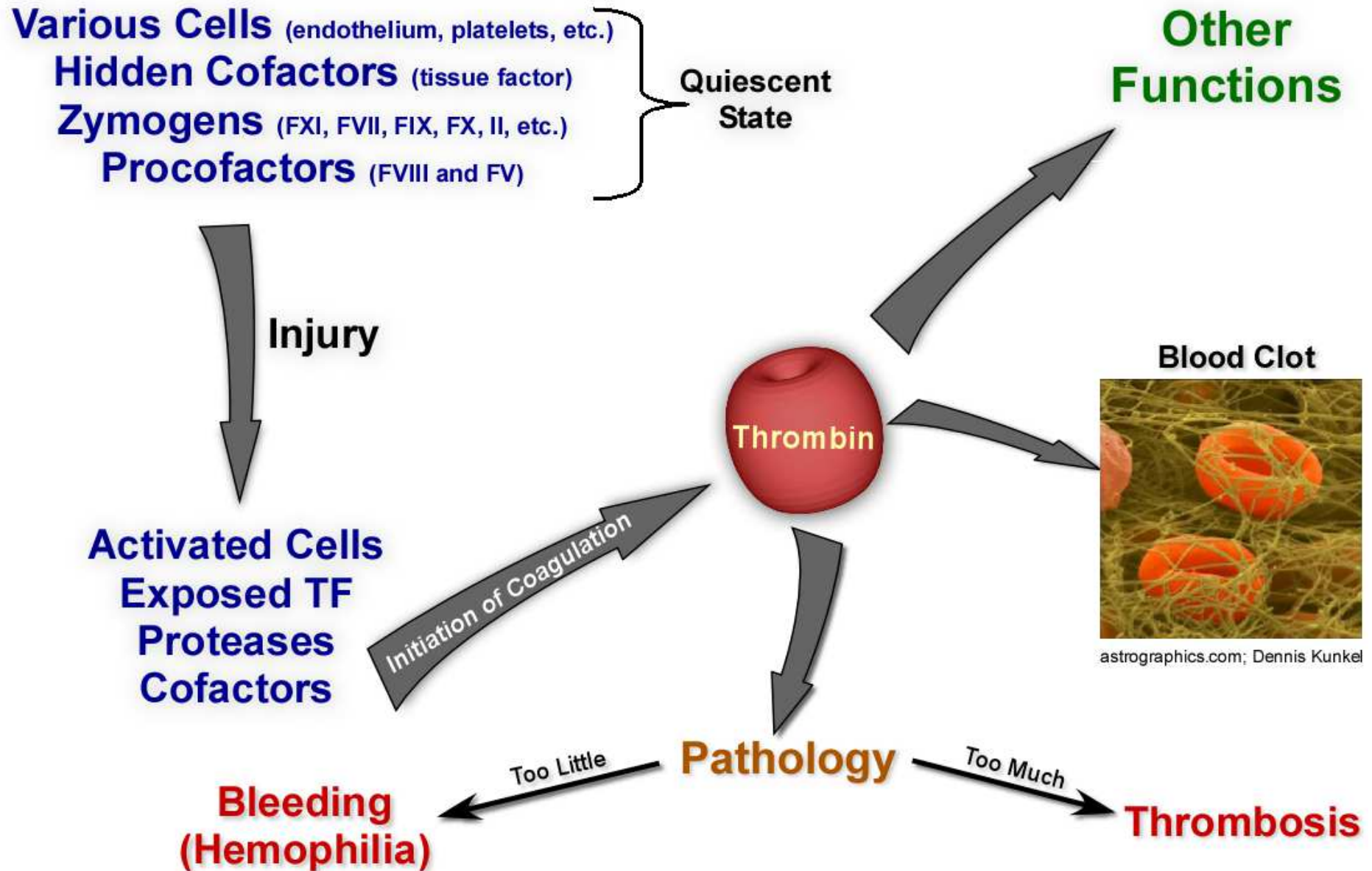


# Activation and specificity of Thrombin

Giulia Pavani

# The Blood Coagulation Response:

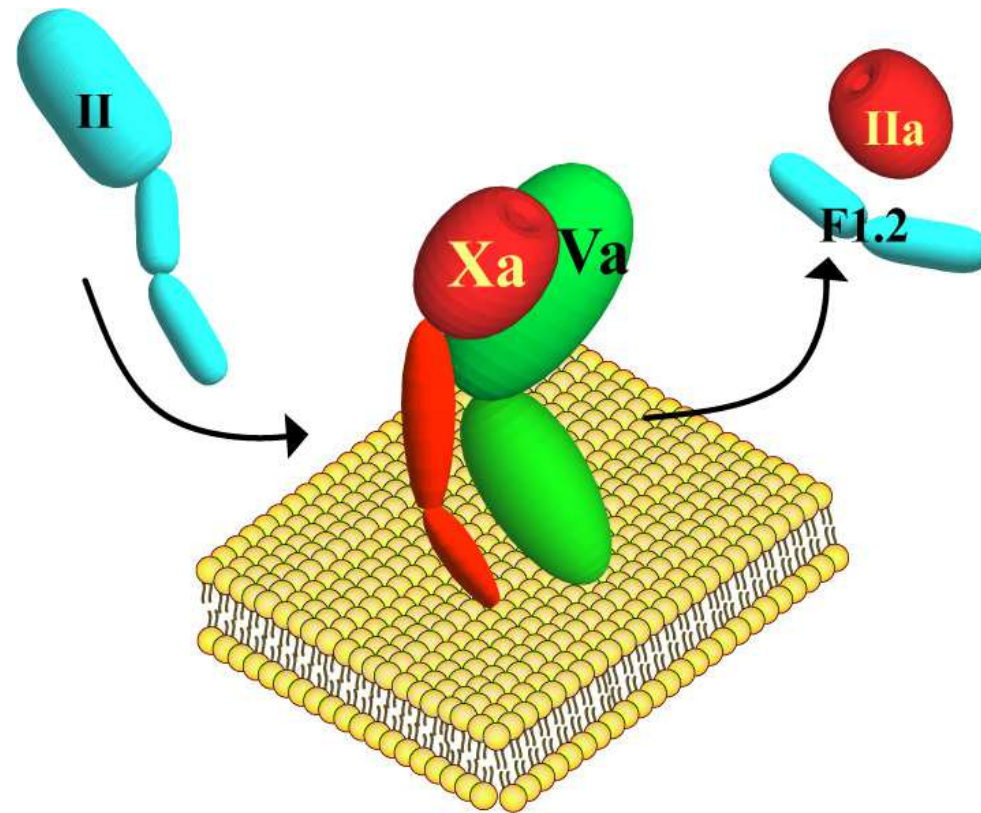


# Summary

- Regulation of a Serine Protease: Thrombin
  - Zymogen → Enzyme → Substrate Specificity
- Staphylocoagulase
  - Bacteria know how a protease works (much more than we do...)
- Diagnostic applications
  - Imaging of Staphylococcus vegetations in the heart

# MECCANISMO ATTIVAZIONE

Prothrombin is activated to thrombin by two proteolytic cleavages



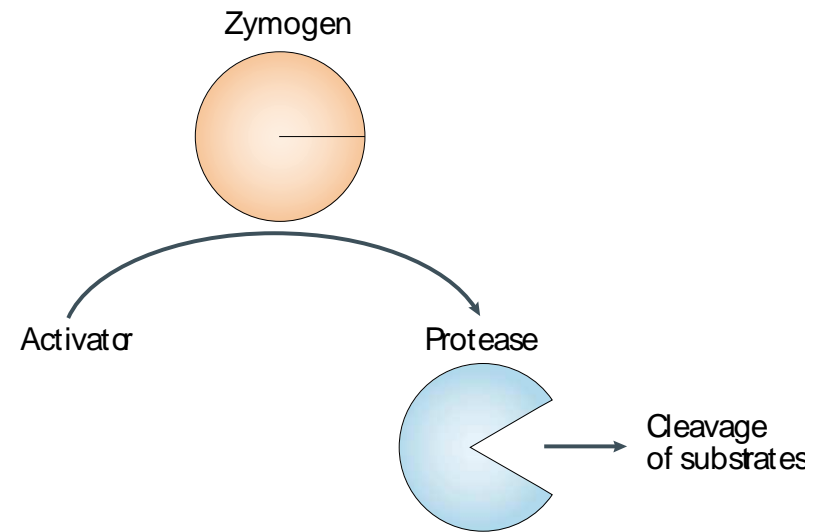
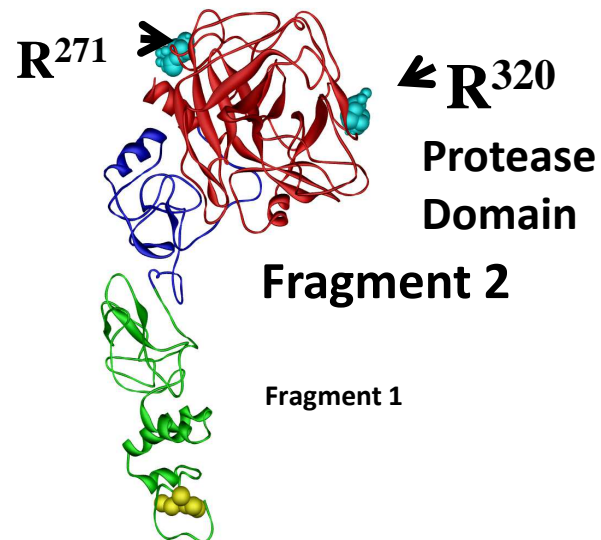
Prothrombinase

# Thrombin is synthesized as a Zymogen: Prothrombin

## Zymogen:

A proenzyme or inactive enzyme. It requires a biochemical change to reveal the active site for it to become an active enzyme.

