AppliedPhotophysics
*Ultrasensitive Spectroscopy for the Life Sciences*

SX SERIES APPLICATION NOTE

# Determination of Enzyme Kinetics Using Stopped-Flow Spectroscopy

***Abstract:*** This study describes the use of an SX20 spectrometer to measure the pre-steady state kinetics of a well-studied enzymatic reaction. The hydrolysis of p-Nitrophenyl acetate catalysed by the enzyme α-chymotrypsin is studied. Kinetic parameters such as rate constants and the Michaelis-Menten constant are evaluated.

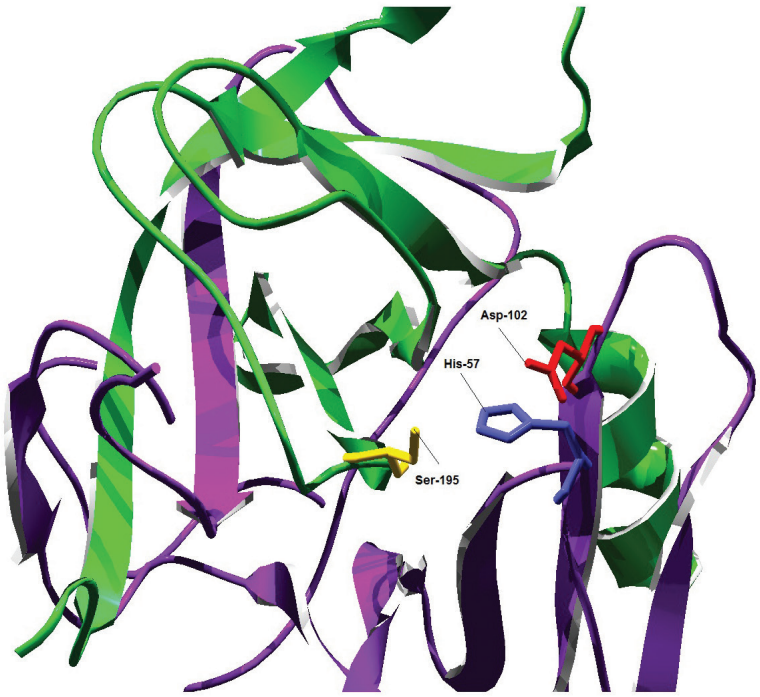


Figure 1. Structural detail of α-chymotrypsin's active site. PDB ID – 4CHA

**Author**
**James Law, PhD**

KEYWORDS

| | |
|---|---|
| ▸ Stopped-Flow | ▸ Rapid Mixing |
| ▸ Enzyme Kinetics | ▸ SX20 |
| ▸ Reaction Kinetics | ▸ Enzymology |
| ▸ Michaelis - Menton | ▸ Protein Studies |

## INTRODUCTION

Stopped-flow systems manufactured by Applied Photophysics Ltd have been used to determine enzyme kinetics for over three decades as can be seen from the thousands of references in the scientific literature. This study demonstrates the application of an SX20 stopped-flow spectrometer to measure the pre-steady state kinetics of a well-known enzymatic reaction. The reaction was one of the first to be characterised by the stopped-flow technique over 50 years ago. This is the hydrolysis of p-Nitrophenyl acetate catalysed by the enzyme α-chymotrypsin.[1] Extensive research over the years has established the mechanistic details of this reaction.

α-chymotrypsin is a member of the serine protease enzyme family; these proteins are ubiquitous throughout life where they carry out their essential function in peptide bond hydrolysis. All serine proteases execute their catalytic role at an active site consisting of a catalytic triad. The triad invariably contains a Histidine, Serine, and an Aspartic acid residue (Figure 1). The Serine residue acts as a nucleophile on the target carbonyl group whereas the Histidine and Aspartic acid residues involve themselves in hydrogen transfer and hydrogen bonding. The steps in the catalysed hydrolysis of p-Nitrophenyl acetate by α-chymotrypsin and trypsin are shown in Figure 2 .[2]

