OBSERVATION OF INHIBITORY THROMBIN-PPACK BINDING AND ALTERNATIVE THROMBIN CONFORMATION EQUILIBRIUM USING RAPID KINETICS AS A FUNCTION OF TEMPERATURE

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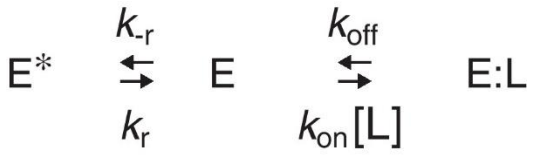
Abstract
The study of enzyme-ligand binding is useful for the understanding of enzyme structure and function. The family of trypsin-like serine proteases are a moderately sized and clinically relevant family of proteins for which to study this class of reaction. The transition between closed and open forms (due to movement of the beta 215-217 residues) of activated thrombin allows for a useful metric to measure PPACK binding via cessation of 215-217 swinging. Using stopped-flow, thrombin-PPACK temperature studies show a relationship between k12 and temperature, and additionally k21, kon and temperature. Thrombin-PPACK binding likely follows the theory of conformational selection, and activation energies and corresponding rate constants calculated by experimentally gathered stopped flow data help to provide more details about the model of equilibrium.

Keywords: thrombin conformation, thrombin inhibition,

Introduction

Enzymes are a broad class of macromolecules responsible for the regulation of chemical reactions throughout the body. They provide a significant source of study for the regulation of metabolic processes that occur throughout the body, as well as for the study of protein structure and function. Enzymes and their respective ligands exist in a state of equilibrium between free enzyme and ligand, and the enzyme-ligand complex. By studying the reaction rates and/or substrate and product concentrations of this type of reaction, information about the equilibrium such as activation energies can be gleaned. Regarding enzyme-ligand binding, there are two main competing theories of the order in which the enzyme conformation is altered for cases in which the model of lock-and-key is insufficiently descriptive. These theories are induced-fit and conformational selection. Induced-fit is characterized by the enzyme altering conformation to a more favorable state after ligand binding, while conformational selection is characterized by the enzyme adopting a more favorable state before binding to its ligand.[1] Pozzi et al. determined that trypsin-like proteases are accurately modeled by conformation where the enzyme changes conformation from one inaccessible to ligand to a conformation accessible to ligand. [2]

Figure 1. Shows Model of Conformational Selection



Trypsin-like proteases are a large group of enzymes present in a variety of metabolic systems in the body. They share a similarity

in that they both cleave peptide bonds following lysine or arginine residues.[2,3] One such trypsin-like protease for study is thrombin, also known as coagulation factor XIIa, which is responsible for the final step in thrombosis: the cleaving of fibrinogen into fibrin. In addition to its clinical relevance, thrombin is used in research studies for modification of proteins. After removal of the Gla and Kringle domains, activated thrombin exists in a state of equilibrium between the closed (E*) and open (E) conformations caused by access to the active site being hindered by beta 215-217 residues in the closed conformation, and unhindered in the open conformation.[2] This change in conformation can be measured through the wavelength given off by the Trp 215 residue once excited by a wavelength of 295nm.

The synthetic molecule PPACK serves as a tool for the observation of rate constants contributing to the conformational equilibrium of thrombin. PPACK irreversibly binds to the active site and prevents the beta 215-217 residues from moving, which can be observed to determine the inactivation rate of thrombin. From that, several rate constants where substrate binding, dissociation, and associated activation energies can be derived from temperature dependence of kcat/km and kcat, as determined by Ayala & Di Cera.[3] This experiment seeks to apply the temperature-dependent method of study established by Ayala & Di Cera to study thrombin-PPACK binding, and more specifically to determine how the change in temperature impacts the k12.