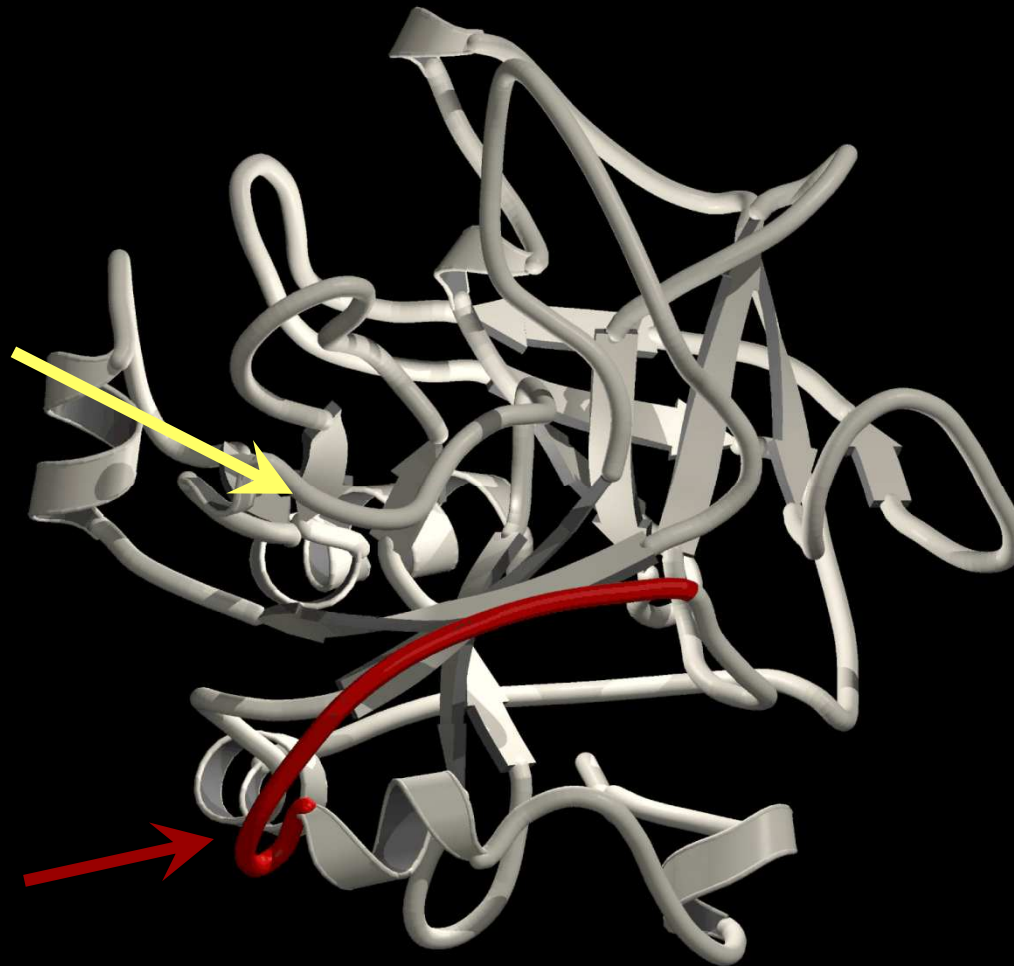
Zymogens lack the structural attributes required for formation of the enzyme-substrate complex.



# Serine Proteases: Conversion Pathway

- Cleavage between Arg<sup>15</sup>-Ile<sup>16</sup> → Exposure of new N-terminus
- New N-terminus (IVGG) forms salt bridge with Asp<sup>194</sup>
- N-terminal insertion leads to a conformational change in the “activation domain”

**Active site  
(184-194)**



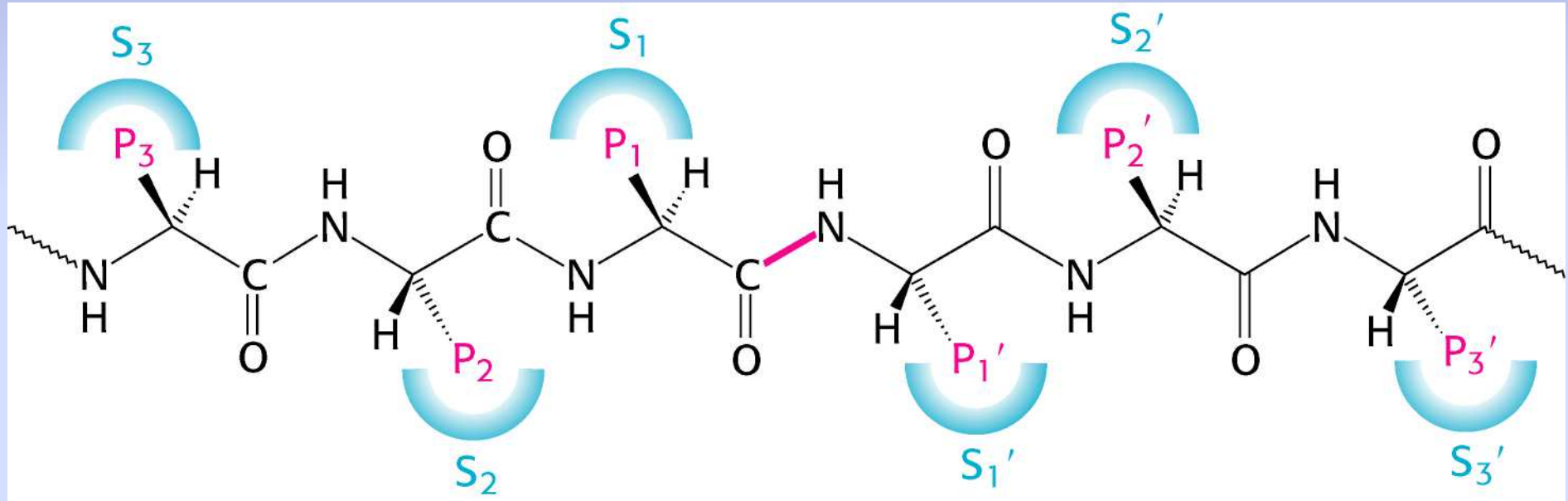
**N-terminus  
(16-19)**

*Courtesy of W. Bode,  
Max Planck  
Institute of Biochemistry*

**SPECIFICITA SUBSTRATO**



## determinanti di specificità di proteasi



# Trypsin and Thrombin have similar structures

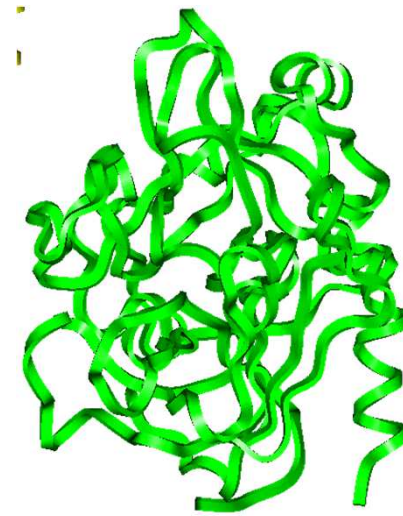
## Trypsin

- Cleaves peptides on the C-term of **Lys** and **Arg** amino acid residues



## Thrombin

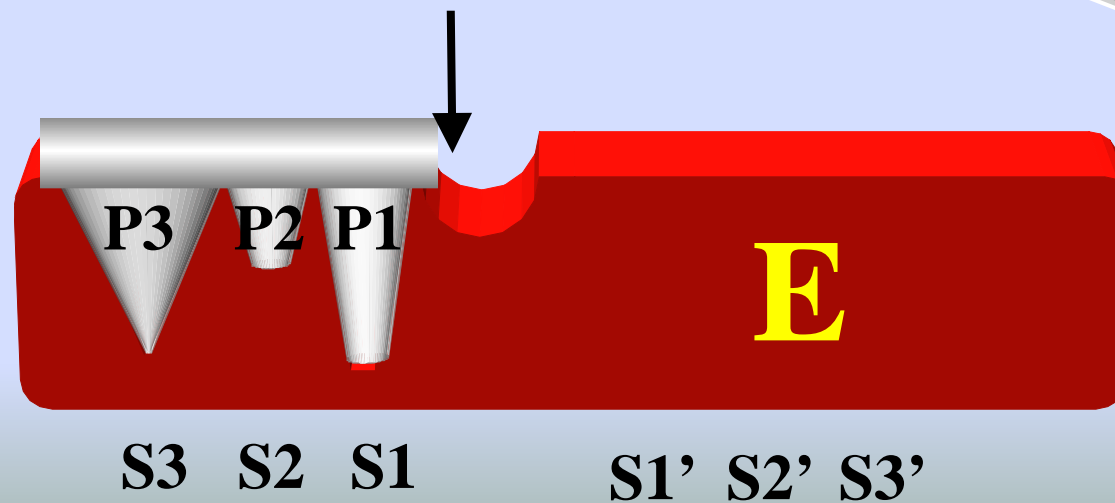
- Cleaves peptides at **Arg** (Pro, **Arg**, Ser/Ala/Gly/Thr, not acidic, Arg)