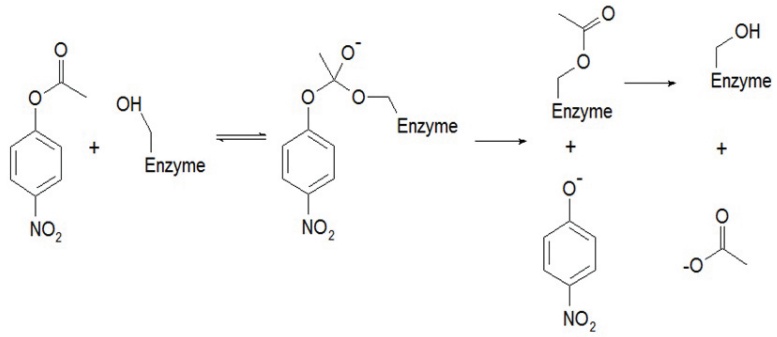


Figure 2. Steps in the serine protease catalysed hydrolysis of p-Nitrophenyl acetate. A nucleophilic serine residue of the enzymes active site initially attacks the carbonyl group of p-Nitrophenyl acetate. A tetrahedral intermediate is formed followed by loss of p-Nitrophenolate. It is the formation of this molecule that allows this reaction to be studied spectroscopically due to its strong absorption of light at 400nm. The acetyl-enzyme intermediate is then deacylated resulting in the formation of acetate as well as regeneration of the enzyme.

This reaction can be summarised in three steps by the following reaction scheme:

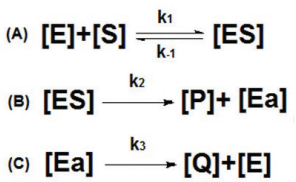


Figure 3. Schematic of serine protease hydrolysis of p-Nitro-phenyl acetate. [E] represents free α-chymotrypsin, [S] represents p-Nitro-phenyl acetate, [ES] represents the tetrahedral intermediate formed in the first step of the reaction, [P] represents the first product p-Nitrophenolate, [Ea] represents the acylated intermediate and [Q] represents acetate.

Step (A) of the reaction is a pre-equilibrium that occurs much faster than the subsequent two steps. The formation of acylated enzyme intermediate is accompanied by the loss of the first product p-Nitrophenolate. It is this step that can be observed in the stopped-flow experiment due to the intense absorption of light at 400 nm by p-Nitrophenolate [Q].

## MATERIALS AND METHODS

Reactions were carried out using an SX20 fitted with a 20μL optical cell (2mm pathlength). Absorbance was measured at 400nm. For each reaction, 1000 points were recorded over a 60s time period.

All chemicals and reagents were purchased from Sigma-Aldrich. 60μM α-chymotrypsinin (20% Isopropyl alcohol, 20mM Phosphate buffer, pH7.3) was mixed with p-Nitrophenolate (250μM to 8mM, 20% Isopropyl alcohol). 6 repeats were performed for each reaction with the averaged trace being taken. The non-catalysed reaction was also recorded for each concentration and subtracted from the equivalent catalysed spectrum.

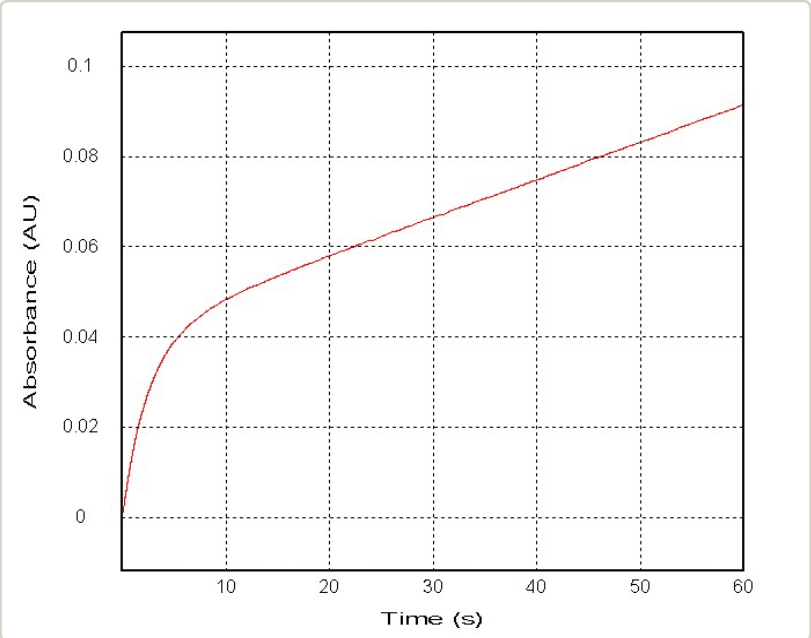


Figure 4. Trace observed at 400nm when chymotrypsin, 60μM, is mixed with an approximate 15 fold excess of p-Nitro-phenyl acetate.

