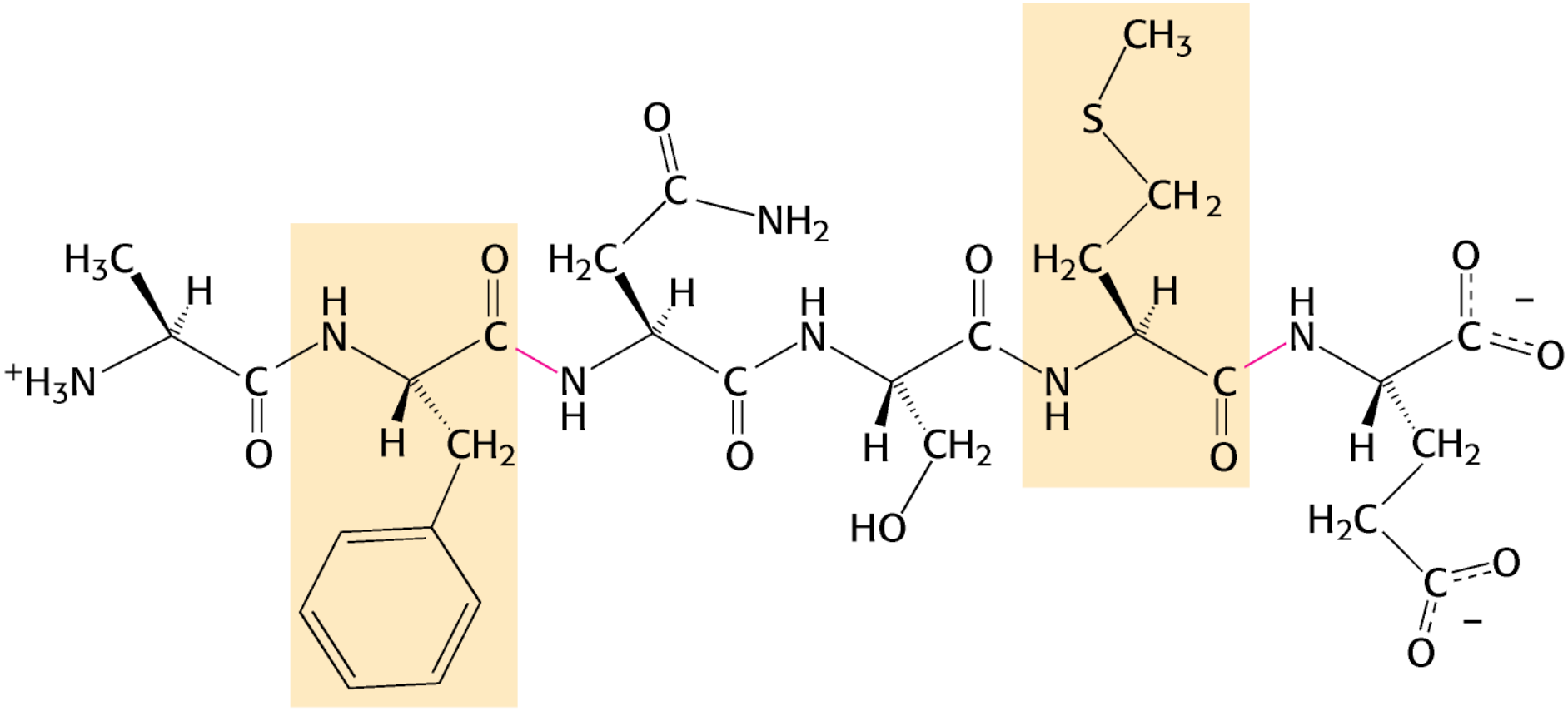


Termodinamicamente favorita ma avverrebbe in 10-1000 anni

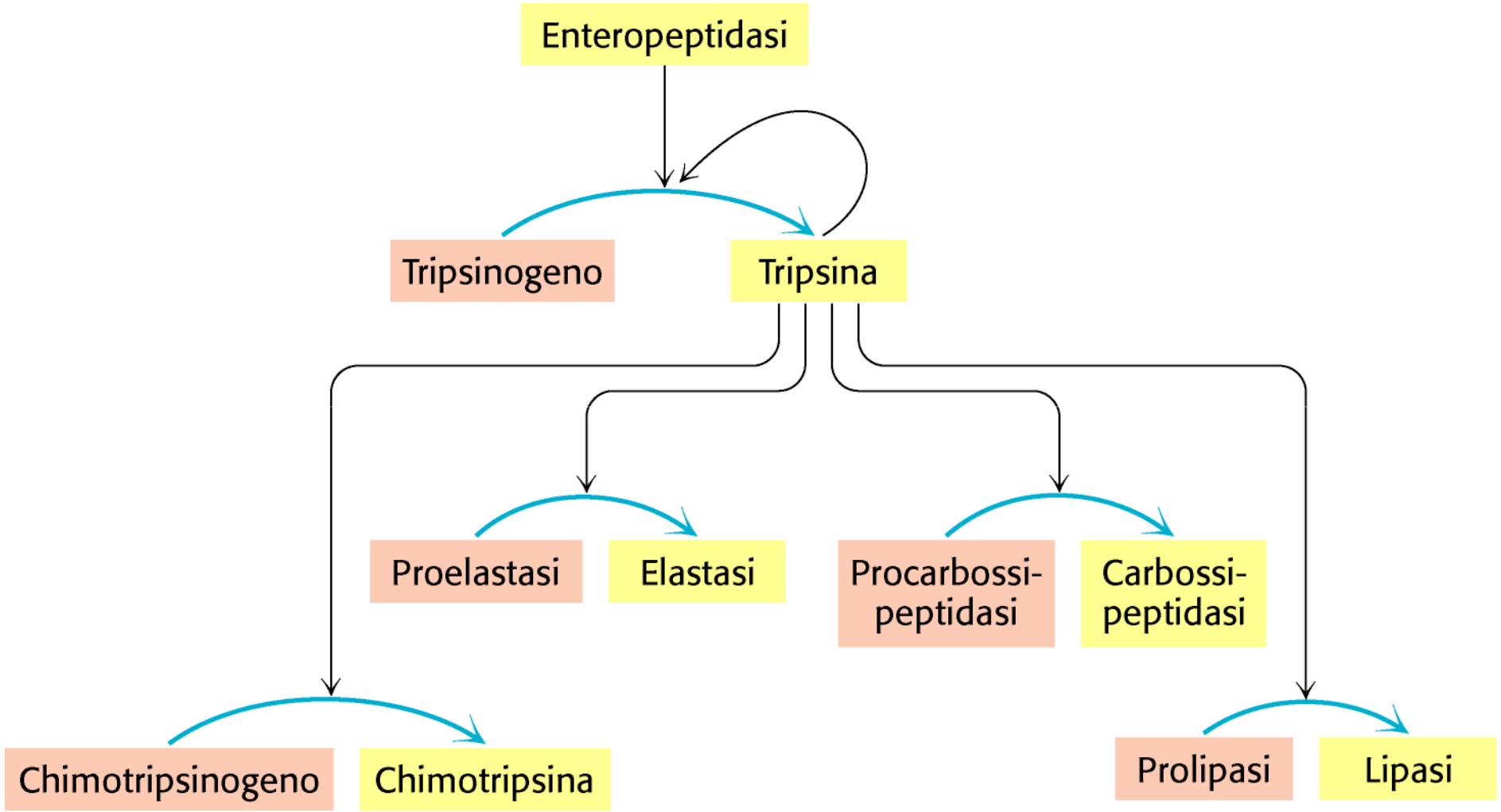
In presenza dell'enzima la reazione avviene in pochi ms


Buco dell'ossianione
Intermedio tetraedrico
Acil-enzima
Acil-enzima
Buco dell'ossianione
Intermedio tetraedrico
Acil-enzima
1
2
3
4
5
6
7
8
H2O

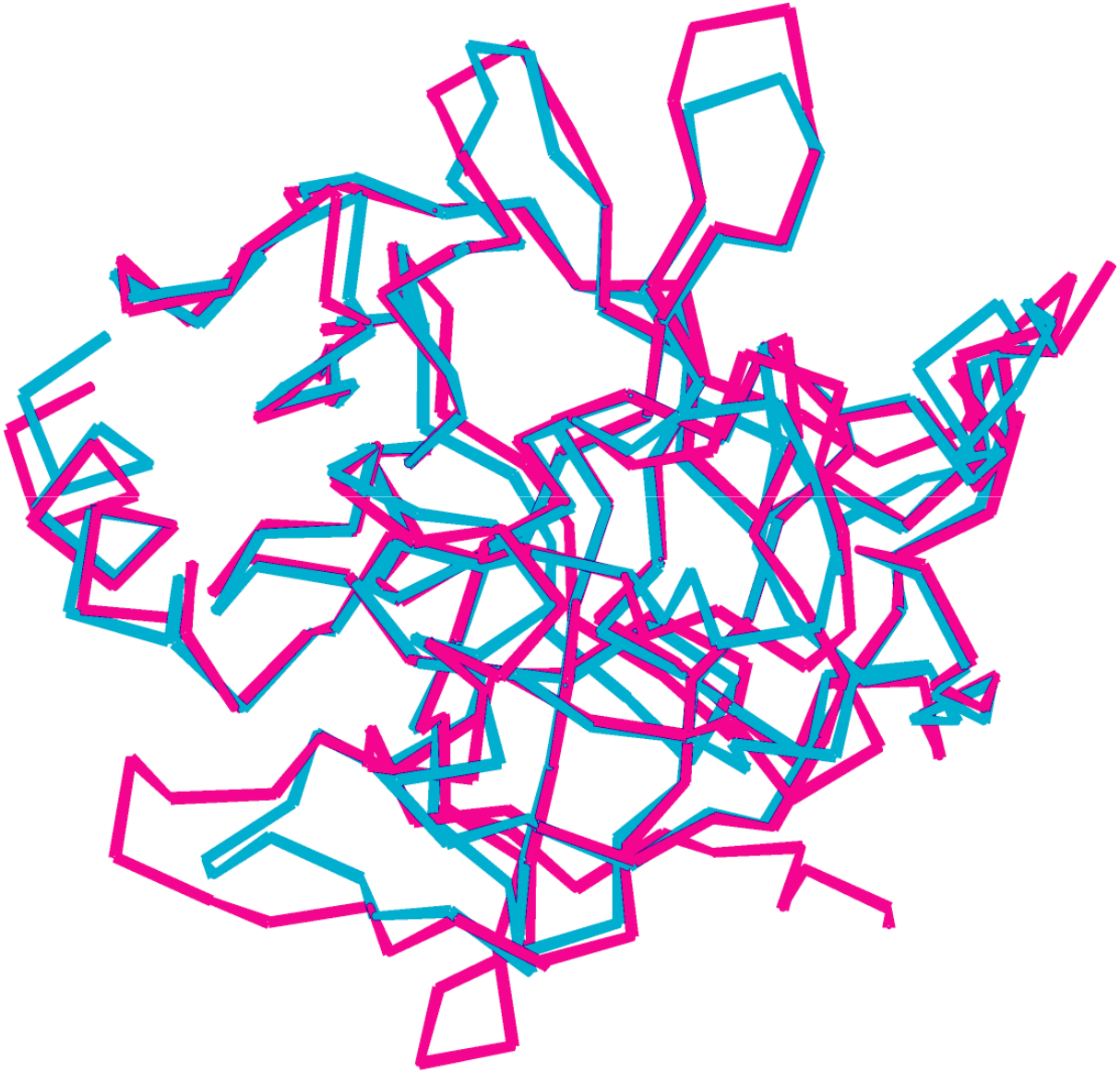
Questo meccanismo spiega l'attività catalitica della chimotripsina ma non la sua specificità!!