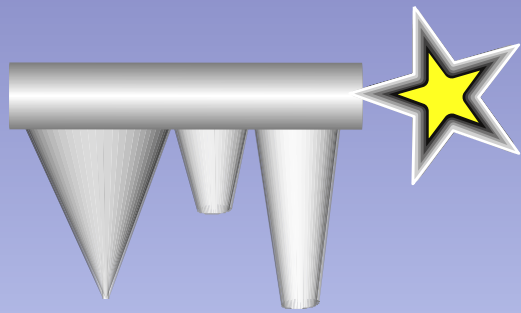


**Table 1** Sites of cleavage in the human vitamin K-dependent zymogens\*

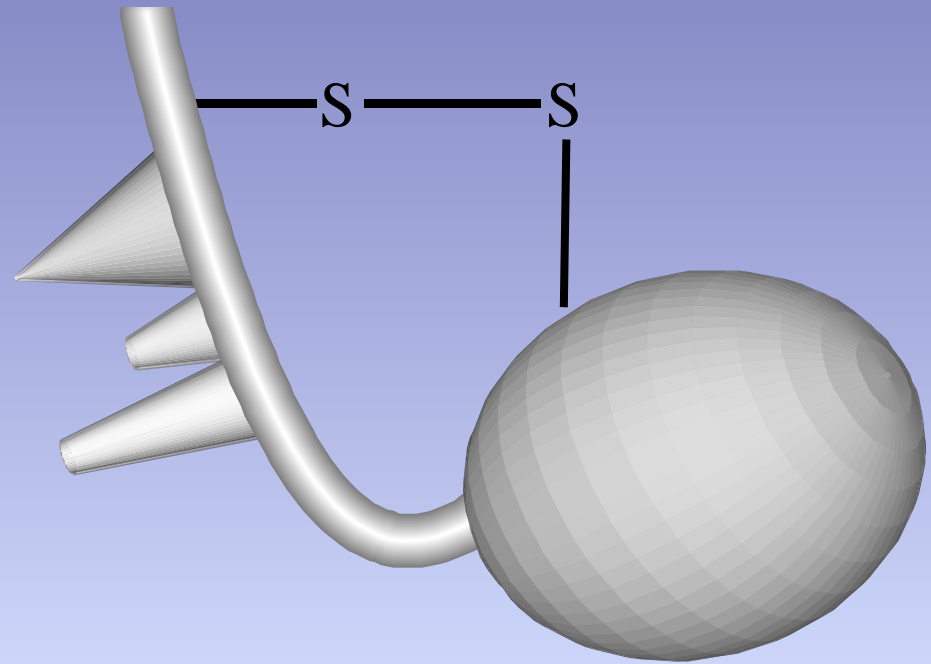
Enzyme	Substrate†	P <sub>4</sub>	P <sub>3</sub>	P <sub>2</sub>	P <sub>1</sub>	↓	P <sub>1</sub> '	P <sub>2</sub> '	P <sub>3</sub> '	P <sub>4</sub> '
Xa/Va	II	I	E	G	R		T	A	T	S
	II <sub>(15-16)</sub>	I	D	G	R		I	V	E	G
VIIa/TF, IXa/VIIIa	X <sub>(15-16)</sub>	N	L	T	R		I	V	G	G
	VIIa/TF, XIa	K	L	T	R		A	E	A	V
VIIa/TF, Xa	IX <sub>(15-16)</sub>	D	F	T	R		V	V	G	G
	VII <sub>(15-16)</sub>	P	Q	G	R		I	V	G	G
IIa/TM	PC <sub>(15-16)</sub>	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].

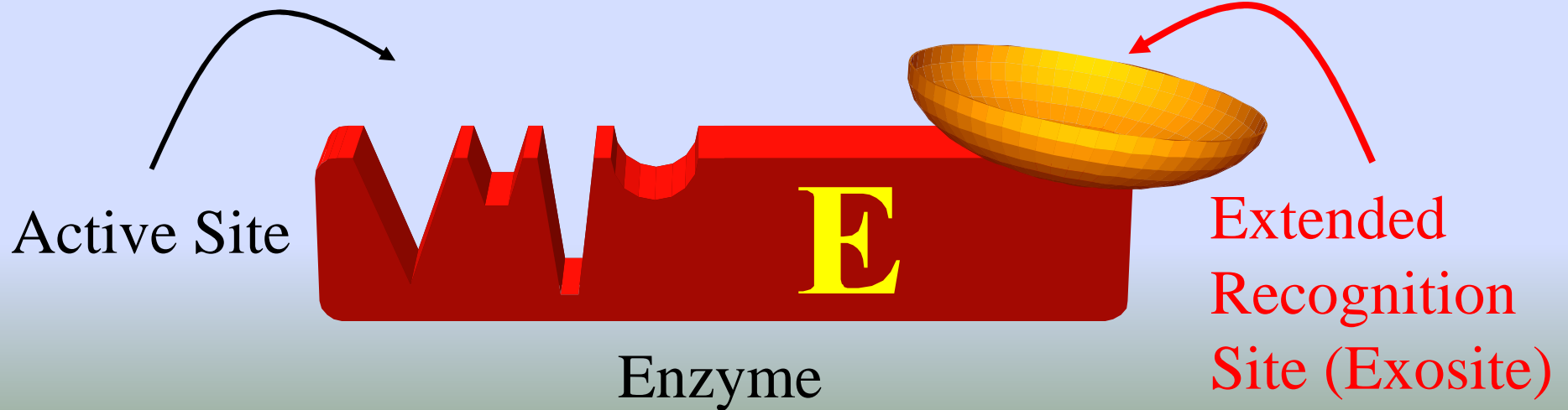




Oligopeptidyl  
Substrate



Protein Substrate

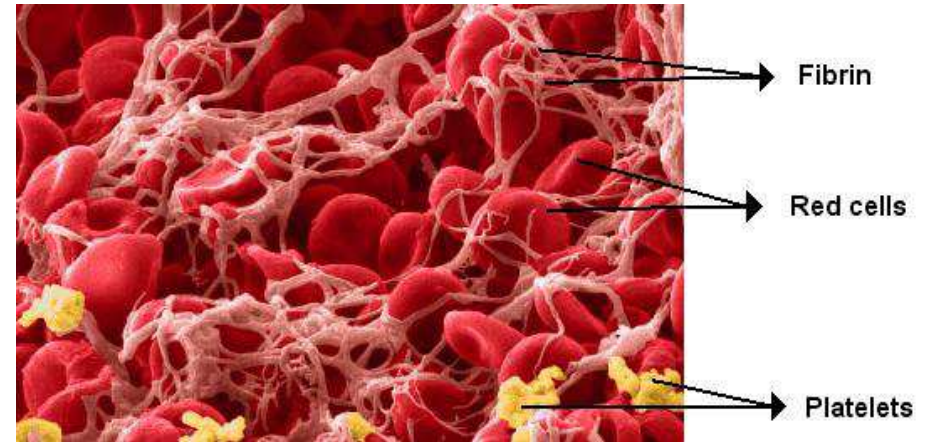
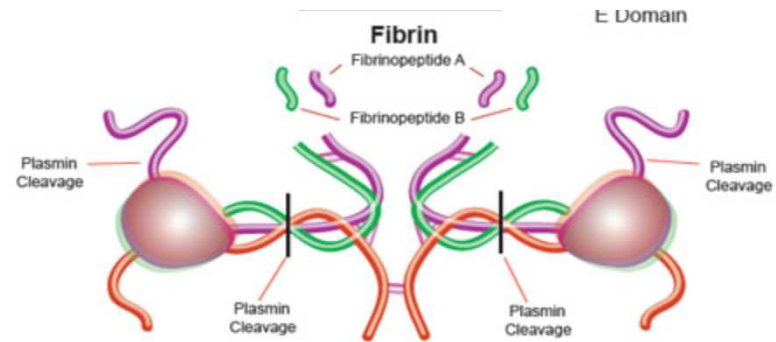
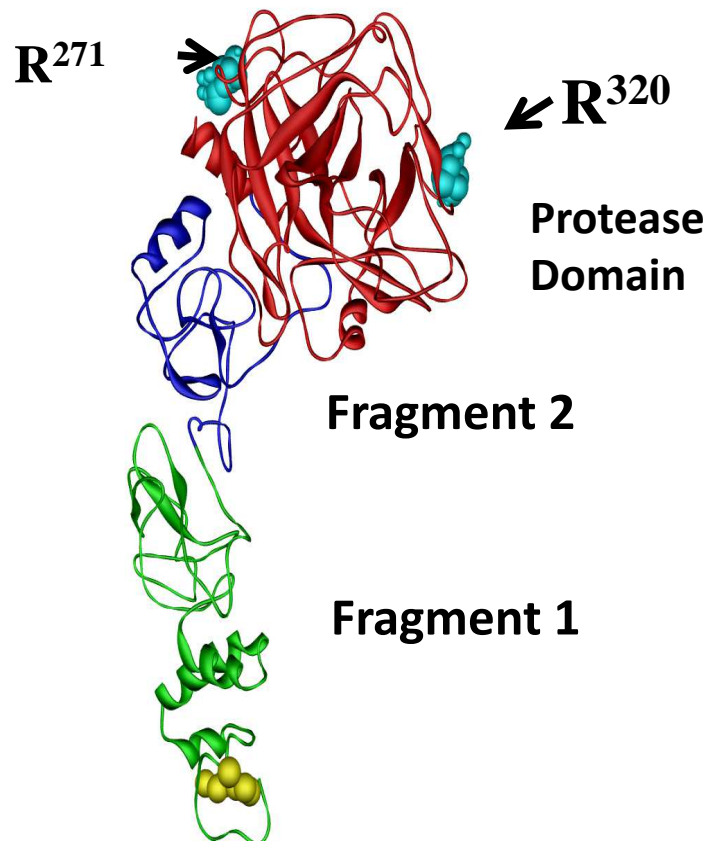