Methods

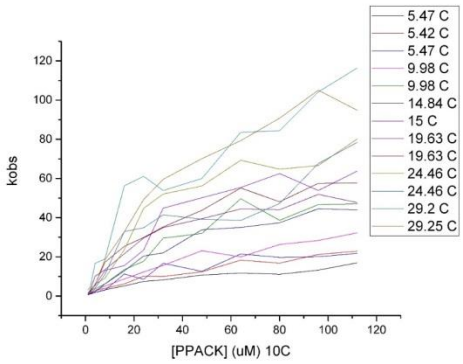
Materials

- Wild-type alpha thrombin, activated
- Buffer – 400mM ChCl, 50mM Tris, 0.1% PEG 8000, pH 8 at experimental temperature
- PPACK
- Applied Photophysics SX20 stopped-flow spectrometer

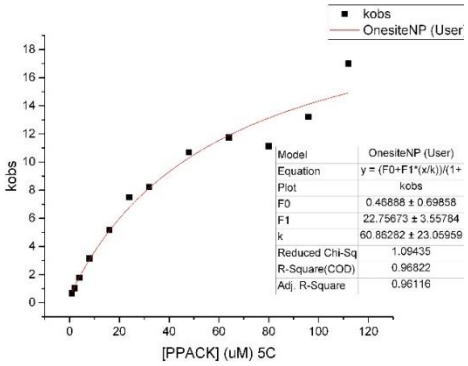
For each temperature study, a series of PPACK dilutions in buffer with a pH of 8 at incubation temperature was created with final concentrations of 1uM, 2uM, 4uM, 8uM, 16uM, 24uM, 32uM, 48uM, 64uM, 80uM, 96uM, and 112uM. A solution of activated wild-type thrombin was also created with a concentration of 250nM in buffer with a pH of 8 at incubation temperature. Solutions were incubated at the desired reaction temperature, then changed in fluorescence over a period of two seconds. The following reagent mixture was recorded for each sample. Protein blanks were recorded prior to reagent mixture for baseline. Each temperature study was completed in duplicate. Raw data was than fitted using single or double exponentials to find the initial velocity for each reaction, then each reaction was compiled for each temperature to find approximate k12 and Xm. Data was also entered into a custom computer program to find experimental Eon, E12, and E21 for calculation of kon, k12, and k21.

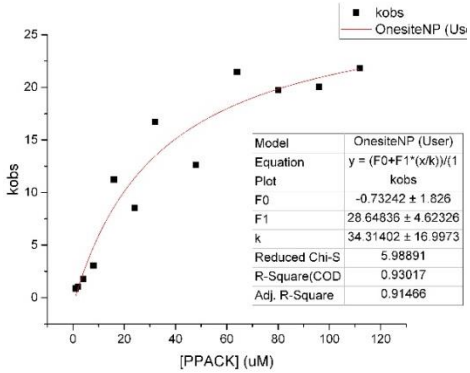
Results

Graph 1. Shows Thrombin-PPACK temperature studies fitted to equation $y=(F0+F1^{(x/k)})/(1+x/k)$. 250nM Thrombin, 400mM ChCl, 50mM Tris, 0.1% PEG 8000, pH 8.

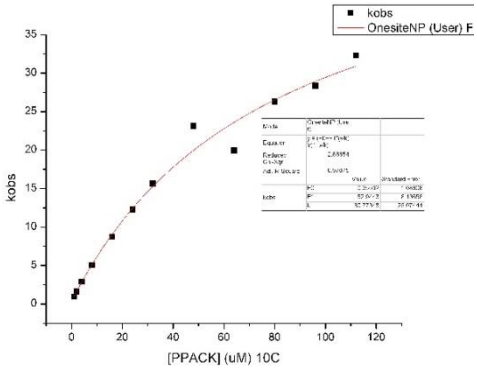


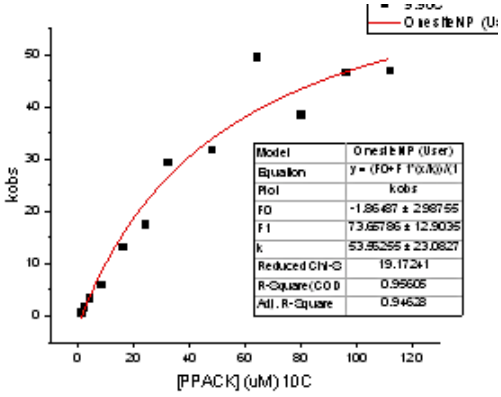
Graphs 2 and 3. Show the Relationship between Concentration PPACK and kobs at 5C, fitted to equation $y=(F0+F1^{(x/k)})/(1+x/k)$. 250nM Thrombin, 400mM ChCl, 50mM Tris, 0.1% PEG 8000, pH 8.