SPECIFICITA' DELLA CHIMOTRIPSINA


Enteropeptidasi
Tripsinogeno
Tripsina
Proelastasi
Elastasi
Procarbossi-
peptidasi
Carbossi-
peptidasi
Chimotripsinogeno
Chimotripsina
Prolipasi
Lipasi

Similitudine strutturale tra chimotripsina e tripsina



La risposta sta nella tasca di specificità

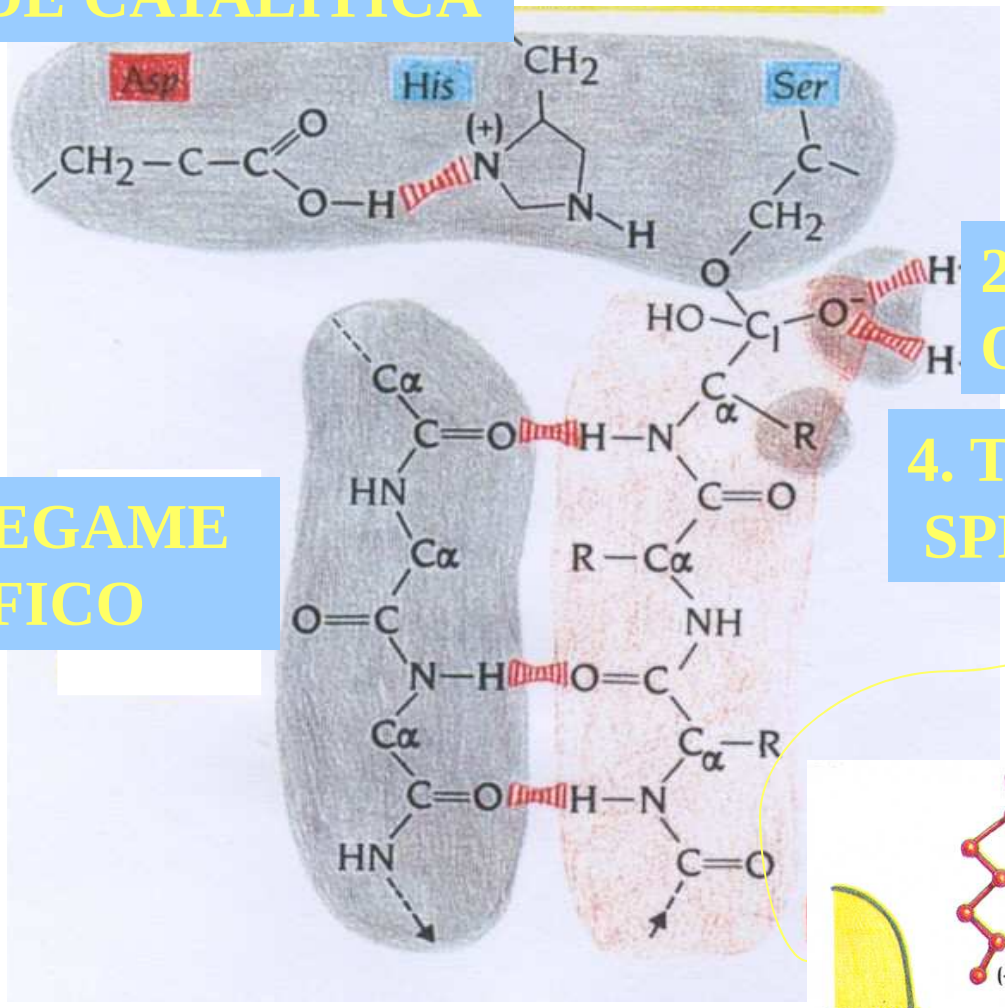
CARATTERISTICHE PER LA CATALISI SERIN PROTEASICA