Active Site

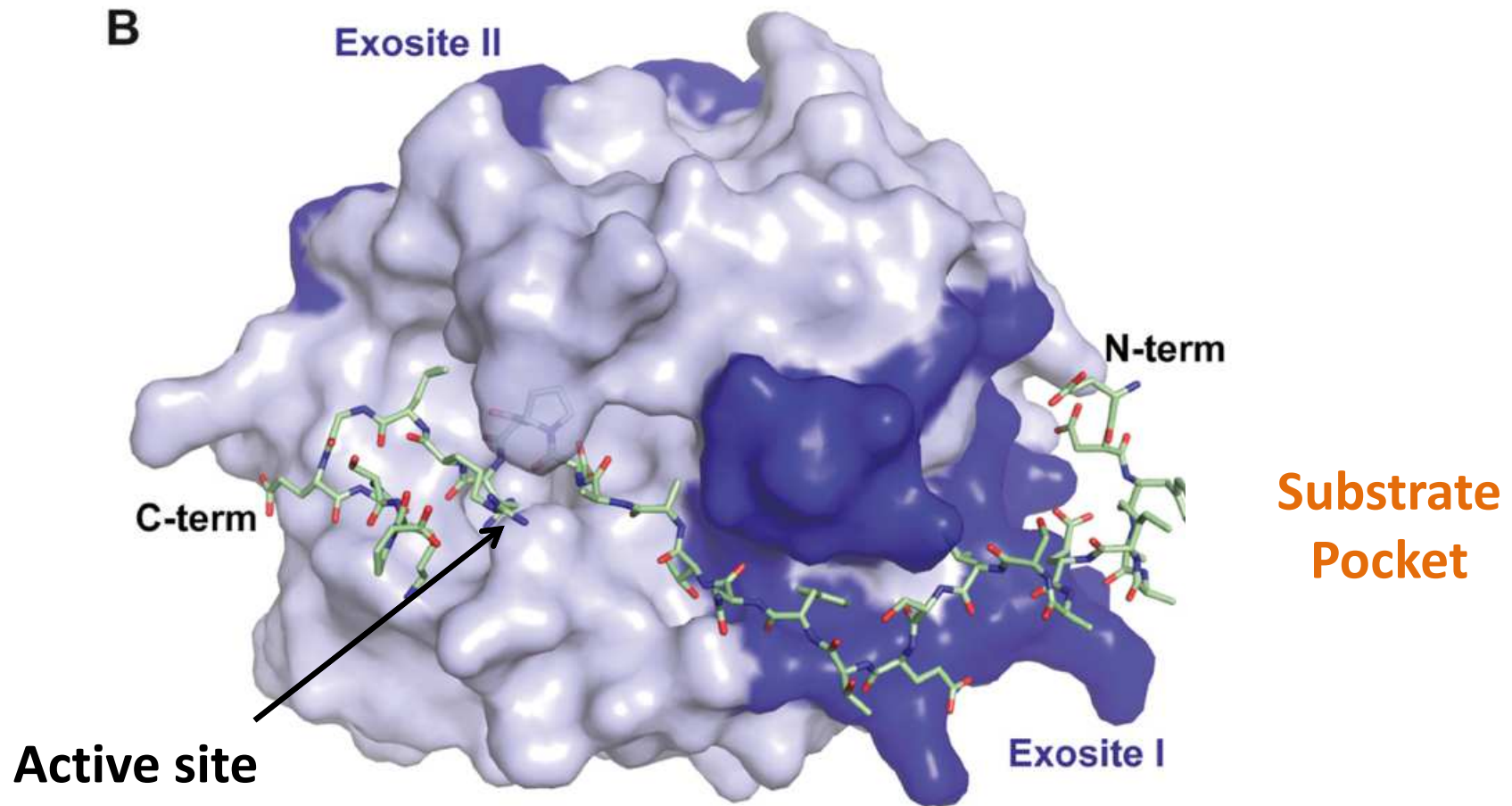
Enzyme

Extended  
Recognition  
Site (Exosite)

# Thrombin cleavage of the plasma protein fibrinogen

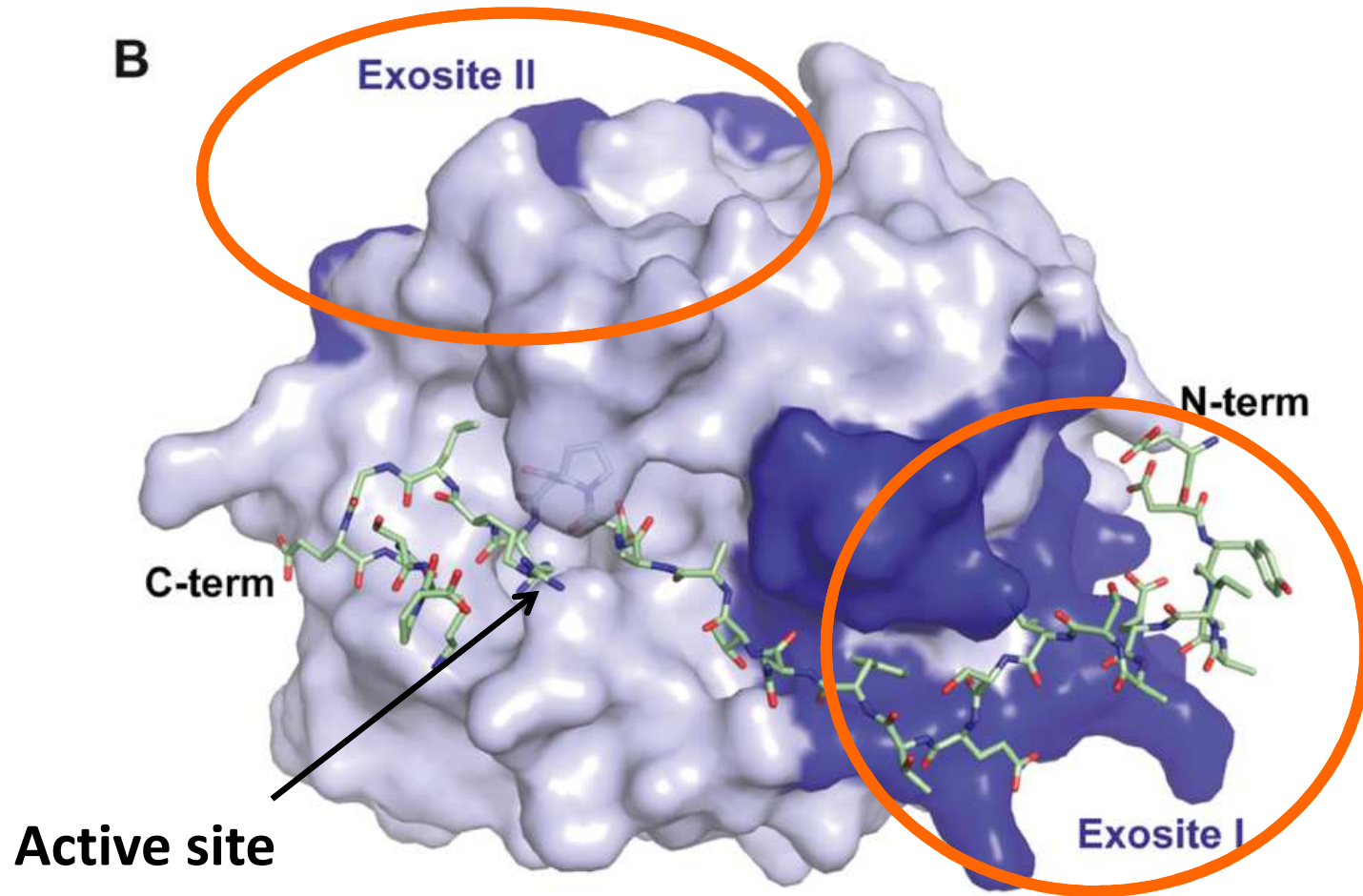


# Thrombin X-ray structure





# Thrombin X-ray structure

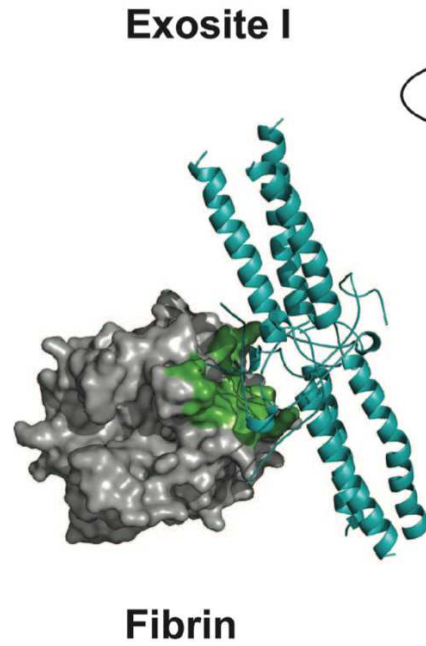


# Exosite binding determines substrate specificity

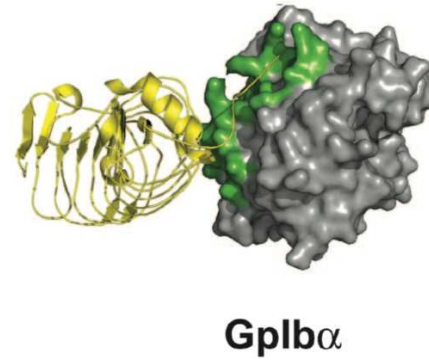
- Thrombin targets are restricted due to specific interactions between the protein substrate and residues outside the catalytic cleft termed **Exosite**
- Extended interactions at exosites drive substrate affinity and contribute to substrate specificity.



