Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2023

Lecture 12

Serine Proteases, Burst Substrates and COVID-19

16 February 2023

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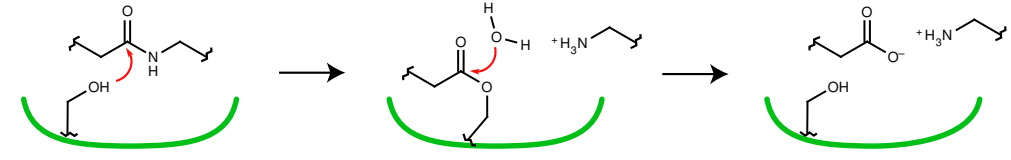
Serine Proteases Employ a Two-Step Mechanism

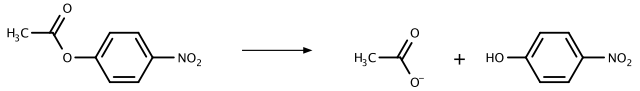


Enzyme-Substrate Complex | Acyl Intermediate | Enzyme + Products

- In step 1, a serine hydroxyl is the nucleophile.
- In step 2, a water molecule is the nucleophile.
- Both steps require activation by a base.
- Both steps require an acid to protonate the leaving group.

Why Do We Think That There Is a Covalent Intermediate?

- An historically important experiment:

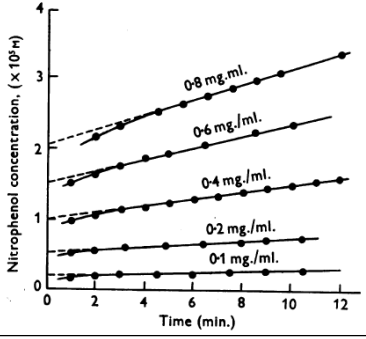


p-nitrophenyl acetate (PNPA) | acetate | *p*-nitrophenol

$\epsilon_{402} = 16,600\,\text{cm}^{-1}\text{M}^{-1}$

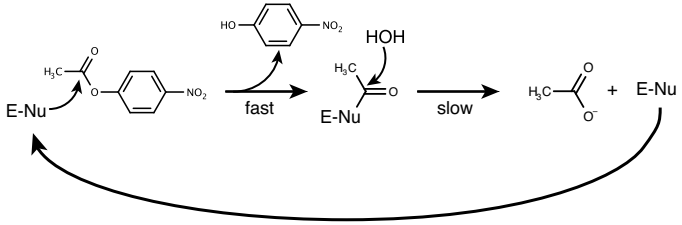
Reaction with chymotrypsin:

- PNPA isn't a very good substrate!
- Reactions are not linear with time.
- Initial displacement of curves increases with enzyme concentration.
- What is going on?



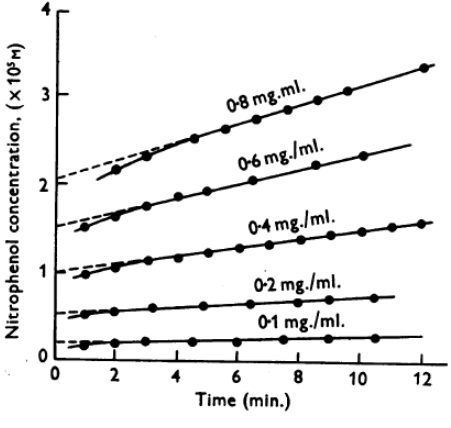
Hartley, B. S. & Kilby, B. A. (1954). The reaction of *p*-nitrophenyl esters with chymotrypsin and insulin. *Biochem. J.*, 56, 288–297. http://dx.doi.org/10.1042/bj0560288

Proposed Mechanism to Explain “Burst Kinetics”



- Enzyme reacts rapidly with substrate to form a covalent intermediate and releases *p*-nitrophenol.
- Each molecule of enzyme produces one molecule of product in burst phase.
- Hydrolysis of the covalent intermediate is much slower and is required to regenerate enzyme (turnover).
- Steady state rate of product formation is determined by the second step.
- Among the first evidence for a covalent intermediate in an enzyme-catalyzed reaction.