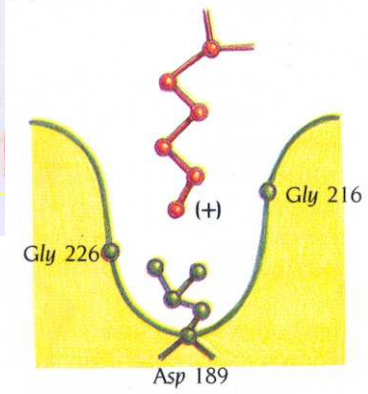
1. TRIADE CATALITICA

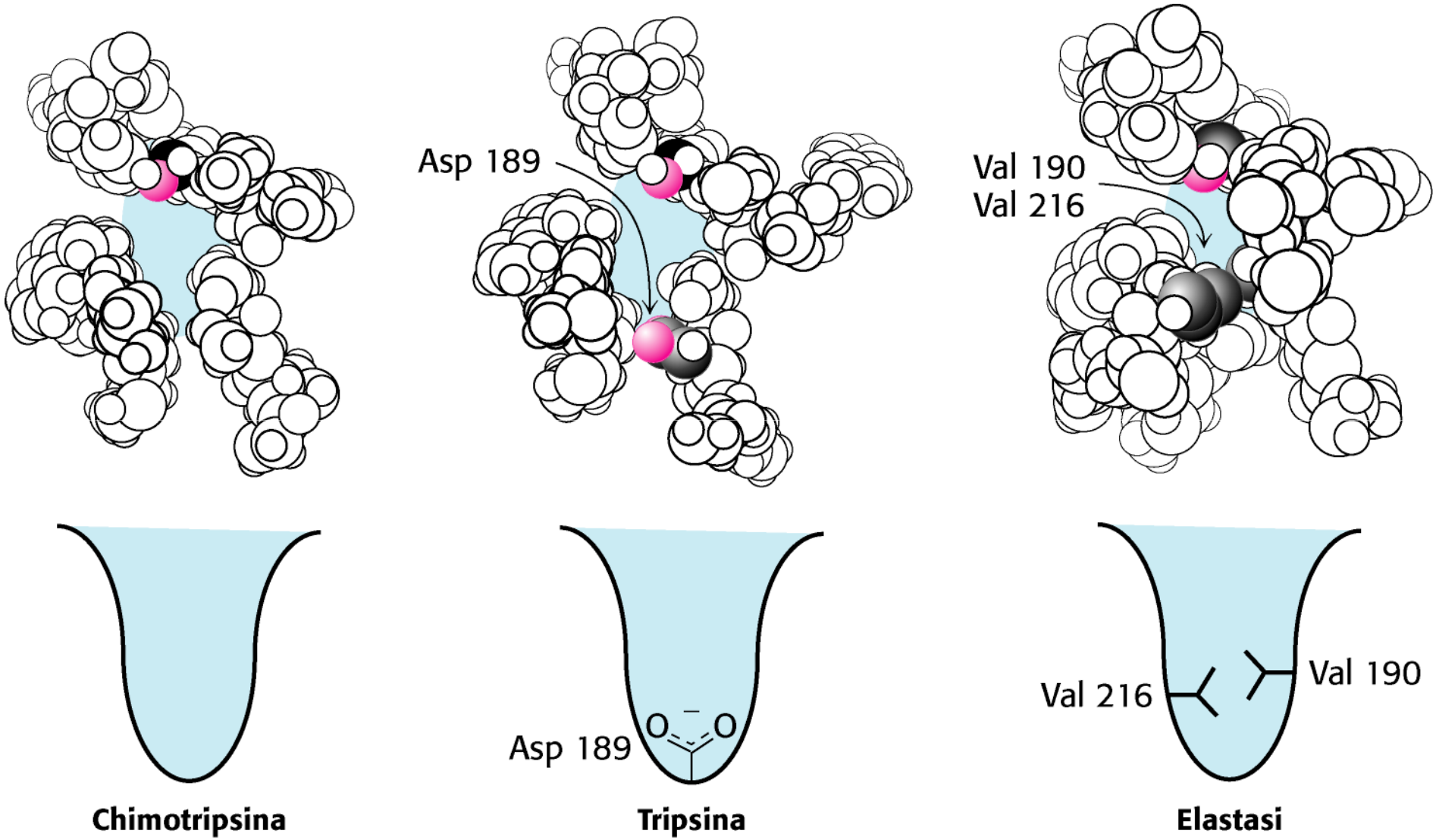
2. INCAVO OSSIANIONICO

3. SITO DI LEGAME ASPECIFICO

4. TASCA DI SPECIFICITA'

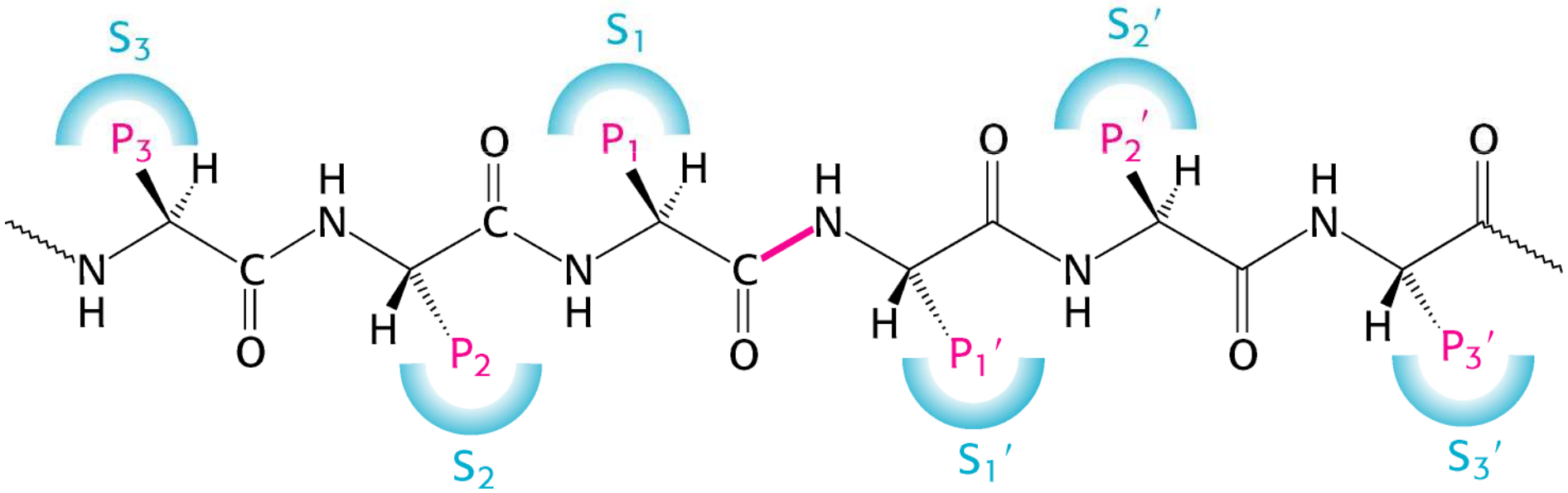




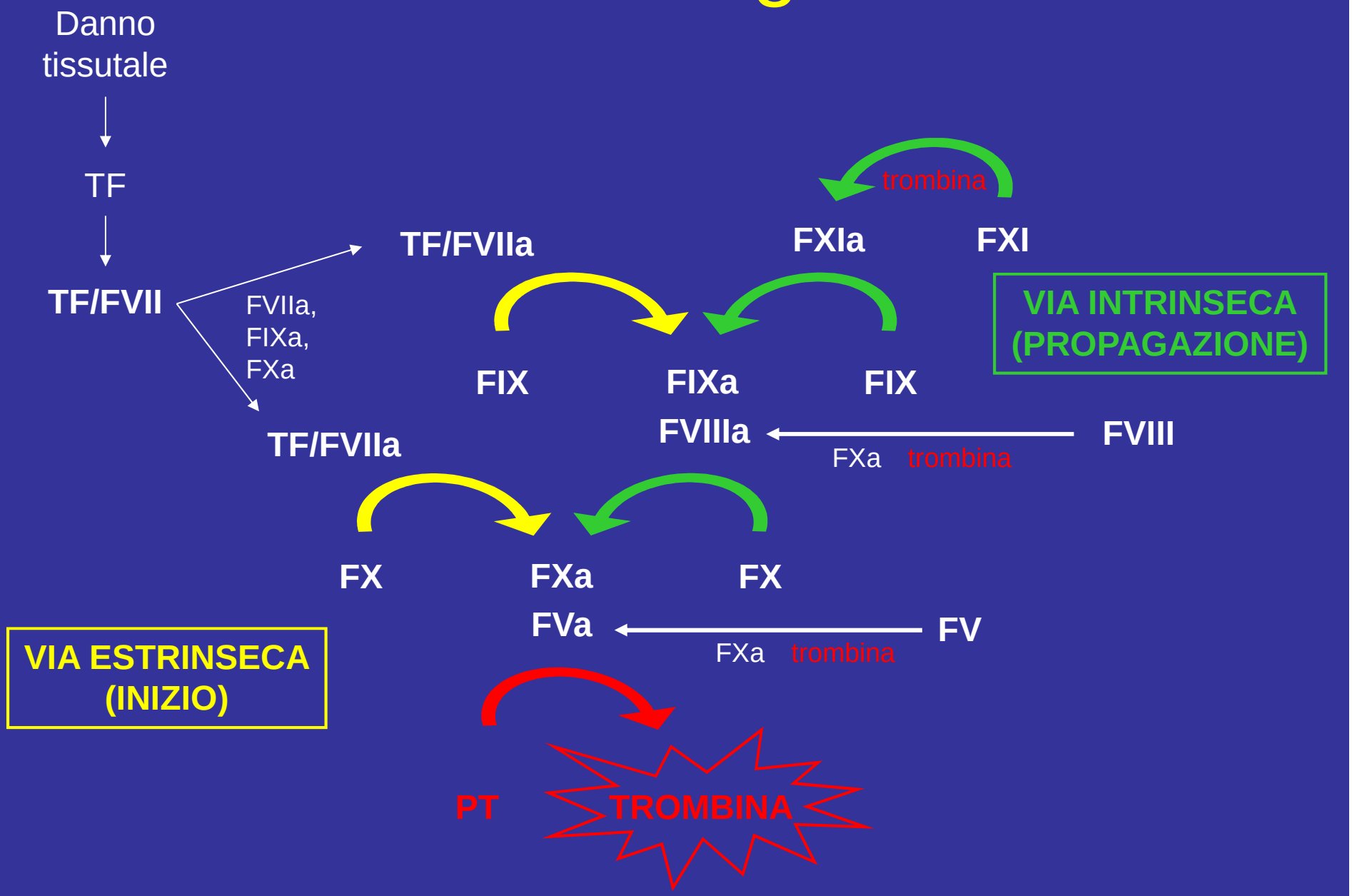

Asp 189
Val 190
Val 216
Asp 189
O
O
Val 216
Val 190
Chimotripsina
Tripsina
Elastasi

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

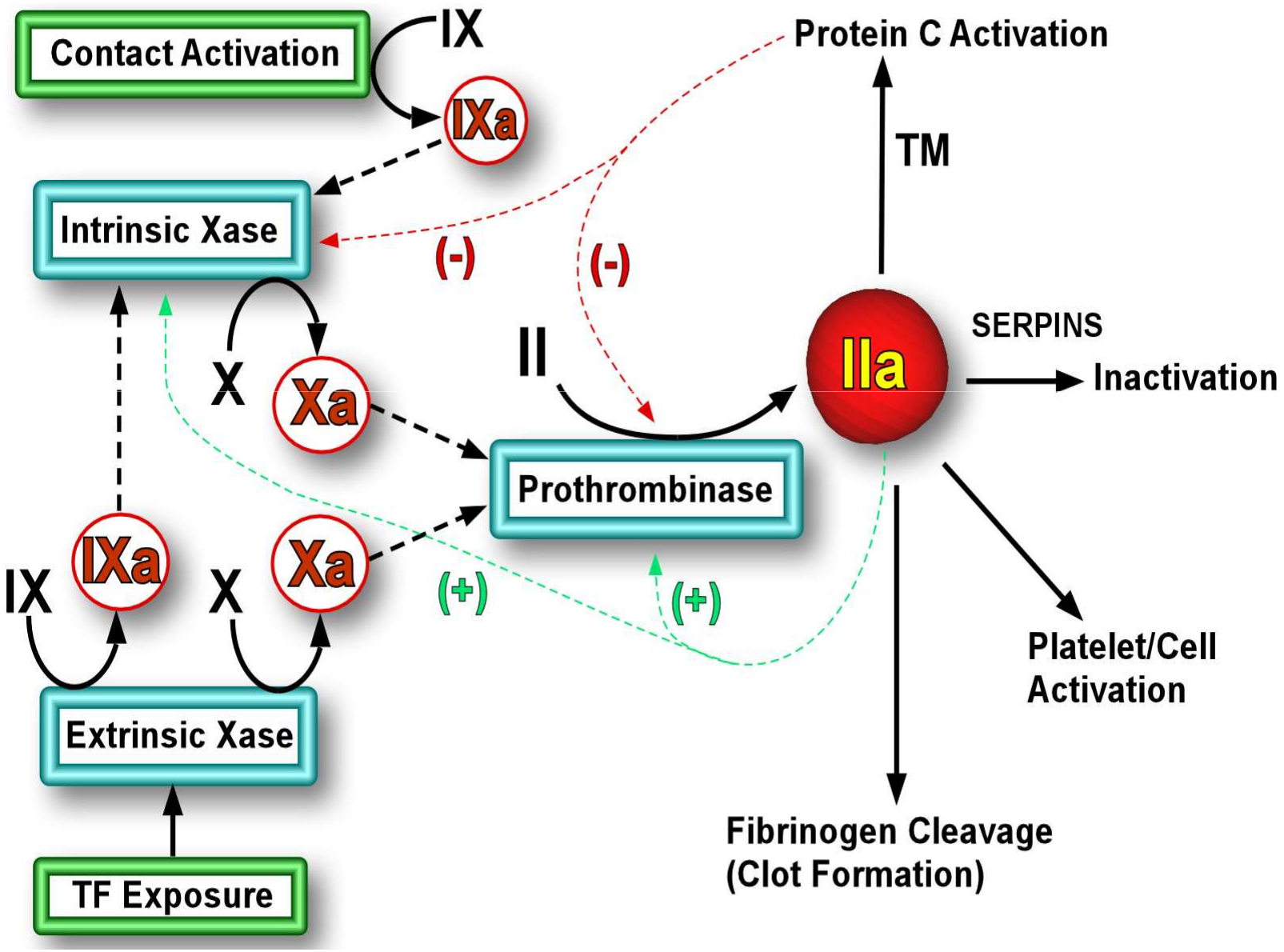
Per molte altre serina proteasi i determinanti di specificità sono molto più complessi