**Clot  
stabilization**

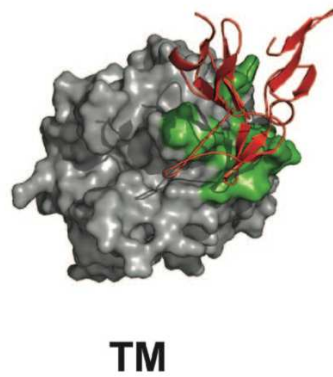


Exosite II

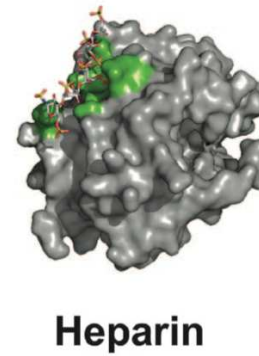


**Platelet  
activation**

**Activation  
of the  
anticoagulant  
pathway**



**Inhibition**



# Thrombin cleaves different substrates

- Thrombin cleaves after Arg residues

Cleavage Sites for Natural Thrombin Substrates

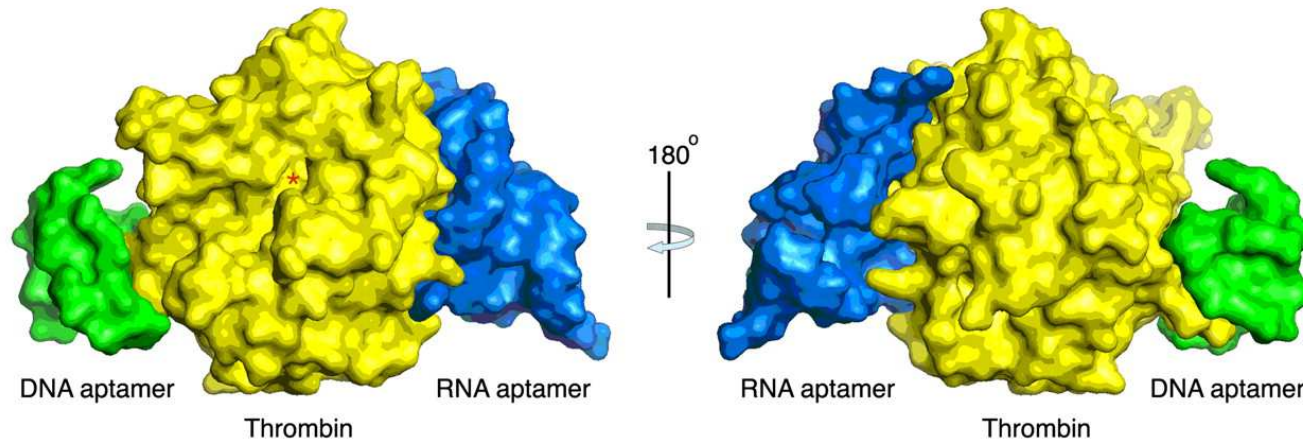


	P4	P3	P2	P1	P1'	P2'	P3'
Fibrinogen (A)	Gly	Gly	Val	Arg	Gly	Pro	Arg
Fibrinogen (B)	Phe	Ser	Ala	Arg	Gly	His	Arg
FV (709)	Leu	Gly	Ile	Arg	Ser	Phe	Arg
FV (1018)	Leu	Ser	Pro	Arg	Thr	Phe	His
FV (1545)	Trp	Tyr	Leu	Arg	Ser	Asn	Asn
FVIII (372)	Ile	Gln	Ile	Arg	Ser	Val	Ala
FVIII (740)	Ile	Glu	Pro	Arg	Ser	Phe	Ser
FVIII (1689)	Gln	Ser	Pro	Arg	Ser	Phe	Gln
FXIII	Gly	Val	Pro	Arg	Gly	Val	Asn
PAR1	Leu	Asp	Pro	Arg	Ser	Phe	Leu
PAR4	Pro	Ala	Pro	Arg	Gly	Tyr	Pro
FXI	Ile	Lys	Pro	Arg	Ile	Val	Gly
PC	Val	Asp	Pro	Arg	Leu	Ile	Asp
TAFI	Val	Ser	Pro	Arg	Ala	Ser	Ala
AT	Ile	Ala	Gly	Arg	Ser	Leu	Asn

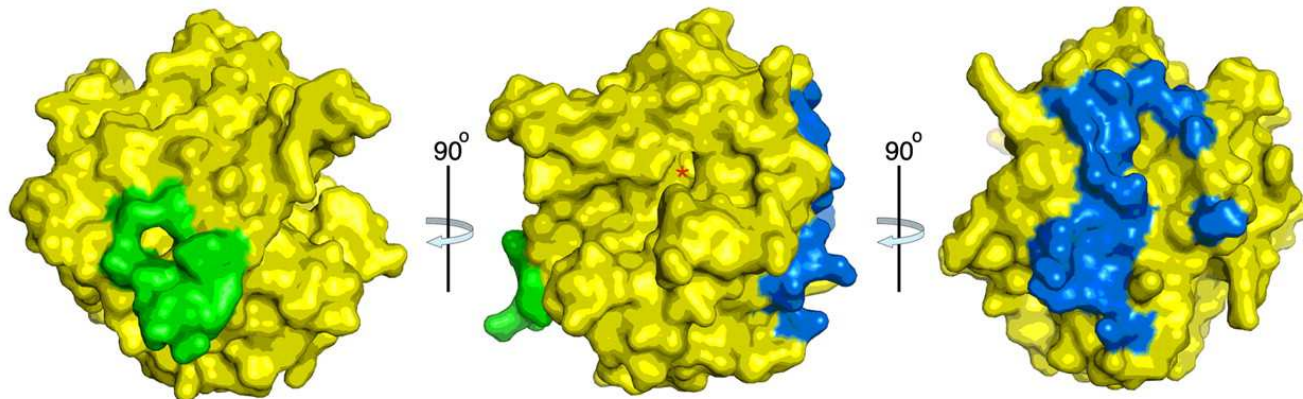
# Exosites are good targets to inhibit specific functions of thrombin

A

\*Active site



B



Nimjee S M et al. *RNA* 2009;15:2105-2111

be briefly stated as follows: The staphylococcus pyogenes aureus has a specific influence in causing coagulation of the blood. Bouillon cultures of the staphylococcus were much more potent than any one of the other organisms. The

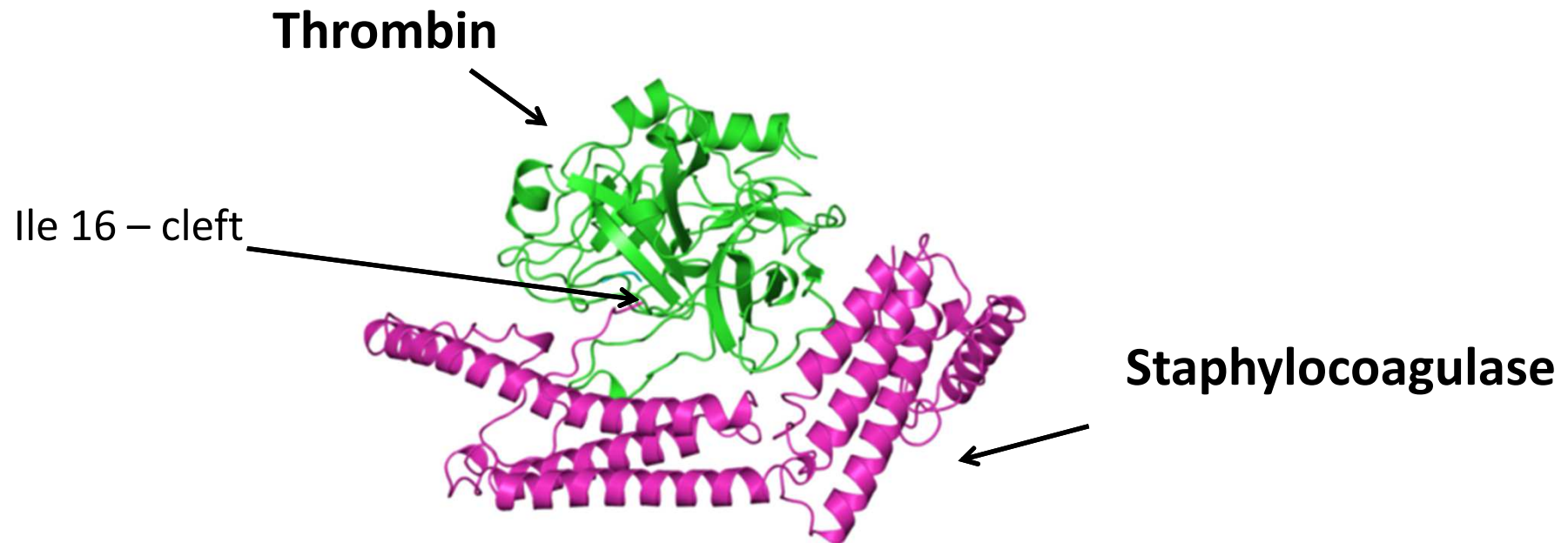
- Certain strains of *Staphylococcus Aureus* trigger coagulation (1903)
- Isolation of a bacterial agent that specifically activates thrombin: Staphylocoagulase (1970)
- SC does not cleave thrombin, No cleavage between Arg<sup>15</sup>-Ile<sup>16</sup>