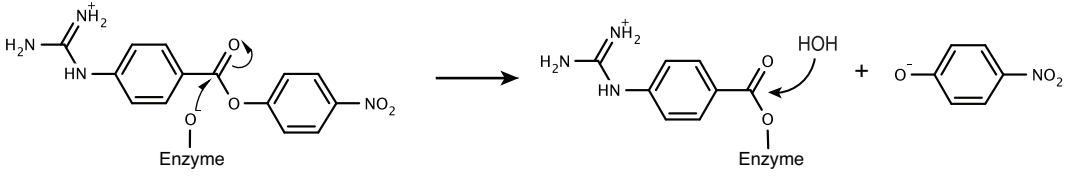
Burst Substrates Can be Used to Measure Active Enzyme Concentration



- Extrapolated product concentration should equal the enzyme concentration.

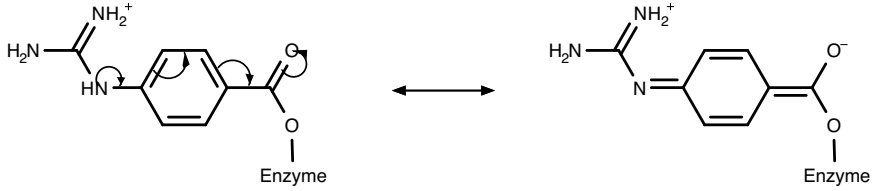
A Designed Burst Substrate for Trypsin

p-nitrophenyl-*p'*-guanido benzoate



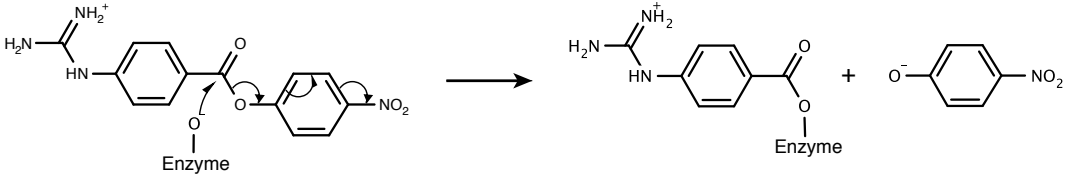
- Guanido group resembles arginine side chain.
- Hydrolysis of covalent intermediate is very slow.
  (half-time greater than $\approx 40\,\mathrm{h}$)
- An (almost) irreversible inhibitor, or “suicide substrate”.
- Why is hydrolysis so slow?

Resonance Stabilization of the Covalent Intermediate



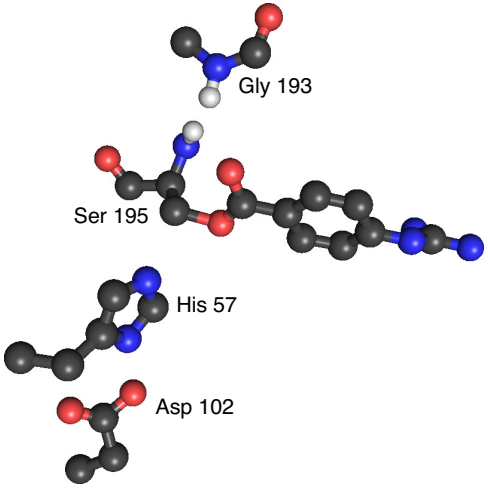
- Resonance structures shift electrons from phenyl ring to carbonyl carbon.
- Electron density on carbonyl carbon disfavors nucleophilic attack by water.
- Why doesn't the same effect prevent *formation* of the intermediate?

Resonance Also Favors Formation of the Covalent Intermediate



- Resonance shifts electrons from carbonyl carbon to nitrophenol ring.
- Makes the nitrophenol group a better leaving group.
- *Favors* nucleophilic attack in the first step of the reaction.