Cascata coagulativa



Blood Coagulation:
A Highly Specific Proteolytic Cascade



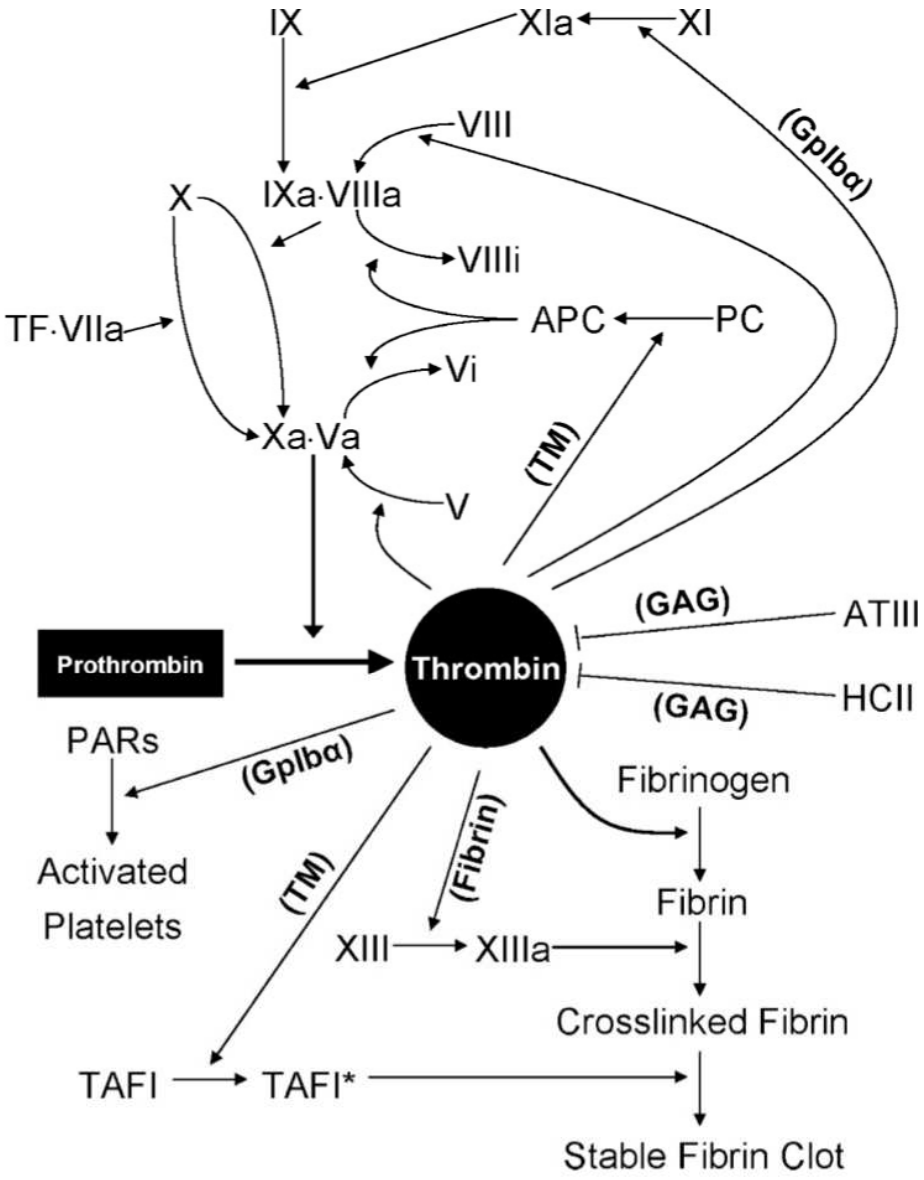


Figure 1. Thrombin activities. Schematic representation of thrombin activities in coagulation, with cofactors indicated in parentheses.

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

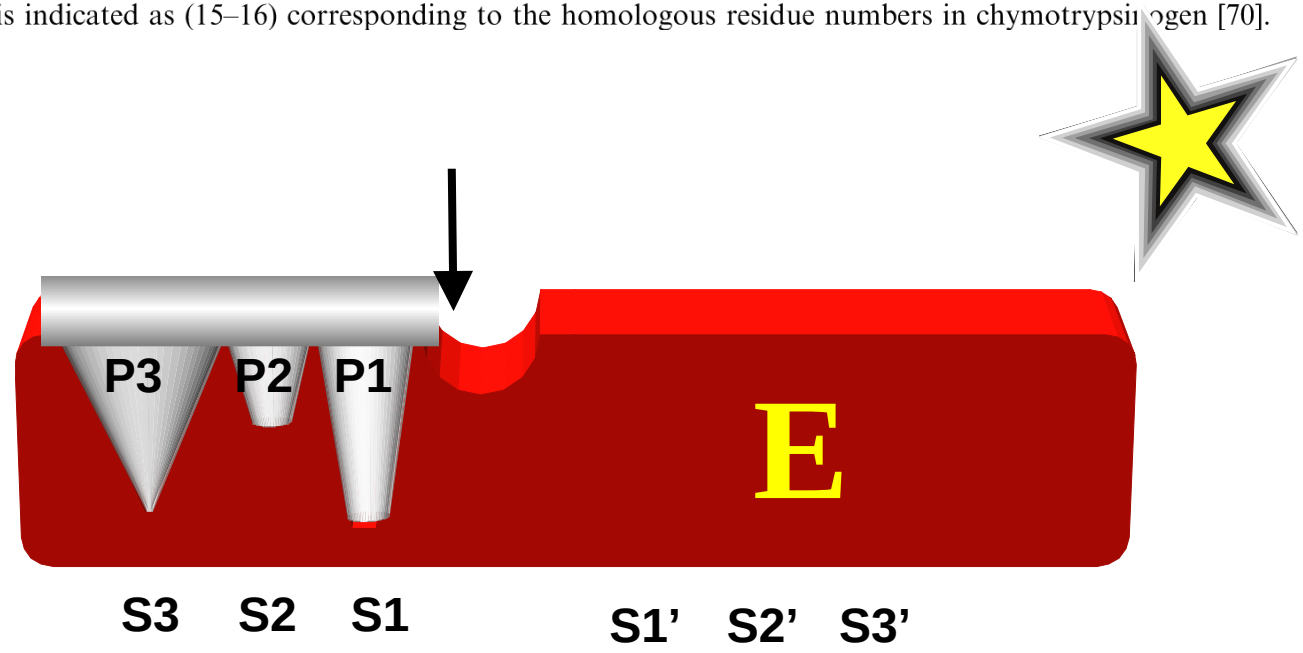
Hirudin

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].



Kinetic studies to understand mechanisms of interaction

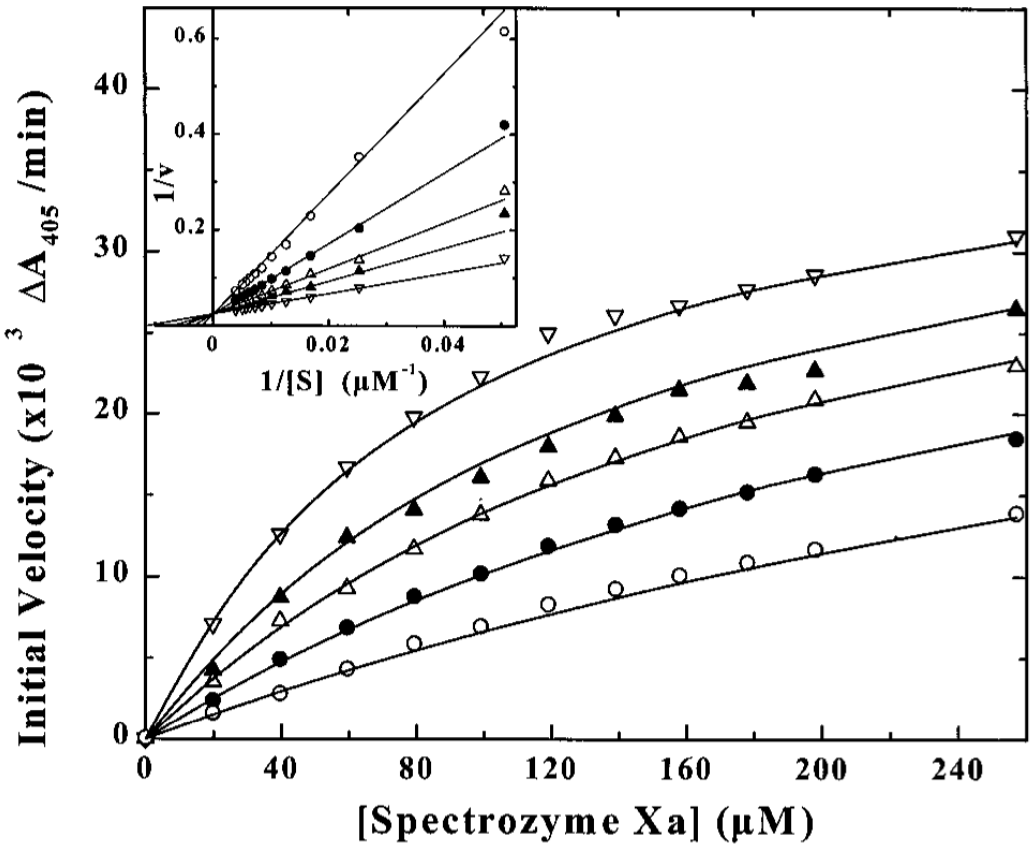
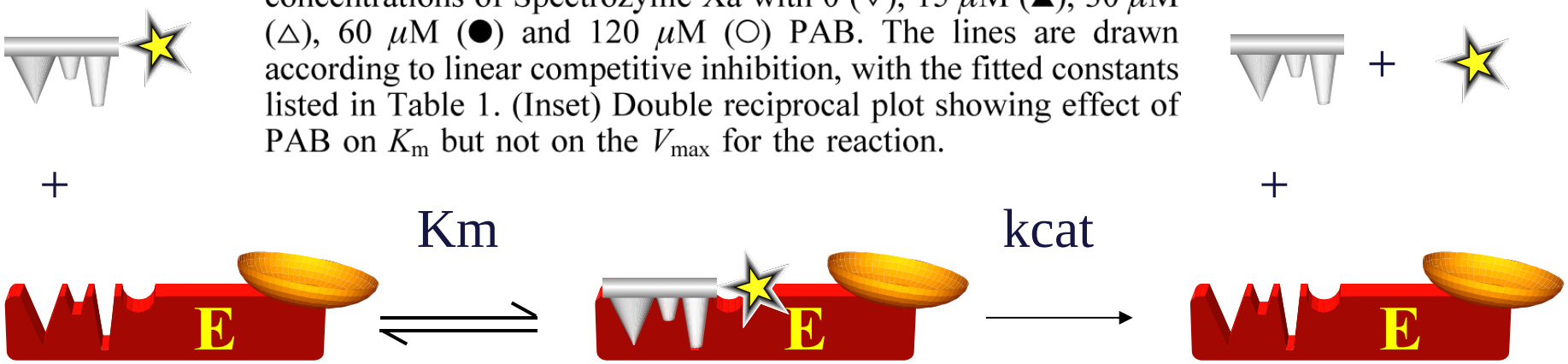