*How is that possible???*

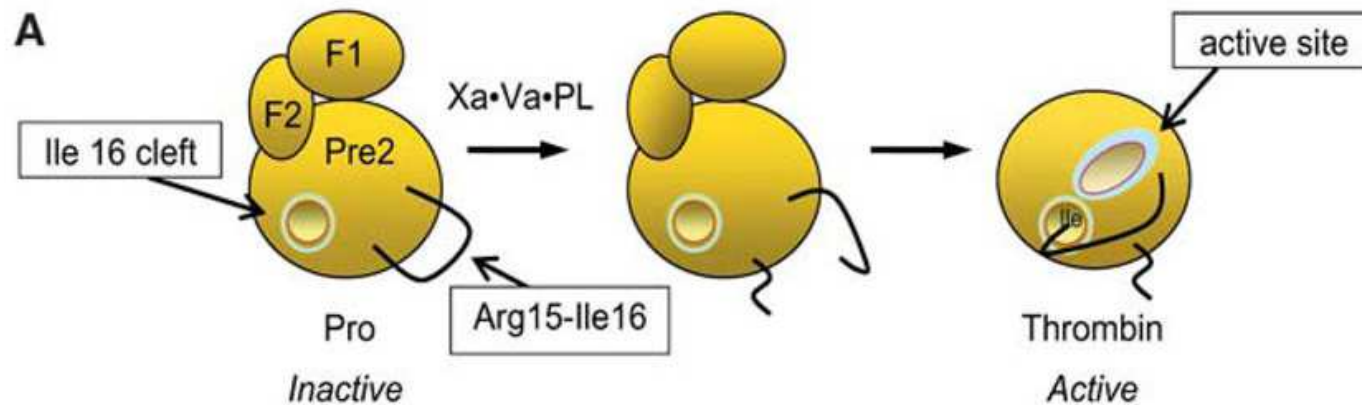
# MECCANISMO ATTIVAZIONE BATTERICA

# Staphylocoagulase (SC) X ray-structure

In 2003 crystal structure of (Pre2)Thrombin-bound Staphylocoagulase was published (Friedrich, et al. *Nature*, 2003)

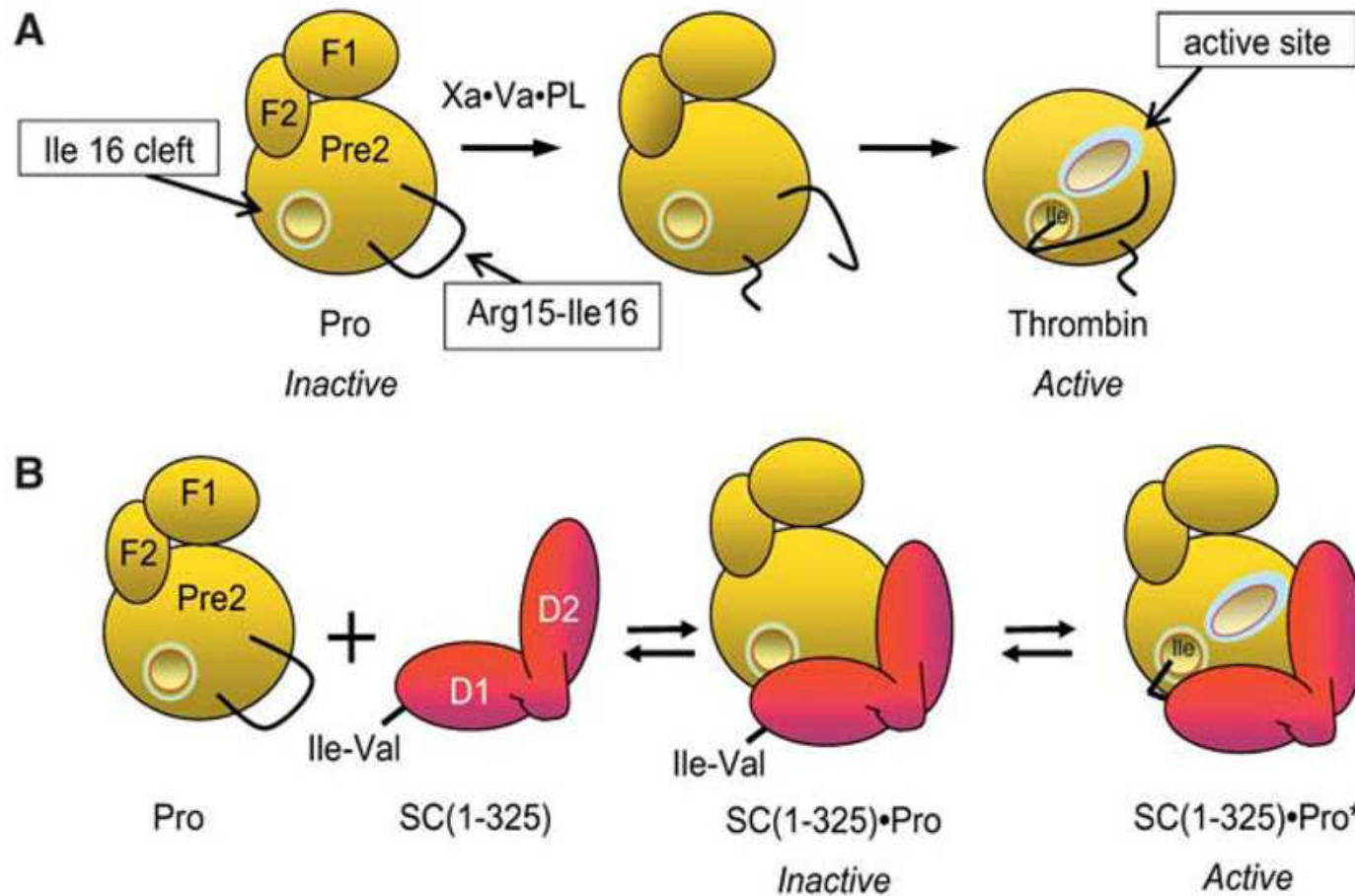


# Non-Proteolytic Activation of Prothrombin by Staphylocoagulase support for the “Molecular Sexuality” Hypothesis



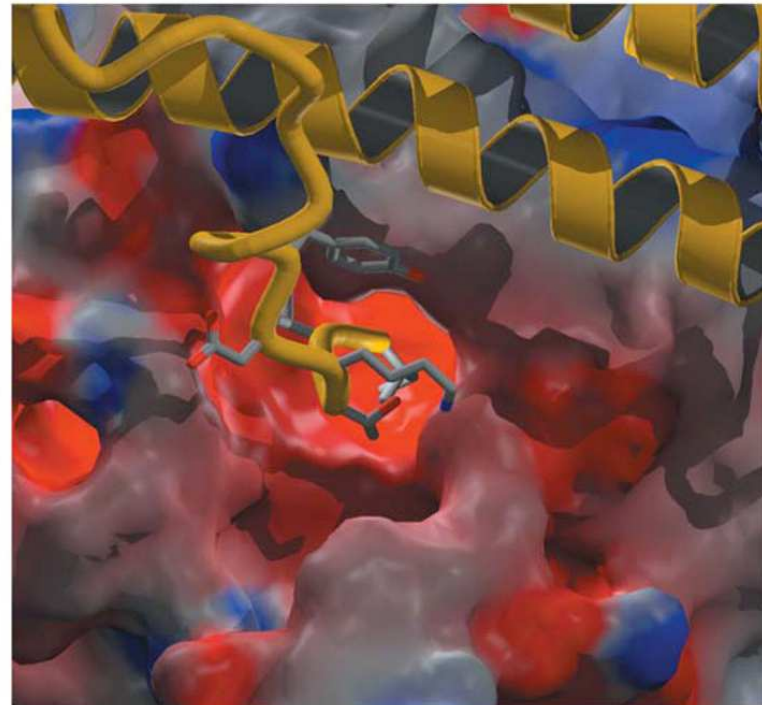
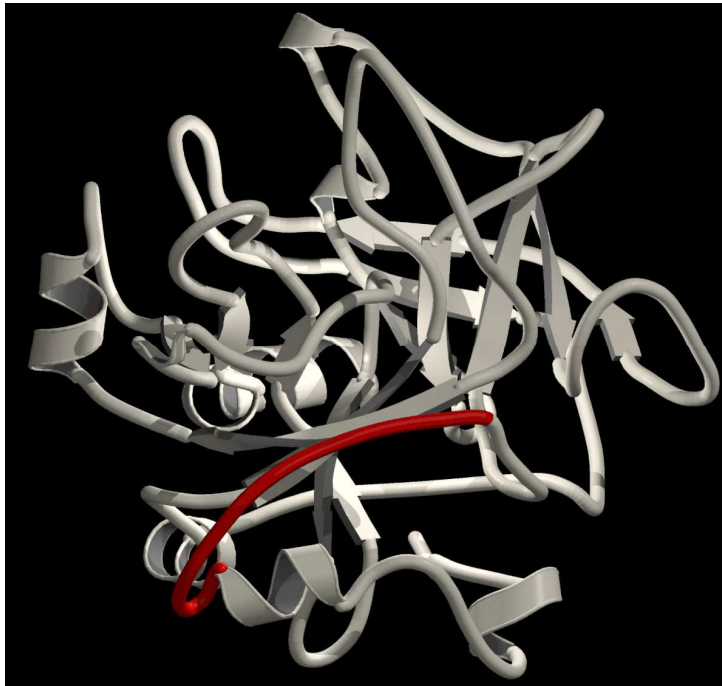


# Non-Proteolytic Activation of Prothrombin by Staphylocoagulase support for the “Molecular Sexuality” Hypothesis





The observed insertion of the SC N-terminus into the Ile<sup>16</sup> cleft of prethrombin 2, which triggers the activating conformational change, provided the first unambiguous structural evidence for the **Molecular Sexuality** mechanism of non-proteolytic zymogen activation.