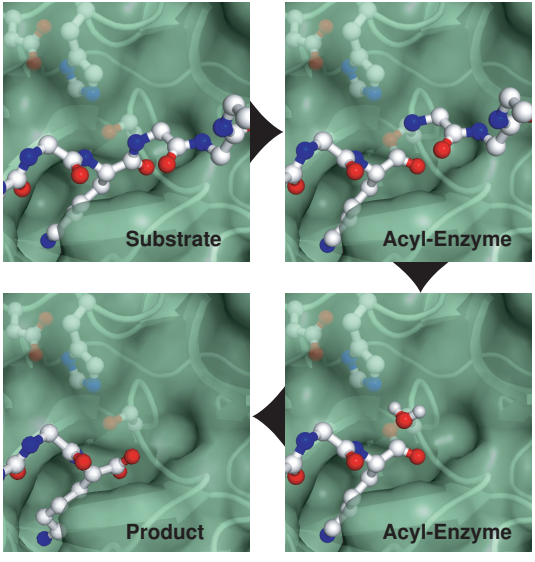
Three-dimensional Structure of *p*-guanido benzoate intermediate



- As predicted, Ser 195 O_γ forms ester with *p*-guanido benzoate.
- Amide groups of Ser 195 and Gly 193 form "oxyanion hole" that stabilizes negative charge on carbonyl oxygen in transition state.
- Where would the water molecule be for hydrolysis of the intermediate?

Mangel, W. F., Singer, P. T., Umland, T. C., Toledo, D. L., Stroud, R. M., Pflugrath, J. W. & Sweet, R. M. (1990). Structure of an acyl-enzyme intermediate during catalysis:(guanidobenzoyl)trypsin. *Biochemistry*, 29, 8351–8357. http://dx.doi.org/10.1021/bi00488a022

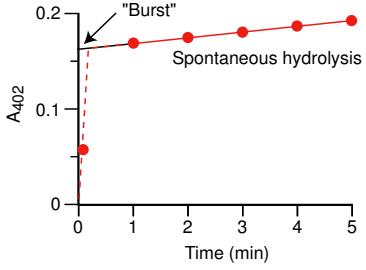
Reconstruction of the Serine Protease Mechanism



- Models built from crystal structures.
  PDB entries: 2FTL, 2AGE and 3FP7.
- Very little motion is required for the catalytic reaction.

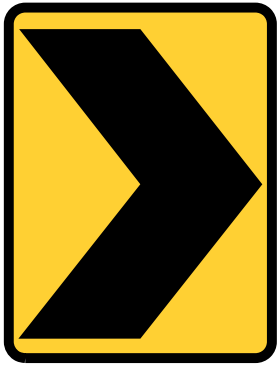
Zakharova, E., Horvath, M. P. & Goldenberg, D. P. (2009). Structure of a serine protease poised to resynthesize a peptide bond. *Proc. Natl. Acad. Sci., USA*, 106, 11034–11039.
http://dx.doi.org/10.1073/pnas.0902463106

Experiment 3, Part C:
Measurement of Trypsin Concentration with Burst Substrate



- Requires high enzyme concentration, since each enzyme molecule generates only one chromophore molecule.
- Tris buffer is bad for this experiment, because amines are nucleophilic and react with *p*-NPGB.
- Need to record both the absolute increase in absorbance and the rate of steady-state increase, in order to extrapolate initial burst phase.
- May need to ignore the first data point, since it is recorded slightly after reaction starts, but possibly before the burst is complete.

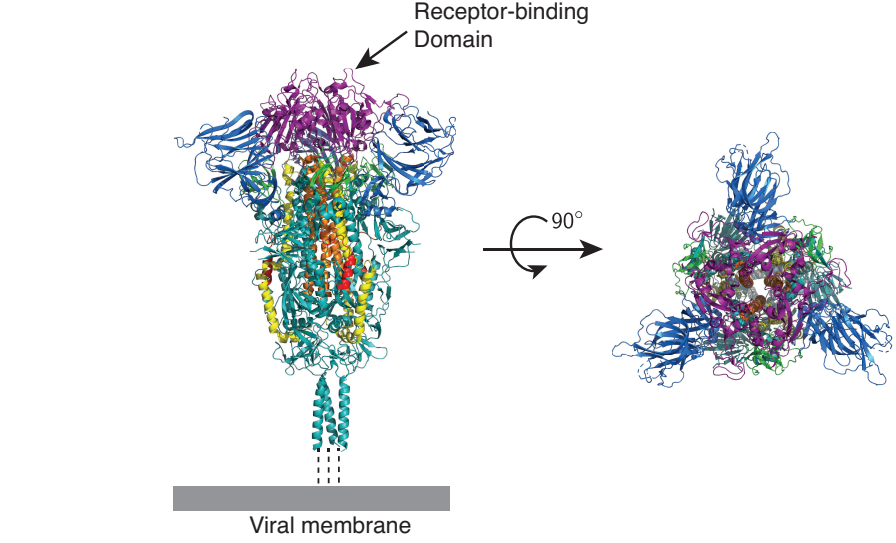
Warning!