FIGURE 1: Inhibition kinetics of peptidyl substrate hydrolysis by prothrombinase. Initial velocities were measured using 0.5 nM prothrombinase (0.5 nM Xa, 20 nM Va, 50 μM PCPS), increasing concentrations of Spectrozyme Xa with 0 (▽), 15 μM (▲), 30 μM (△), 60 μM (●) and 120 μM (○) PAB. The lines are drawn 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

Km increases



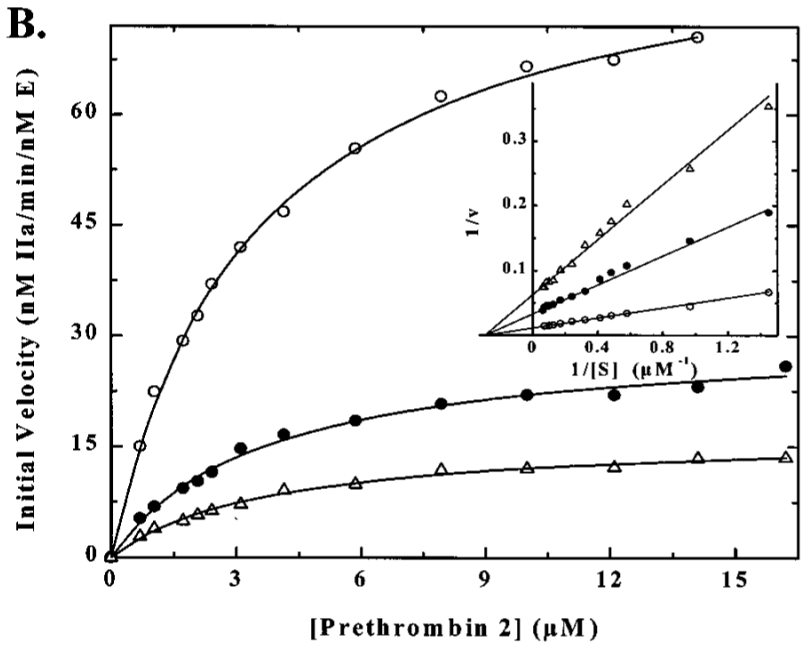
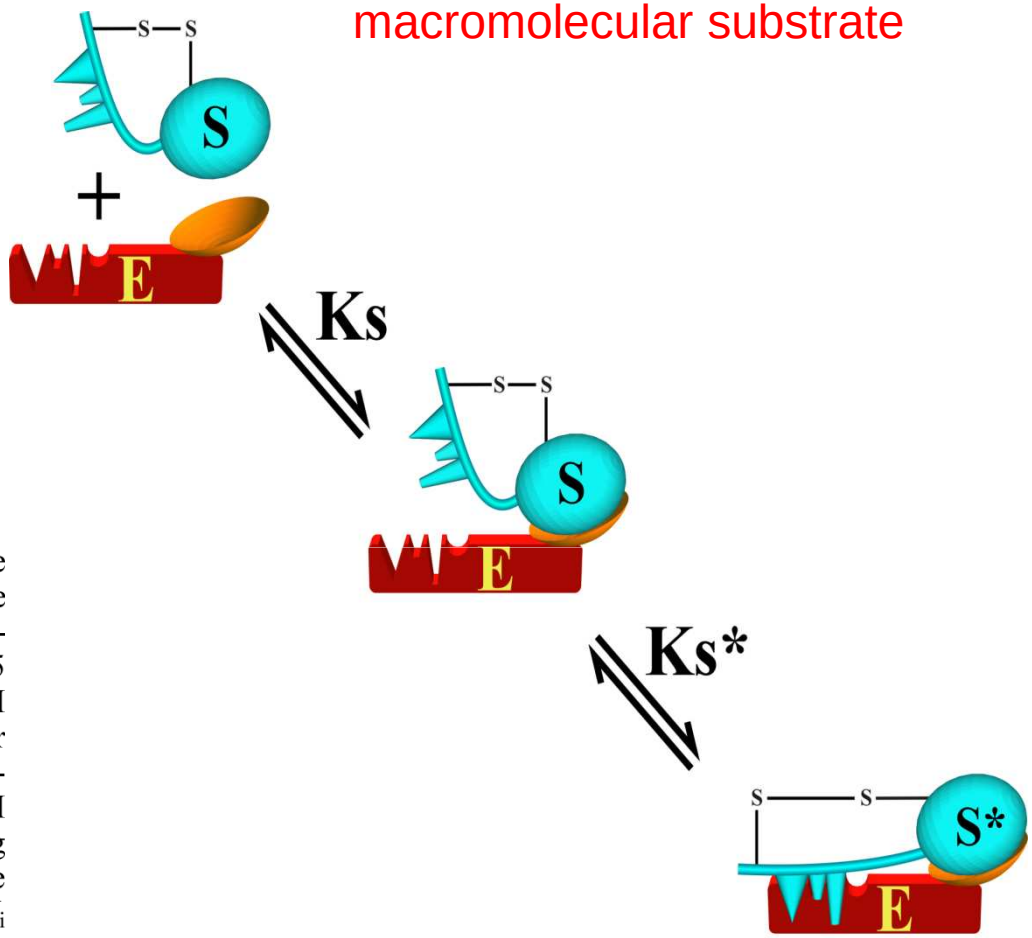
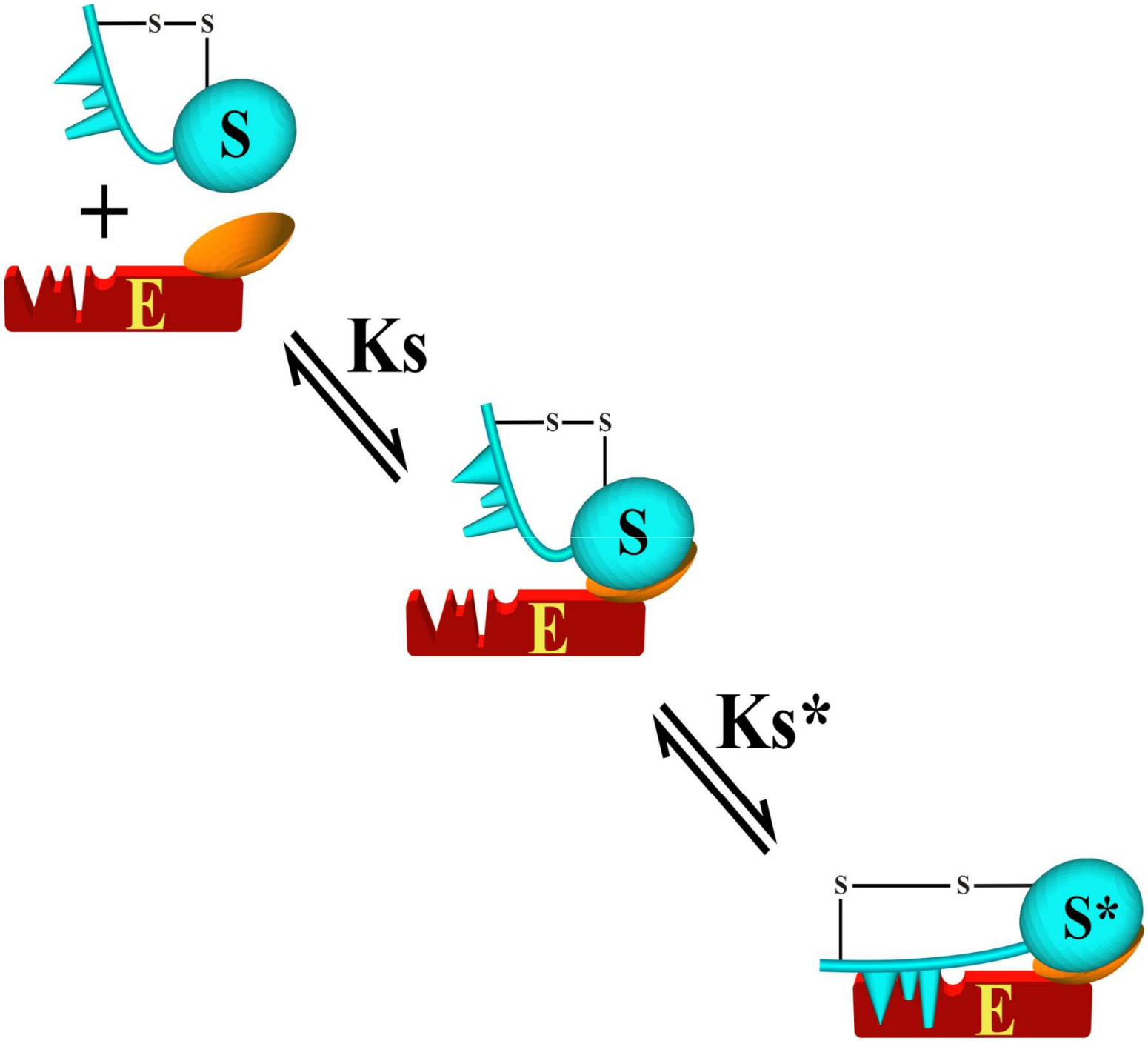


FIGURE 2: Inhibition kinetics of macromolecular substrate cleavage by prothrombinase. The initial velocity for thrombin formation (rate normalized/nanomolar prothrombinase) was determined at increasing concentrations of prethrombin 2 *plus* fragment 1.2 with 0.25 nM prothrombinase (0.25 nM factor Xa, 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.

