Serine Proteases of Physiological Importance

<table>
<thead>
<tr>
<th>Protease</th>
<th>Cleavage Site</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>Arg-X, Lys-X</td>
<td>Digestion</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>Tyr-X, Phe-X, Trp-X</td>
<td>Digestion</td>
</tr>
<tr>
<td>Elastase</td>
<td>Val-X</td>
<td>Tissue degradation</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Arg-Gly</td>
<td>Blood coagulation</td>
</tr>
<tr>
<td>Factor Xa</td>
<td>Arg-Ile, Arg-Gly</td>
<td>Blood coagulation</td>
</tr>
</tbody>
</table>

Other Important Serine Proteases:
- Tryptase
- Lipases
- Phospholipases
- Subtilisin
Catalytic Mechanism of Serine Proteases

Asp            His              Ser

Substrate

"oxygen anion hole"

"tetraedric transition state"

Ser

acyl-enzyme complex

products
Dissection of the Catalytic Triad of Subtilisin
(substrate: N-succinoyl-L-Ala-L-Ala-L-Pro-L-Phe-\(p\)-nitroanilide).

<table>
<thead>
<tr>
<th>Subtilisin, wild type and mutants</th>
<th>(K_m) ((\mu)M)</th>
<th>(k_{cat}/K_m) (s(^{-1})mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp AA 32, Ala AA 64, Ser AA 221</td>
<td>220</td>
<td>250 000</td>
</tr>
<tr>
<td>Ala AA 32, His AA 64, Ser AA 221</td>
<td>480</td>
<td>5</td>
</tr>
<tr>
<td>Asp AA 32, Ala AA 64, Ser AA 221</td>
<td>390</td>
<td>0.1</td>
</tr>
<tr>
<td>Asp AA 32, His AA 64, Ala AA 221</td>
<td>420</td>
<td>0.1</td>
</tr>
<tr>
<td>Ala AA 32, Ala AA 64, Ala AA 221</td>
<td>330</td>
<td>0.1</td>
</tr>
</tbody>
</table>

uncatalysed reaction: \(k_{Ala,Ala,Ala} \approx 0.0003\)

Why is the Ala-Ala-Ala mutant still enzymatically active?

Substrate-Assisted Catalysis in a Subtilisin His64Ala Mutant

Subtilisin cleaves structurally different peptide substrates. The His64Ala mutant cleaves XXX-His-XXX peptides 4 orders of magnitude slower than wild-type subtilisin but about 200 times faster than substrates without His.

Convergent Evolution

His
Asp
Ser
Convergent Evolution of Serine Proteases

Asp, His and Ser are the amino acids of the catalytic triad. Ser, Leu/Trp and Gly interact with the substrate.

J. D. Robertus et al., Biochemistry 11, 2439-2449 (1972)
Protease Binding Pockets for a Peptide Substrate

Comparison of the P₁ Pockets of Trypsin, Chymotrypsin and Elastase

The binding pockets of trypsin and thrombin accommodate positively charged amino acid side chains by the negatively charged Asp 189. The P₁ pocket of chymotrypsin is designed for large, lipophilic side chains. Elastase has a relatively small lipophilic P₁ pocket; it binds only small hydrophobic amino acids, like alanine and valine.
Specificity of a Trypsin Asp189Lys Mutant

Trypsin, wild-type enzyme
Hugo Kubinyi, www.kubinyi.de

Trypsin Asp189Lys Mutant

Asp 189

Gly 216

Gly 226

Lys 189

What is the reason for this surprising selectivity?