Direction Change

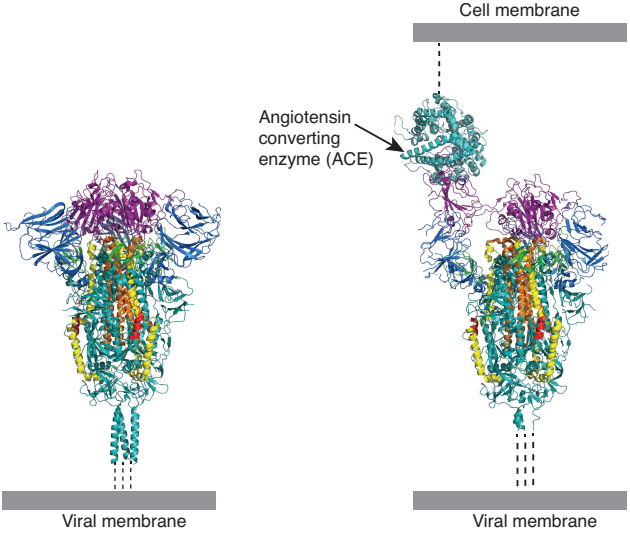
The COVID-19 Connection

Structure of SARS-CoV-2 Spike



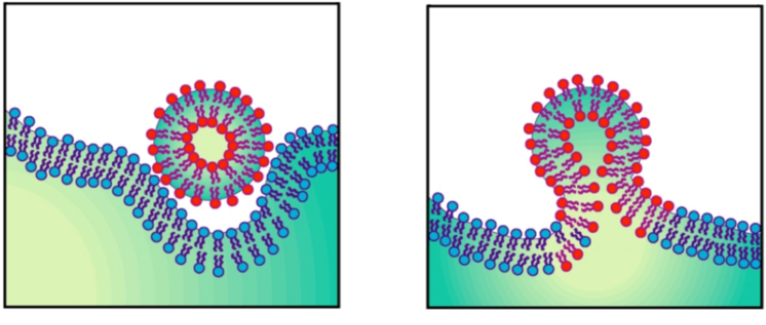
Cai, Y., Zhang, J., Xiao, T., Peng, H., Sterling, S. M., Walsh Jr., R. M., Rawson, S., Rits-Volloch, S. & Chen, B. (2020). *Science*, 369, 1586–1592. http://doi.org/10.1126/science.abd4251, PDB entry 6XR8