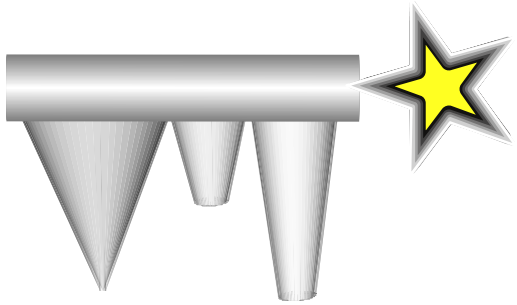
Non competitive inhibition when using a macromolecular substrate



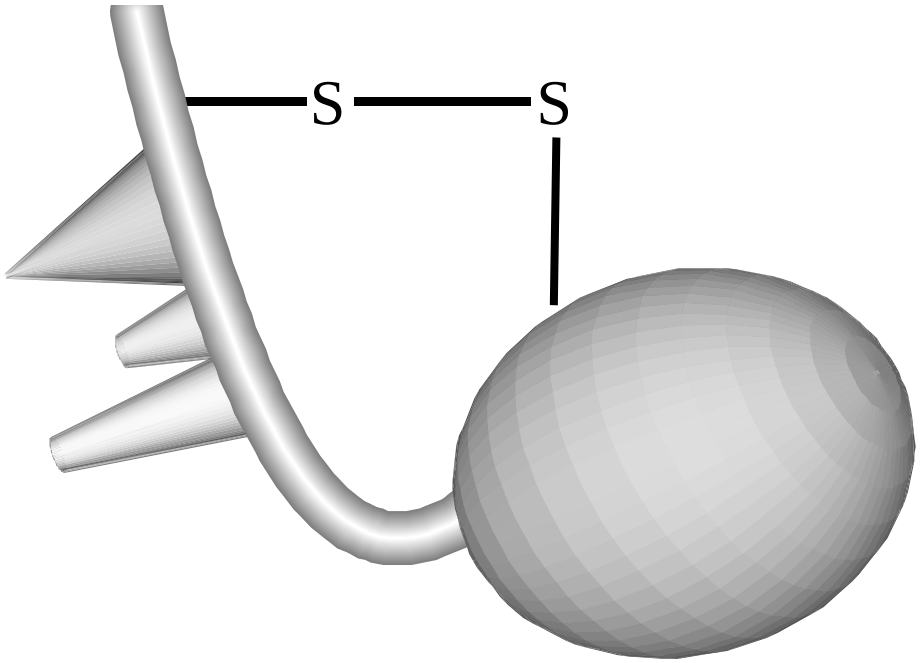

S
S
S
+
E
Ks
S
S
S
E
Ks*
S
S
S*
E

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}).



Oligopeptidyl
Substrate



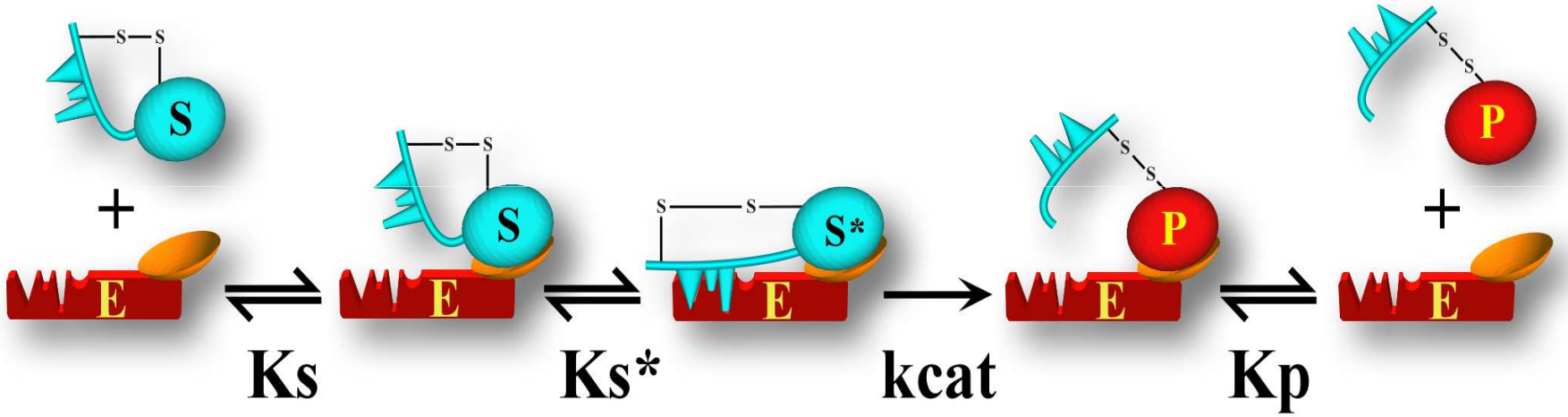
Protein Substrate

Active Site

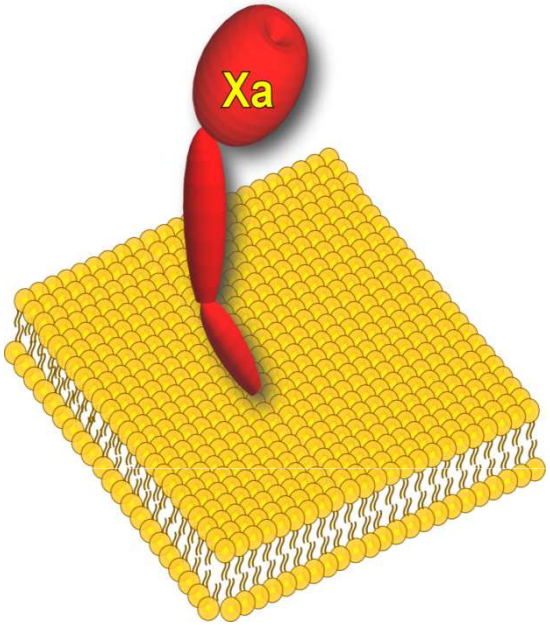
Enzyme

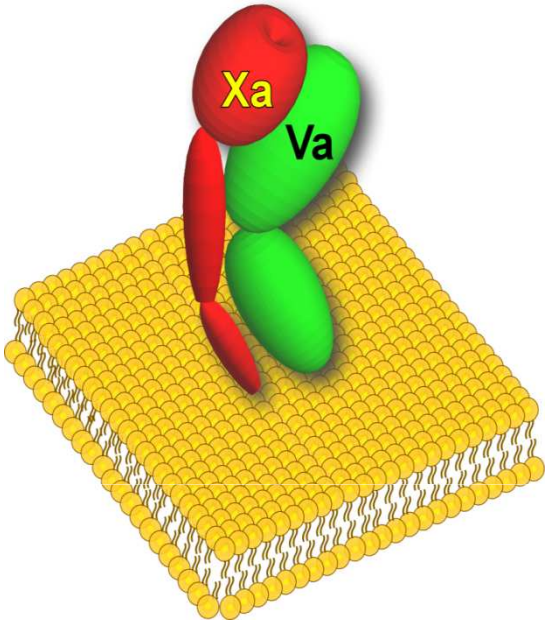
Extended
Recognition
Site (Exosite)

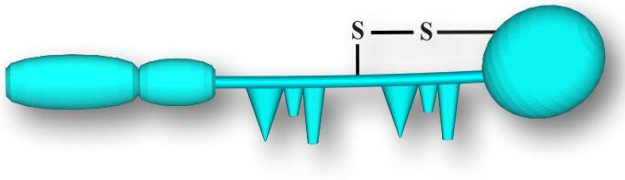
Protein Substrate Recognition by Prothrombinase is a Multi-Step Process



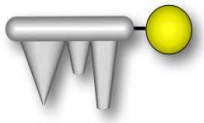
Complex Assembly Selectively Increases Catalytic Efficiency for Protein Substrate Cleavage