Hugo Kubinyi, www.kubinyi.de
Trypsin and Thrombin Show Different Substrate Selectivity

open pocket, non-selective  closed pocket, selective

Blood Coagulation

Intrinsic pathway
F XII
F XI
F IX
F X
Thrombin
Fibrin
F XIII
Thrombus

Extrinsic pathway
F VIII
Fibrinolysis
Plasmin
Plasminogen
Relative Inhibitory Activities of Tripeptide Aldehydes vs. Thrombin

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Relative inhibitory activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Val-Arg-H</td>
<td>1</td>
</tr>
<tr>
<td>Gly-Pro-Arg-H</td>
<td>9</td>
</tr>
<tr>
<td>Phe-Pro-Arg-H</td>
<td>57</td>
</tr>
<tr>
<td>D-Ala-Pro-Arg-H</td>
<td>469</td>
</tr>
<tr>
<td>D-Val-Pro-Arg-H</td>
<td>1273</td>
</tr>
<tr>
<td>D-Phe-Pro-Arg-H</td>
<td>7370</td>
</tr>
</tbody>
</table>

Arg-H = arginine aldehyde

Why is the D-Phe analog more active than the L-Phe analog?
Affinity of Inhibitors to Thrombin and Trypsin

\[
\begin{align*}
K_i (\text{thrombin}) &= 220 \ \mu\text{M} \\
K_i (\text{trypsin}) &= 35 \ \mu\text{M}
\end{align*}
\]

\[
\begin{align*}
K_i (\text{thrombin}) &= 150 \ \mu\text{M} \\
K_i (\text{trypsin}) &= 360 \ \mu\text{M}
\end{align*}
\]

Benzamidine binds specifically to trypsin, whereas N-amidino-piperidine has a slightly higher specificity for thrombin.

Thrombin 189-200: DACEGDSGGPFV
Trypsin 189-200: DSCQGDSGGPVV

Hemiketal Formation of Phenylpyruvic Acid

p-Amidinophenyl-pyruvic acid (1AHT)
Groups that Covalently Interact With the Catalytically Active Serine

<table>
<thead>
<tr>
<th>Inhibitor Type</th>
<th>Functional Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irreversible</td>
<td></td>
</tr>
<tr>
<td>Inhibitors</td>
<td>Chloromethylketones -COCH₂Cl</td>
</tr>
<tr>
<td></td>
<td>Sulfonylfluorides -SO₂F</td>
</tr>
<tr>
<td></td>
<td>Esters -COOR</td>
</tr>
<tr>
<td></td>
<td>Boronic Acids -B(OR)₂</td>
</tr>
<tr>
<td>Reversible</td>
<td></td>
</tr>
<tr>
<td>Inhibitors</td>
<td>Aldehydes -CHO</td>
</tr>
<tr>
<td></td>
<td>Arylketones -CO-Aryl</td>
</tr>
<tr>
<td></td>
<td>Trifluoromethylketones -COCF₃</td>
</tr>
<tr>
<td></td>
<td>Ketocarboxylic acids -COCOOH</td>
</tr>
</tbody>
</table>

"Classical" Thrombin Inhibitors

R = H
\[ K_d = 180 \text{ nM} \]

R = CHO
D-Phe-Pro-Arg-H
\[ K_d = 1.8 \text{ nM} \]
(reversible covalent inhibitor)

R = COCH₂Cl
(irreversible covalent inhibitor)
1DWE, 1PPB,
(cf. 1Al8, 1AlX, 1HAI)
"Classical" Thrombin Inhibitors

- D-NAPAP (1DWD, 1ETS)
  \[ K_i = 2.1 \text{ nM} \]

- meta: 3-TAPAP

- para: 4-TAPAP (1ETT)

Argatroban, MD-805
(1DWC, 1ETR)
\[ K_i = 19 \text{ nM} \]

NAPAP-Thrombin Complex

1DWD
Binding Mode of NAPAP (1DWD)

Hydrophobic P3 pocket
Hydrophobic P2 pocket
P1 pocket

Gly-216
Asp-189

Binding Modes of Thrombin Inhibitors

("hydrophobic collapse")

NAPAP (1DWD)
Argatroban (1DWC)
Binding Modes of Different Thrombin Inhibitors

(coordinates from X-ray structure analyses and from docking)