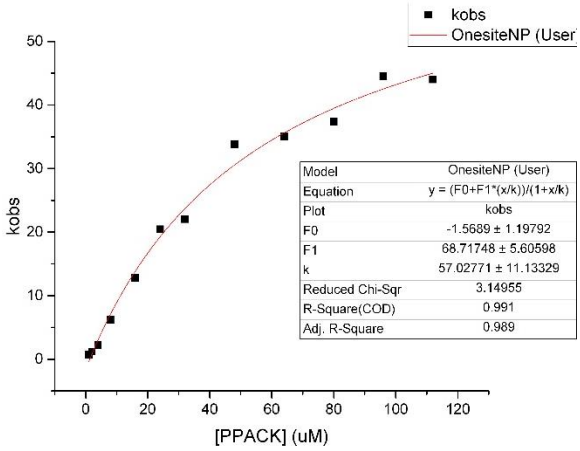


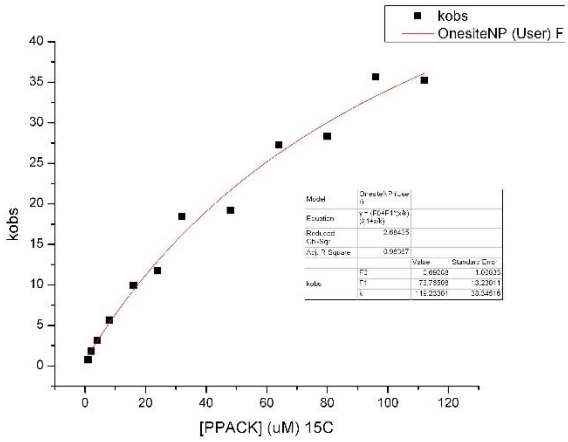
Graphs 4 and 5. Show the Relationship between Concentration PPACK and kobs at 10C, fitted to equation $y=(F0+F1^{(x/k)})/(1+x/k)$. 250nM Thrombin, 400mM ChCl, 50mM Tris, 0.1% PEG 8000, pH 8.



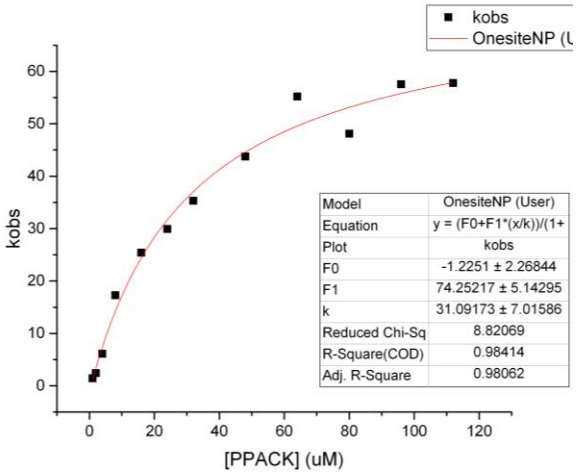


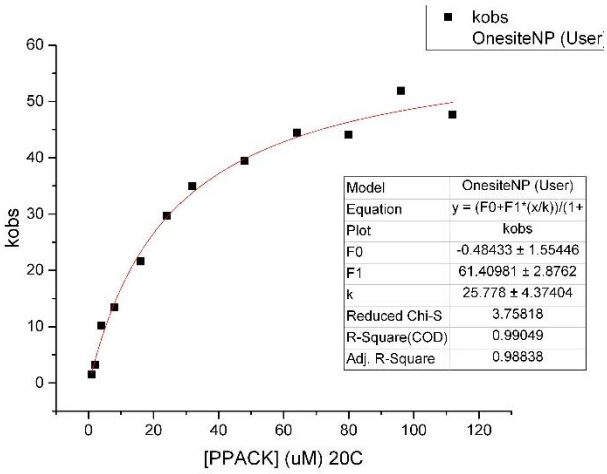
Graphs 6 and 7. Show the Relationship between Concentration PPACK and kobs at 115C, fitted to equation $y=(F0+F1^{(x/k)})/(1+x/k)$. 250nM Thrombin, 400mM ChCl, 50mM Tris, 0.1% PEG 8000, pH 8.