1 **12,640**



1 **~0.9**

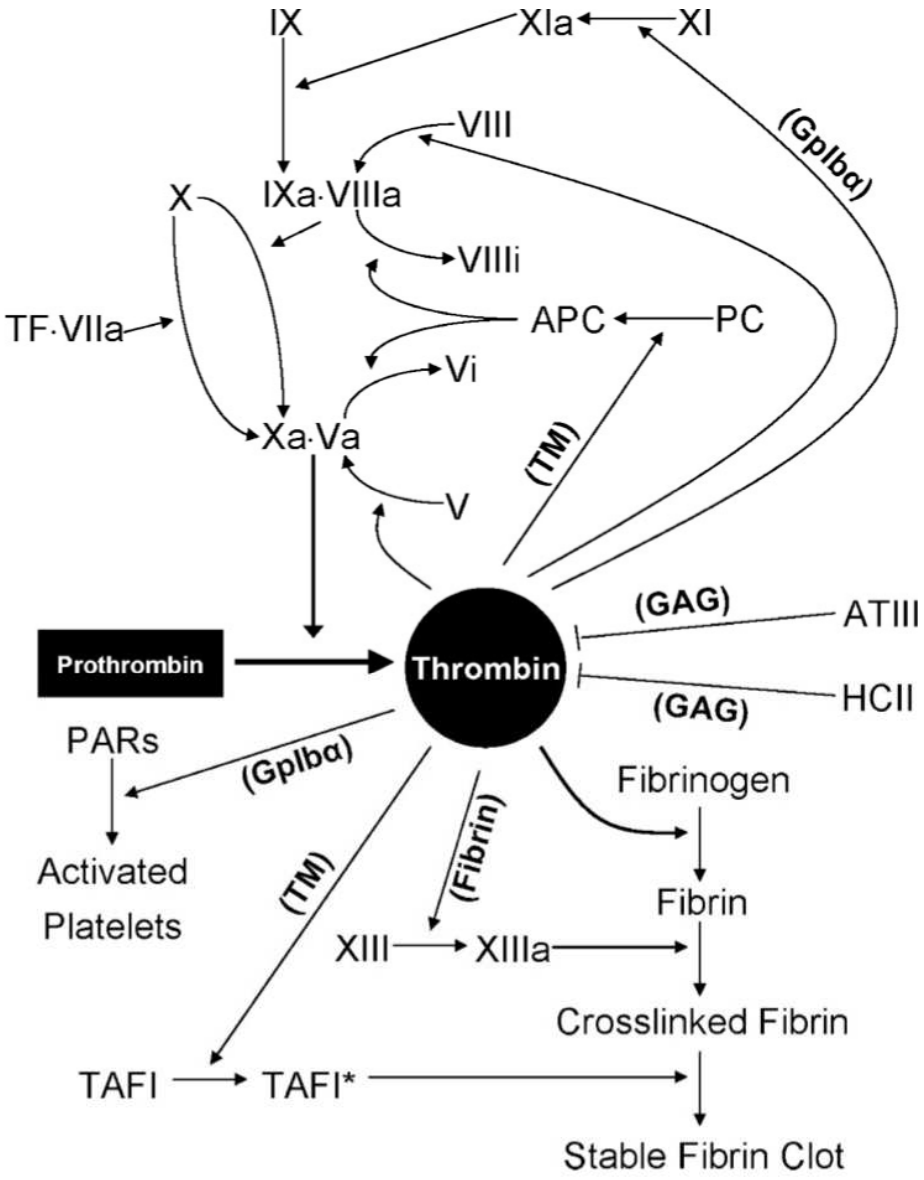


Figure 1. Thrombin activities. Schematic representation of thrombin activities in coagulation, with cofactors indicated in parentheses.

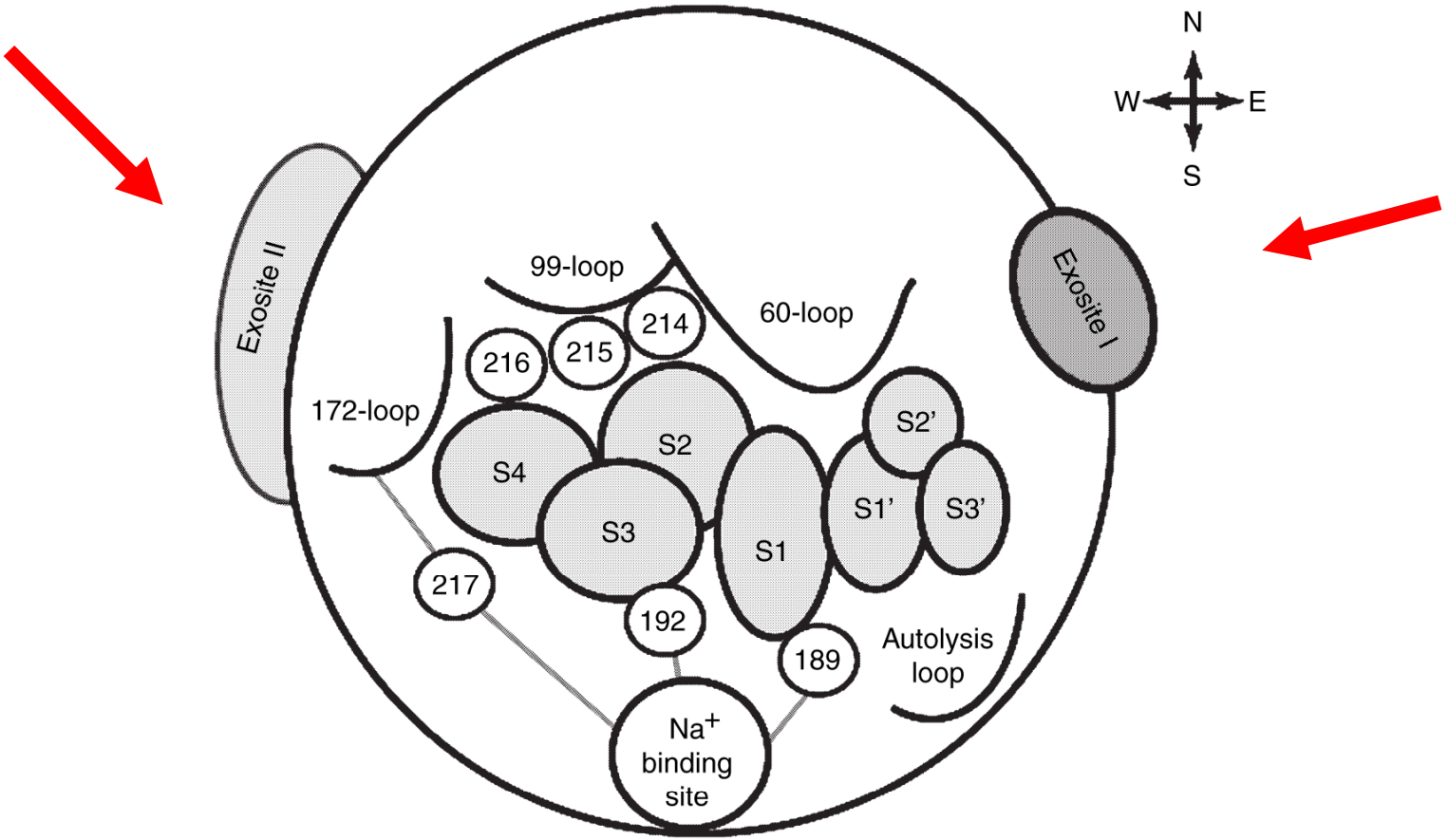


Fig. 1. Schematic representation of the specificity determinants of coagulation proteases. Loops at positions 60, 99, and 172 influence the active site specificity toward small peptide substrates and can impact macromolecular specificity. Exosites I and II, located to the east and west of the active 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.

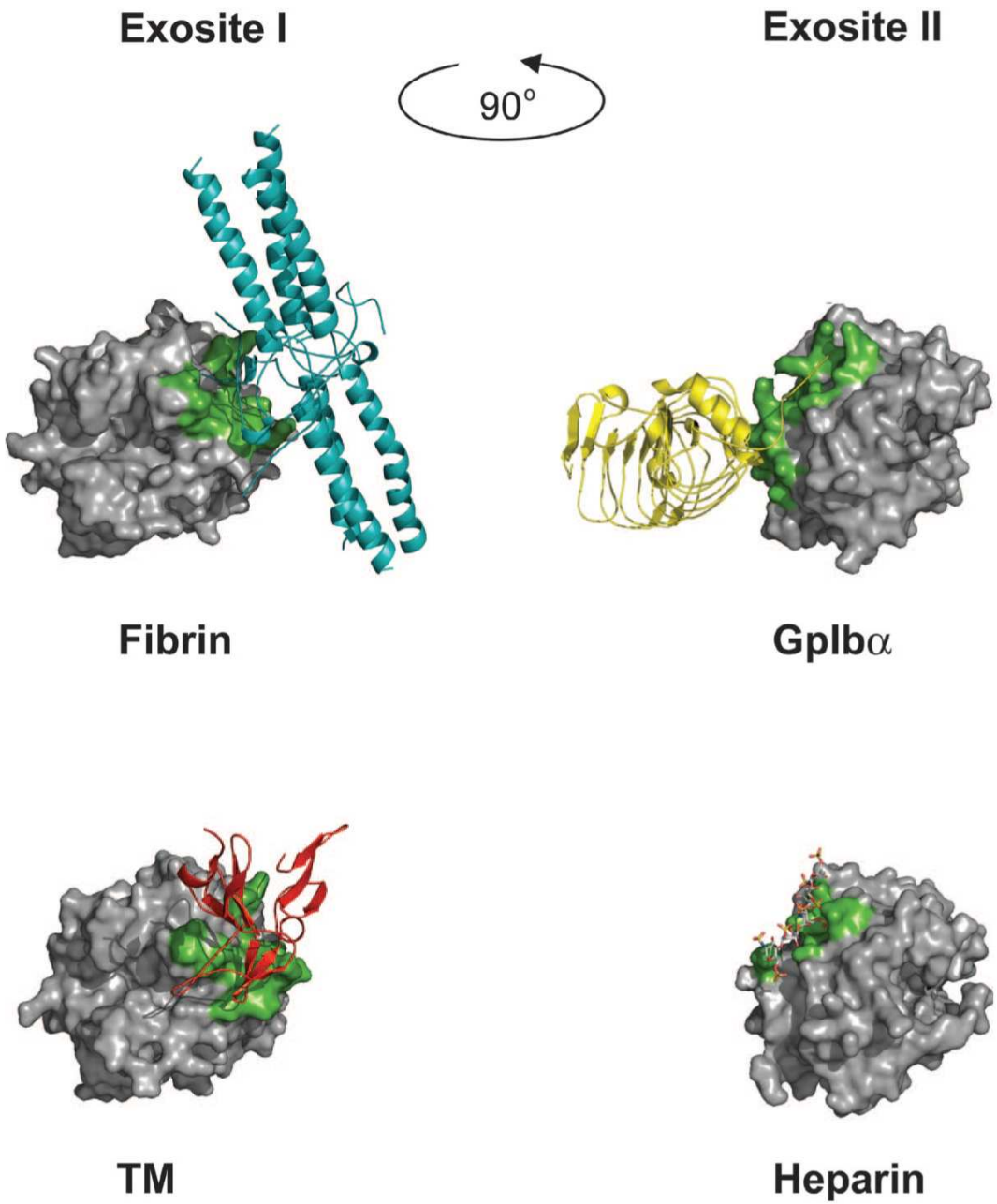
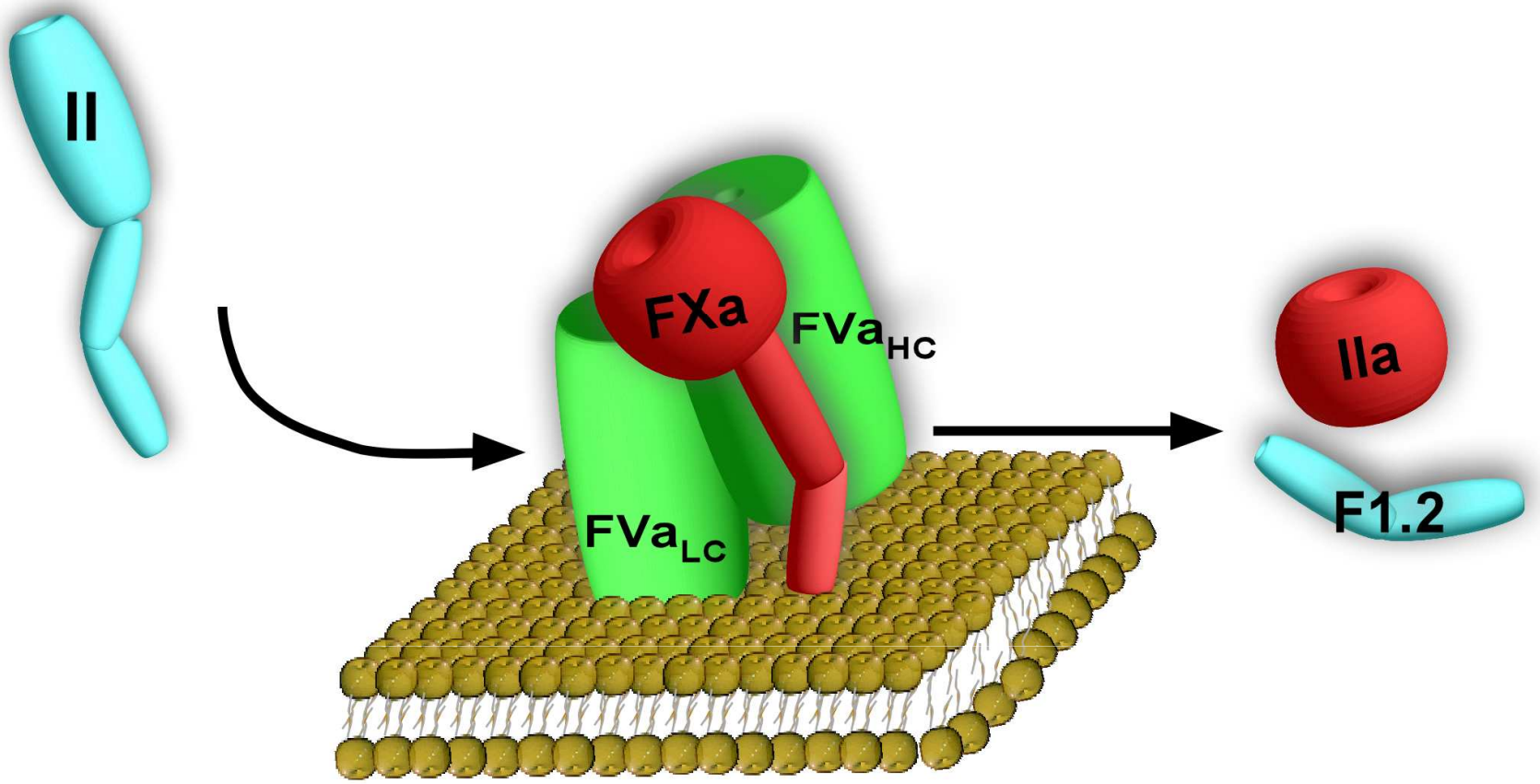