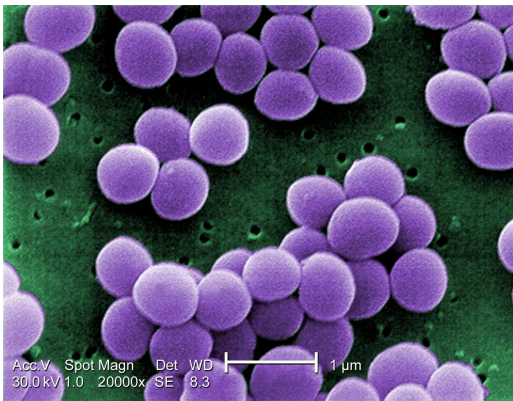


# ***S. Aureus* causes Endocarditis**

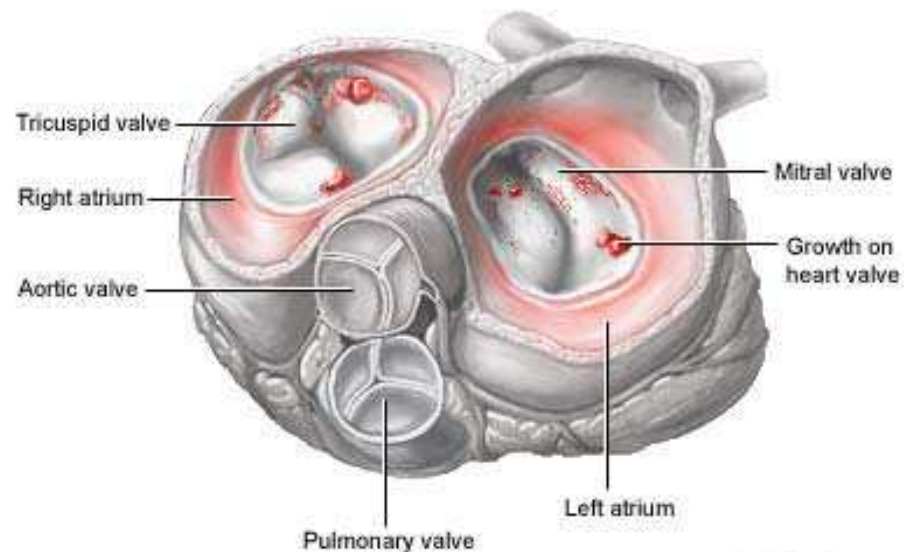
- Severe infection of the heart valves
- More than 50% of patients dies within days or weeks despite treatment
- Difficult diagnosis
  - new heart murmur, fever and the detection of circulating bacteria in blood cultures
- Coagulase-positive *S. aureus* causes 40–50% of neonatal endocarditis and 30–40% of endocarditis in adults

# Acute bacterial endocarditis is characterized by vegetations on heart valves consisting of bacteria, platelets and fibrin

*S. Aureus*



Infective endocarditis is an infection of the heart chambers or valves



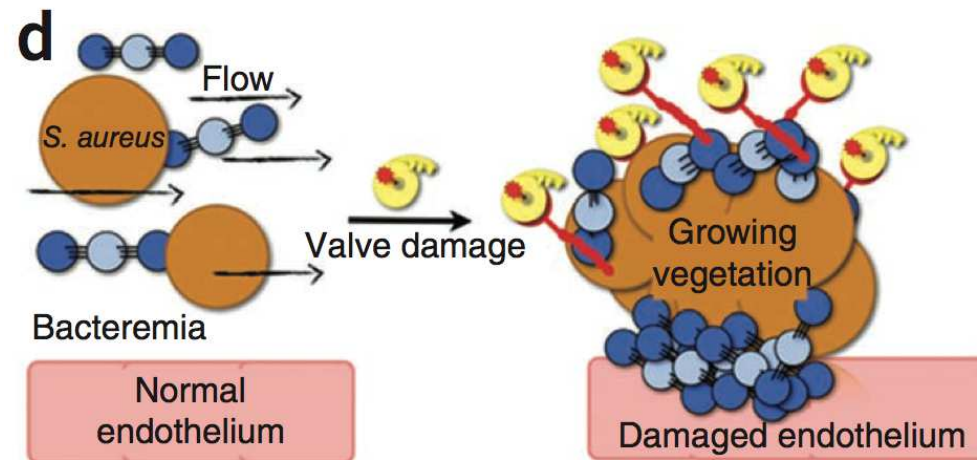
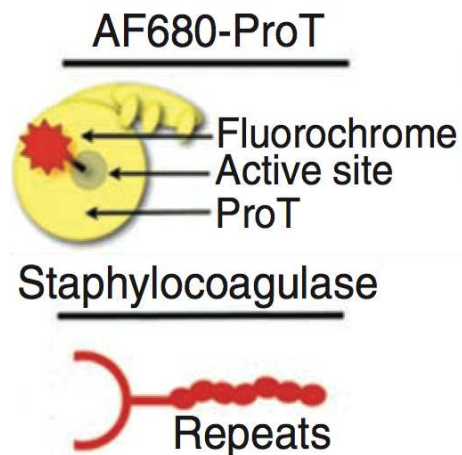
- Growth and fortification of the vegetation by SC-induced fibrin deposition protects the bacteria in the vegetation from clearance by leukocytes and macrophages
- Heart valves are not easily accessible to the immune system

*In vivo* detection of *Staphylococcus aureus*  
endocarditis by targeting pathogen-specific  
prothrombin activation

Peter Panizzi<sup>1,2,9</sup>, Matthias Nahrendorf<sup>1,9</sup>, Jose-Luiz Figueiredo<sup>1</sup>, Jennifer Panizzi<sup>3</sup>, Brett Marinelli<sup>1</sup>,  
Yoshiko Iwamoto<sup>1</sup>, Edmund Keliher<sup>1</sup>, Ashoka A Maddur<sup>4</sup>, Peter Waterman<sup>1</sup>, Heather K Kroh<sup>4</sup>, Florian Leuschner<sup>1</sup>,  
Elena Aikawa<sup>1</sup>, Filip K Swirski<sup>1</sup>, Mikael J Pittet<sup>1</sup>, Tilman M Hackeng<sup>5</sup>, Pablo Fuentes-Prior<sup>6</sup>, Olaf Schneewind<sup>7</sup>,  
Paul E Bock<sup>4</sup> & Ralph Weissleder<sup>1,8</sup>