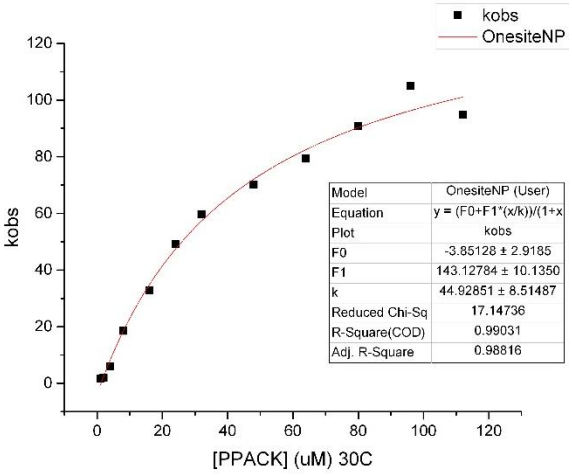


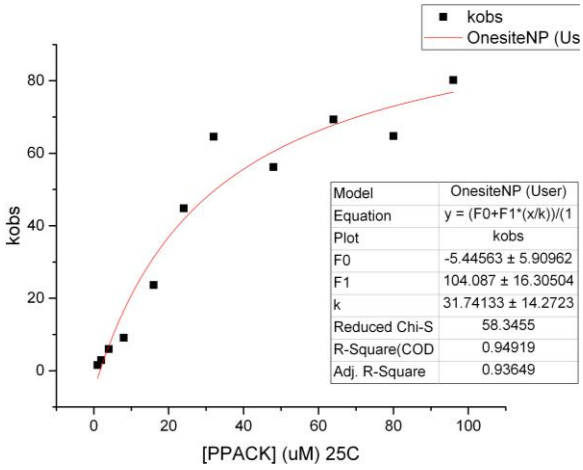
Graphs 8 and 9. Show the Relationship between Concentration PPACK and kobs at 20C, fitted to equation $y=(F0+F1^{(x/k)})/(1+x/k)$. 250nM Thrombin, 400mM ChCl, 50mM Tris, 0.1% PEG 8000, pH 8.



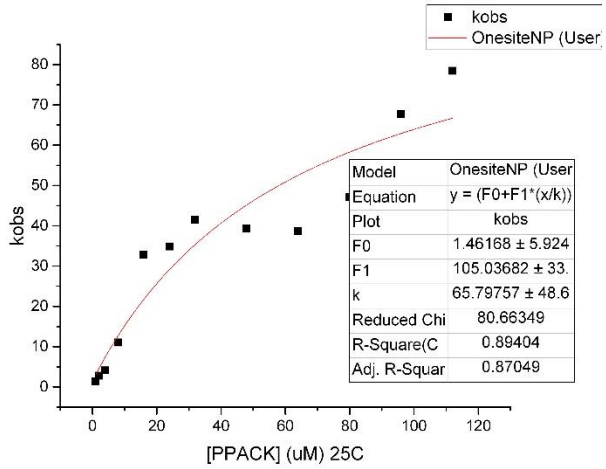


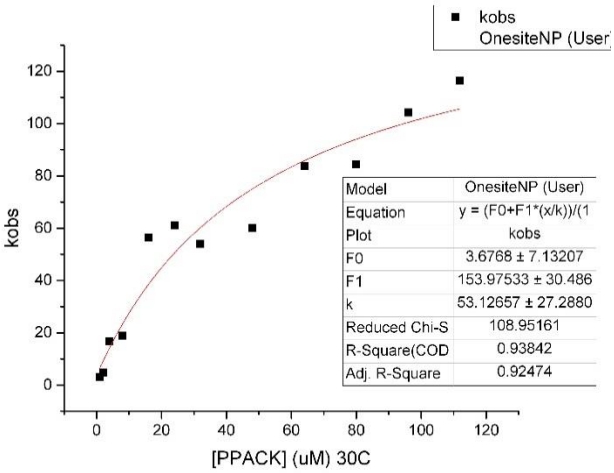
Graphs 10 and 11. Show the Relationship between Concentration PPACK and kobs at 25C, fitted to equation $y=(F0+F1^{(x/k)})/(1+x/k)$. 250nM Thrombin, 400mM ChCl, 50mM Tris, 0.1% PEG 8000, pH 8.