Structure of the Spike Bound to its Receptor



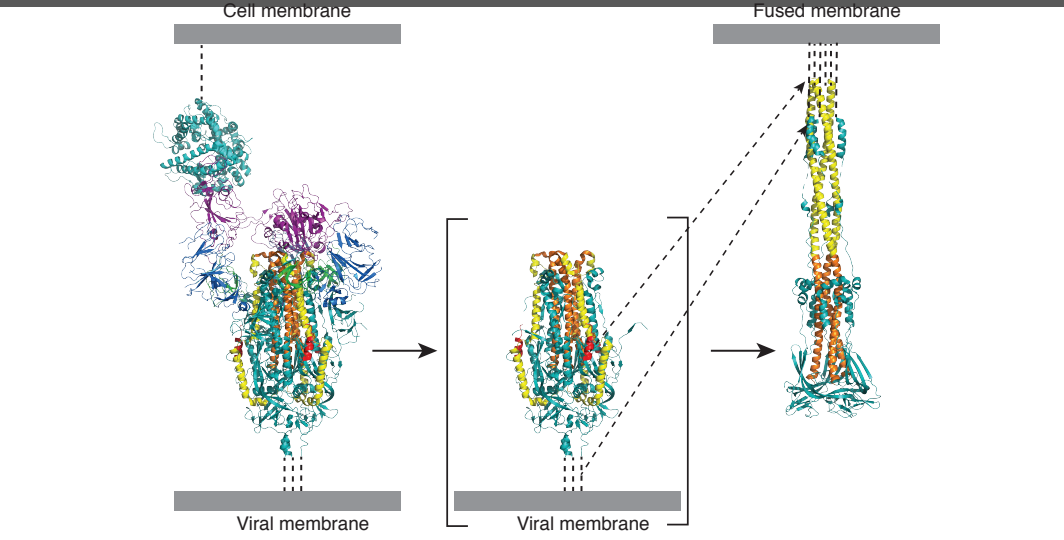
ACE-spike complex: Zhou, T., Tsybovsky, Y., Gorman, J., Rapp, M., Cerutti, G., Chuang, G.,*et al.*. *Cell Host & Microbe*, 28, 867–879. https://doi.org/10.1016/j.chom.2020.11.004, PDB entry: 7KNB.

How Do the Viral and Cell Membranes Fuse?



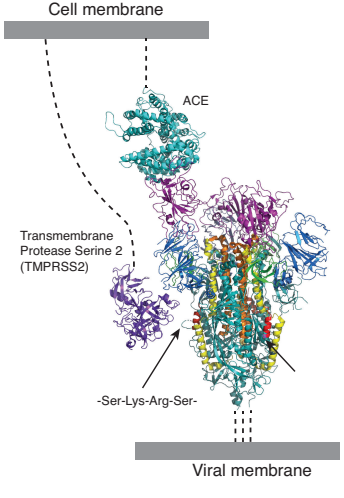
Drawing from laboratory of Peter S. Kim, Stanford University, https://peterkimlab.stanford.edu/

Conversion of the Spike to the Post-fusion State



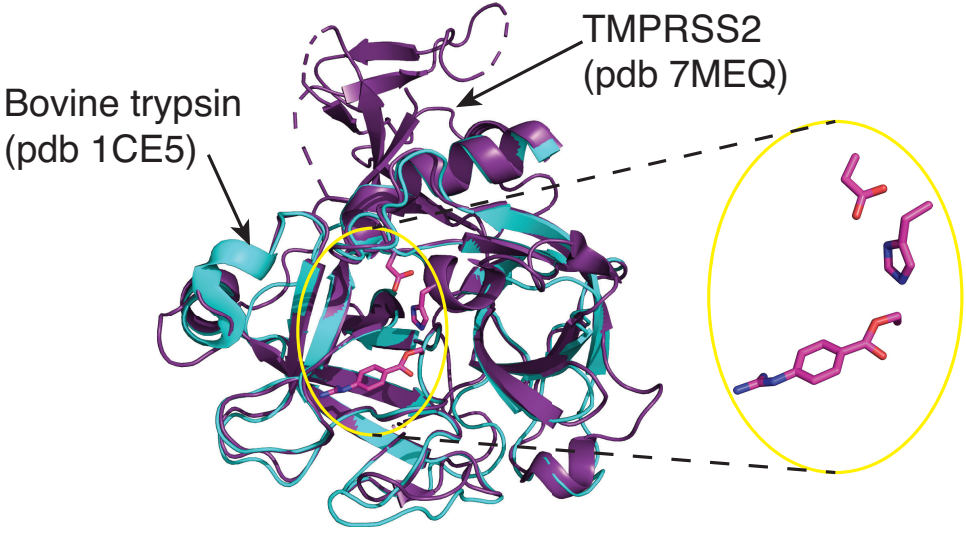
Post-fusion structure: Cai, Y., Zhang, J., Xiao, T., Peng, H., Sterling, S. M., Walsh Jr., R. M., Rawson, S., Rits-Volloch, S. & Chen, B. (2020). *Science*, 369, 1586–1592. http://doi.org/10.1126/science.abd4251, PDB entry 6XRA