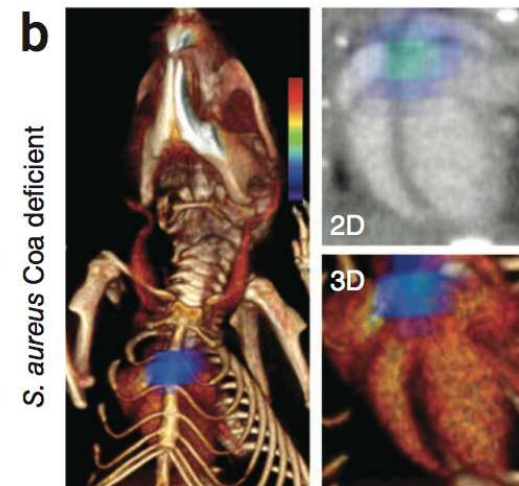
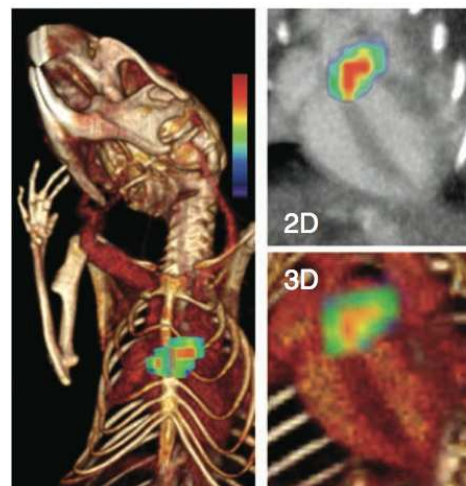
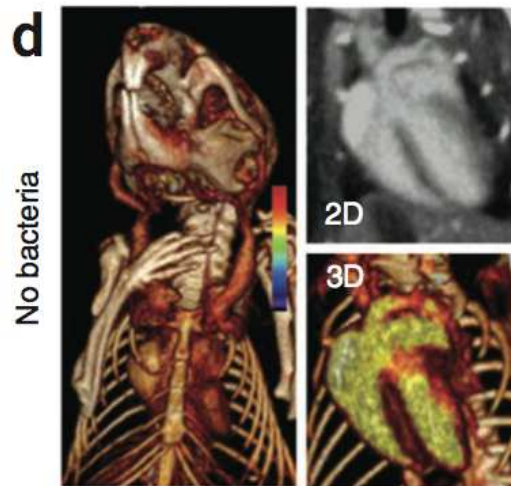
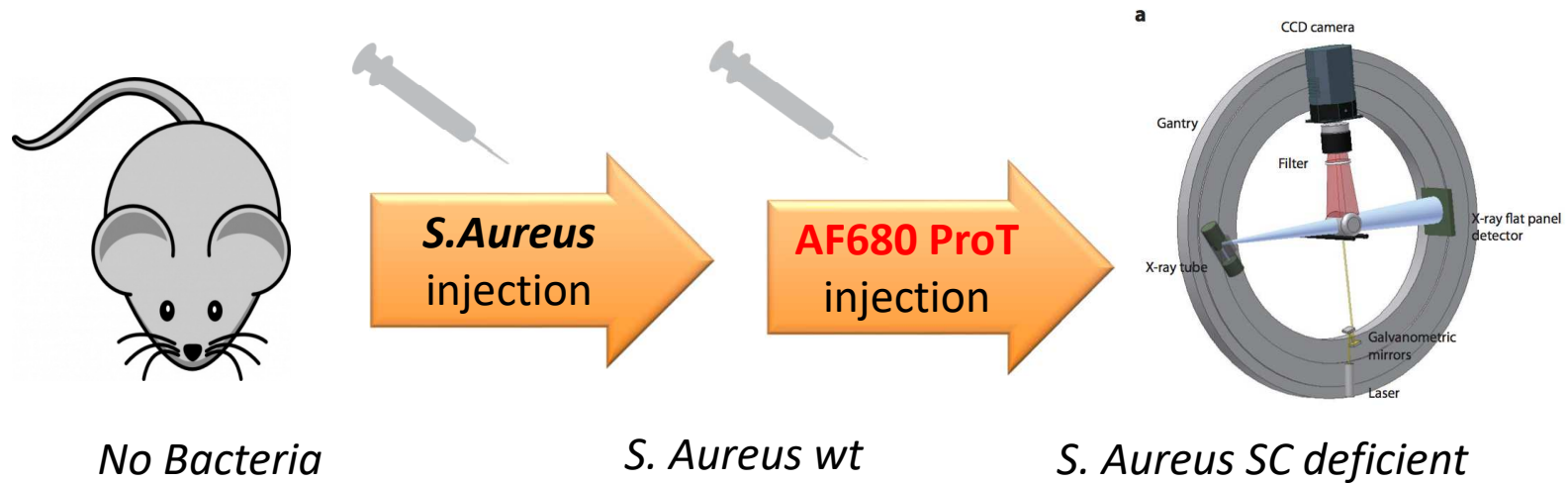
# SC Prothrombin as a probe for *S. Aureus*

- SC binds prothrombin with high affinity and activates it through a conformation change
- SC-Prothrombin complex clots fibrinogen but is impervious to physiologic thrombin inhibitors.
- SC-Prothrombin is present in the vegetation
- Labeled Prothrombin can be used as a probe to detect bacterial vegetation in the heart





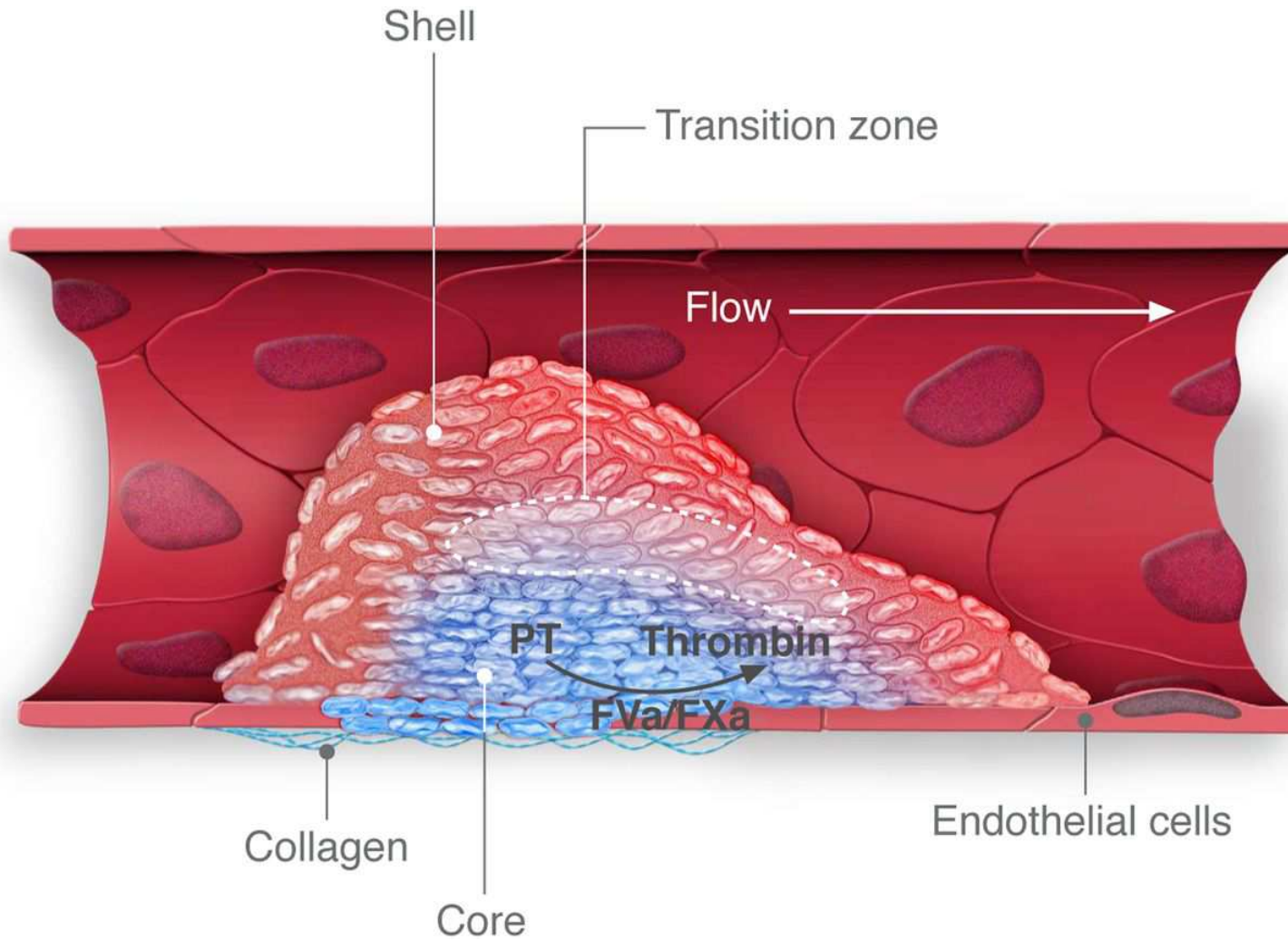
# Fluorescent prothrombin co-localise with SC positive bacteria



# Conclusion

- Zymogen activation requires conformational changes and maturation of the active site. This can be achieved even in the absence of canonical proteolysis.
- Exosite-Substrate interactions determine enzyme specificity.
- AF680ProT detects *S.Aureus* in vivo and can be used as a diagnostic tool to determine site, bacterial load and activity of the infection.

## Thrombus formation following penetrating injury.



Robert Flaumenhaft *Blood* 2014;124:1697-1698



# Bibliography

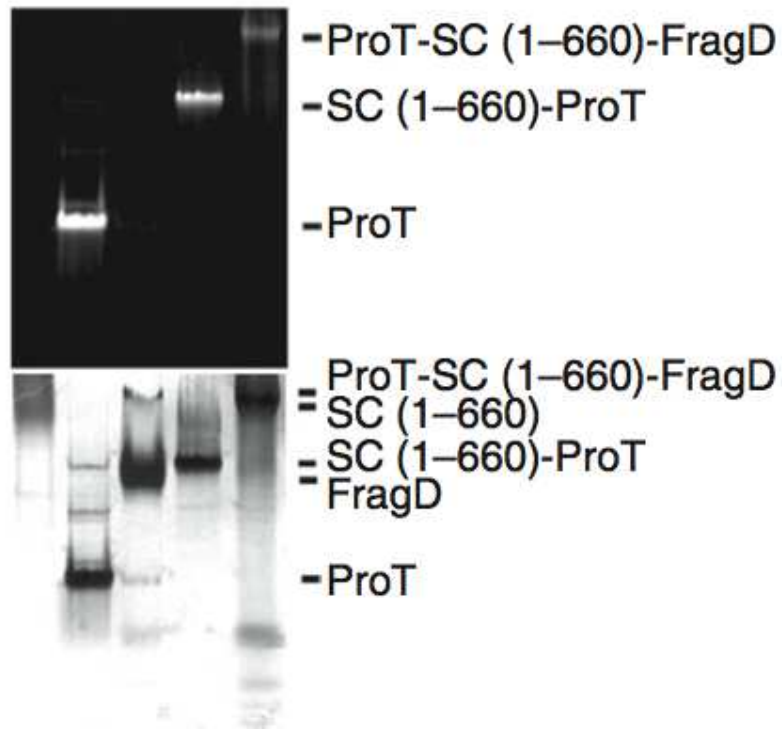
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# Visualisation of *S. Aureus* in vivo using Near Infrared Imaging

## The PROBE

**AF680**- Prothrombin



## The DETECTOR

**Fluorescence molecular tomography**  
- Computer Tomography