Figure 3. Thrombin-cofactor exosite interactions. The surface representation of thrombin is shown in the standard orientation (left) for exosite I interactions (Protein DataBank entries: fibrin-1QVH, TM-1DX5) and rotated 90° (right) to show exosite II interactions (PDB entries: GPIbα-1P8V, heparin-1XMN).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

Interazioni macromolecolari estese rendono conto di queste differenti specificità

↓

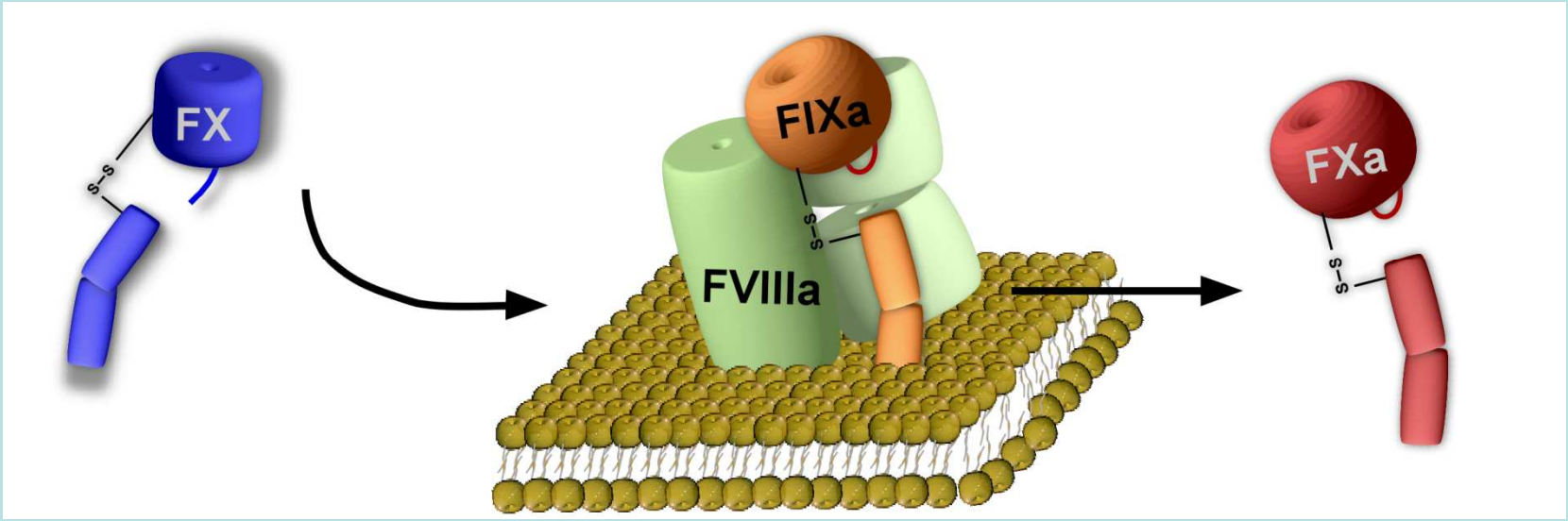
COMPLESSI MACROMOLECOLARI



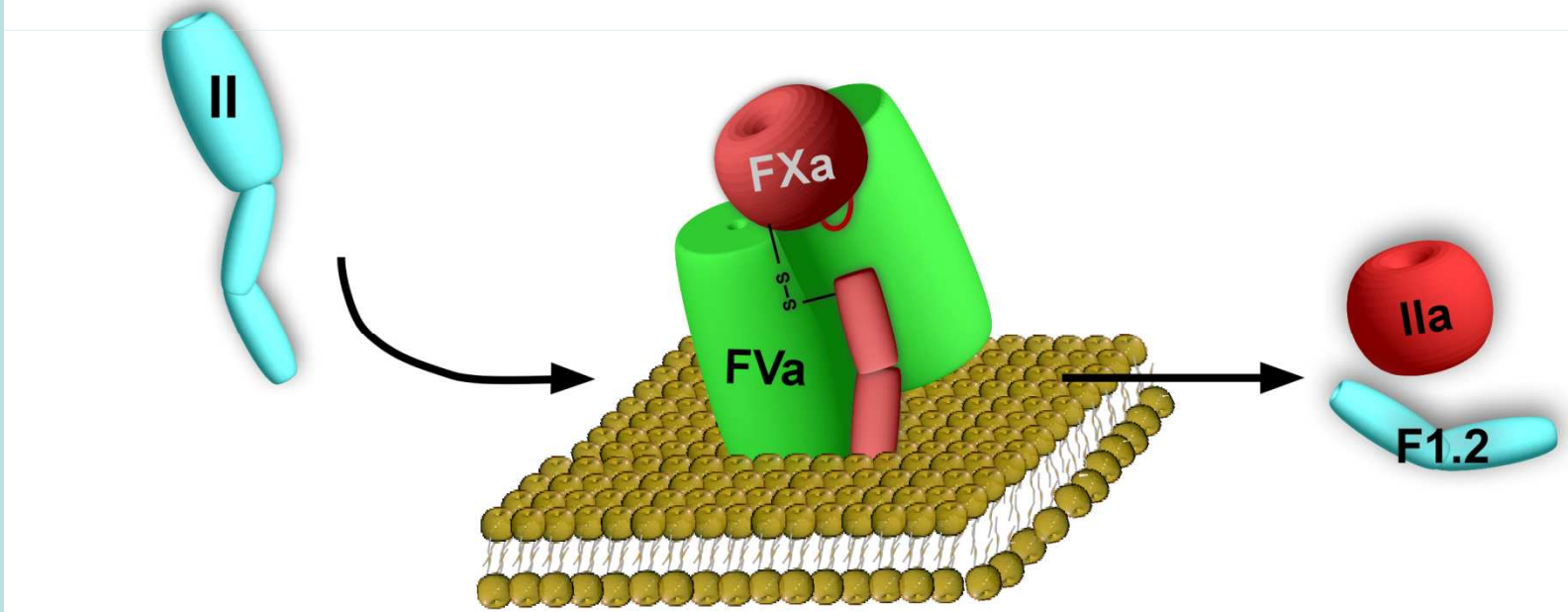
Prothrombinase

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

So, the importance of cofactors



Intrinsic Xase



Prothrombinase

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, $\mu M^{-1} \cdot min^{-1}$
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, $\mu M^{-1} \cdot sec^{-1}$	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 TABLE 3
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.