Cleavage of the Spike by TMPRSS2



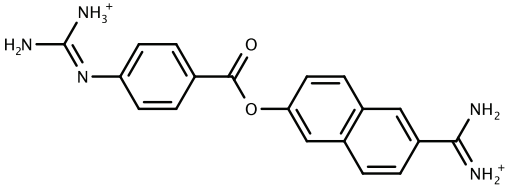
TMPRSS2 structure, PDB entry 7MEQ:
Fraser, *et al.* (2022). Structure and activity of human TMPRSS2 protease implicated in SARS-CoV-2 activation. *Nature Chemical Biology*, 18, 963–971.
https://doi.org/10.1038/s41589-022-01059-7

TMPRSS2 and Trypsin

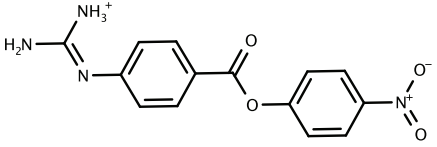


Nafamostat: A Potential Antiviral Agent

Nafamostat



4-nitrophenyl-4-guanidobenzoate



- Forms stable guanidobenzoate esters with active-site serine of trypsin, TMPRSS2 and other serine proteases, just like *p*-NGPB.
- Approved in Japan and used for pancreatitis, and as anti-coagulant.
- Shown to inhibit SARS-CoV-2 viral entry in vitro.
- Was being tested as a treatment for COVID-19 in 2021.
- Some differences among variants may be due to differences in ability of TMPRSS2 to cleave the spike.

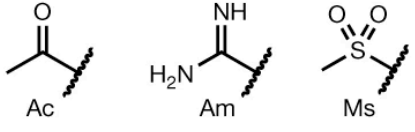
Hoffmann, M., Kleine-Weber, H., Schroeder, S., Krüger, N., Herrler, T., Erichsen, S., Schiergens, T. S., Herrler, G., Wu, N.-H., Nitsche, A., Müller, M. A., Dorsten, C. & Pöhlmann (2020). SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor. *Cell*, 181, 271–280.

Ketobenzothiazole-based Inhibitors of TMPRSS2

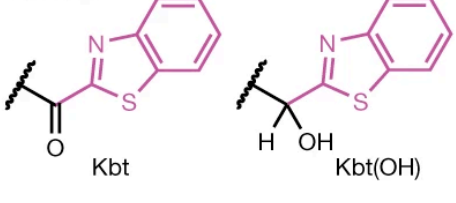
a

Compound	Peptide sequence
N-0100	Arg-Gln-Ala-Arg-kbt
N-0130	(H)Arg-Gln-Phe-Arg-kbt
N-0438	(H)Arg-Glu-Phe-Arg-kbt
N-0678	(H)Arg-Gln-Cha-Arg-kbt
N-0676	Ac-Gln-Cha-Arg-kbt
N-0386	Ac-Gln-Phe-Arg-kbt
N-1296	Am-Gln-Phe-Arg-kbt
N-0385	Ms-Gln-Phe-Arg-kbt
N-0385(OH)	Ms-Gln-Phe-Arg-kbt(OH)

N-terminal caps

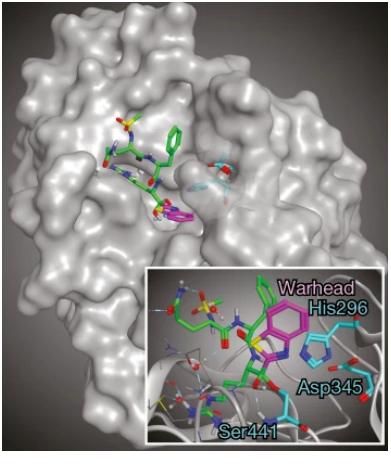


Warhead



Shapira, T. *et al.* (2022). A TMPRSS2 inhibitor acts as a pan-SARS-CoV-2 prophylactic and therapeutic. *Nature*, 605, 340–348.
https://doi.org/10.1038/s41586-022-04661-w

Model of Inhibited TMPRSS2



Shapira, T. *et al.* (2022). A TMPRSS2 inhibitor acts as a pan-SARS-CoV-2 prophylactic and therapeutic. *Nature*, 605, 340–348.
https://doi.org/10.1038/s41586-022-04661-w