Graphs 12 and 13. Show the Relationship between Concentration PPACK and kobs at 30C, fitted to equation $y=(F0+F1^{(x/k)})/(1+x/k)$. 250nM Thrombin, 400mM ChCl, 50mM Tris, 0.1% PEG 8000, pH 8.





Graph 14. Shows Linearity of Thrombin-PPACK temperature study-derived logk12/1000T

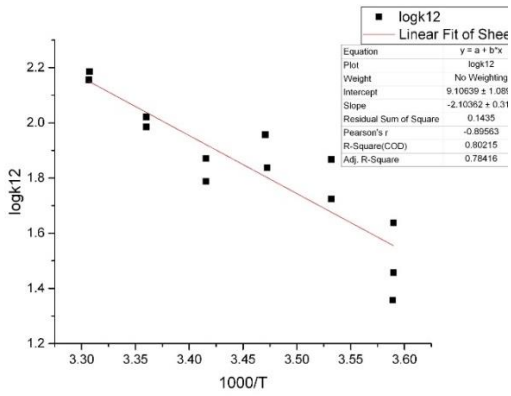
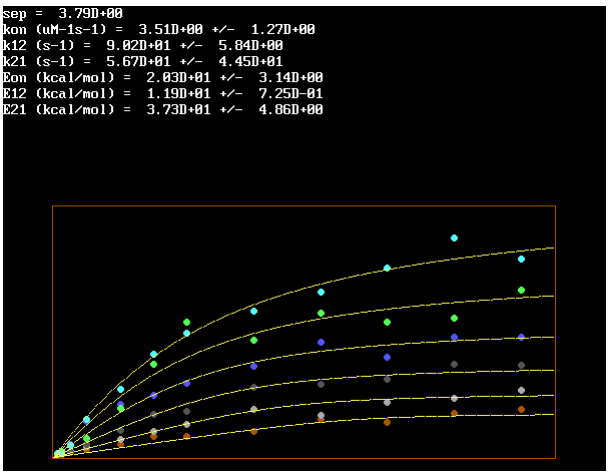


Image 1. Shows Experimentally Determined Activation Energies for Wild-Type Thrombin-PPACK Eon, E12, E21, and corresponding rate constants kon, k12, and k21 at reference temperature.



Discussion

As the general trend in the data suggests, the initial rate at which PPACK binds to thrombin was found to increase with temperature, and the increase in the initial rate stops increasing exponentially at approximately 60uM PPACK, although this point shifts left with an increase in temperature.

When the log of the k12 is compared to the 1000-fold inverse of the temperature in Kelvin, a linear relationship forms between the magnitude of the k12 and the temperature of the experiment. This is consistent with serine protease substrate binding established by Ayala & Di Cera.

When the comprehensive temperature study data was entered into a program that determined the activation energies and reference temperature rate constants of substrate binding, the resulting activation energies Eon (20.3±3.14 kcal/mol) E12 (11.9±.725 kcal/mol) E21 (37.3±4.86 kcal/mol) can be relatively accurately determined as compared to literature values. Resulting reference temperature rate constants of kon (3.51±1.27 $uM^{-1}s^{-1}$) k12 (90.2±5.84 s^{-1}) and k21 (56.7±44.5 s^{-1}) are found from experimentally determined activation energies.

Conclusion

Using temperature as a parameter for the rate of inactivation results in the experimental determination of activation energies. Therefore, rate constants of conformational selection change from closed to open; thrombin-PPACK binding also occurs. Closed thrombin (E*) becomes more populated than open thrombin (E) at higher temperatures due to a higher k21 in comparison to k12.

References

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Ayala, Y. M., & Di Cera, E. (2000). A simple method for the determination of individual rate constants for substrate hydrolysis by serine proteases. *Protein Science*, (9), 1589-1593.