## RESULTS

Figure 4 shows the trace observed when α-chymotrypsin, 60μM, is rapidly mixed with a 100-fold excess of p-Nitrophenyl acetate in a SX20 spectrophotometer. The curve shows some interesting features. Initially (i.e. 0-5s into the reaction), the rate of production is relatively quick. This initial “burst” of p-Nitrophenolate formation is followed by a linear steady state rate of production.

The shape of the curve can be explained if step (B) occurs rapidly compared to the final deacylation step (C) which is 'rate limiting'. The pre-equilibrium in step (A) occurs too quickly to be observed in the stopped-flow trace and therefore the initial observed rate occurs when [ES] is at its highest concentration. At this point, the rate of formation of p-Nitrophenolate depends only on step (B) of the mechanism. This gives rise to the initial observable 'burst phase' of the reaction.

Like most enzymes, serine proteases display saturation kinetics; that is, the initial rate of reaction approaches a hypothetical maximum velocity, $V_{max}$, as [S] is increased. $V_{max}$ is therefore the rate of reaction when the enzyme is fully saturated i.e. all the enzyme is in the form of [ES]. The above mechanism therefore follows the Michaelis-Menten model of enzyme kinetics and the initial rate of reaction ($v_0$) can be describe by equation (1):

$$v_0 = \frac{V_{max}[S]}{K_M + [S]} \quad (1)$$

Where $K_M$ represents the Michaelis-Menten constant. $K_M$ is defined as the concentration of substrate at which the initial rate of reaction is half that of $V_{max}$ and is a commonly used parameter for comparing substrate affinities in enzyme-substrate systems. The initial rate of reaction can be described by a rate constant, $k_{obs}$, which represents the rate constant of the initial exponential phase of the reaction. This constant can be easily obtained by fitting the observed trace to the equation (2) using the SX's Pro-Data Viewer's fitting function. (Single exponential plus slope)

$$Ae^{-kt} + bt + c \quad (2)$$

Figure 5 shows how $k_{obs}$ varies with substrate concentration, exemplifying the enzymes saturation kinetics, i.e. $k_{obs}$ approaches a maximum value (that of which would be obtained at $V_{max}$ and is equal to $k_2$).

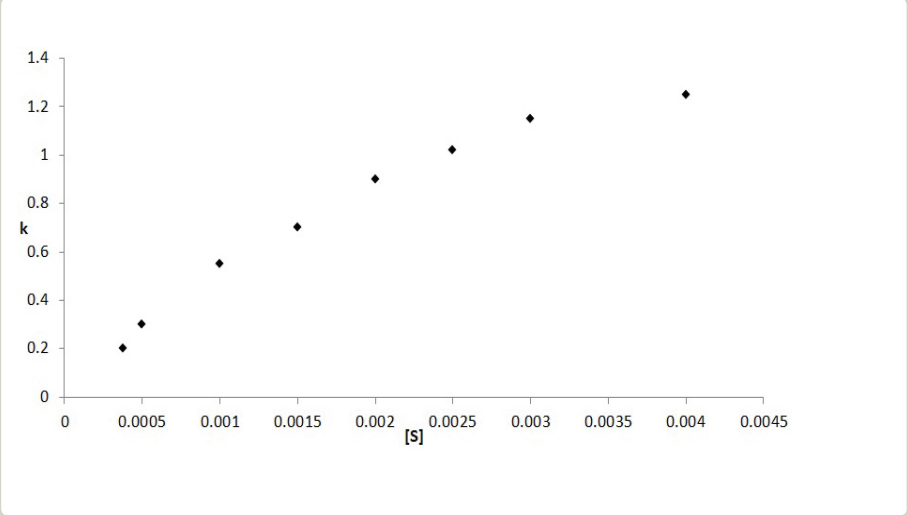


Figure 5. Variation of $k_{obs}$ with substrate concentration

It can be shown that under these conditions ([S]>>[E]) the value of $k_{obs}$ can be described by equation (3): note the similarity to equation (1).

$$k_{obs} = \frac{k_2 [S]}{K_M + [S]} \qquad (3)$$

We can fit the data to this equation using non-linear least squares regression to obtain parameters for $k_2$ and $K_M$. This was performed using the Solver Add in in Microsoft Excel™. The values extracted are shown in Table 1.

Alternatively, this equation can be rearranged to the following:

$$\frac{1}{k_{obs}} = \frac{K_M}{k_2} \quad \frac{1}{[S]} + \frac{1}{k_2} \qquad (4)$$

The values of $K_M$ and $k_2$ can be evaluated by measuring the first order rate constant at increasing initial substrate concentrations. Plotting a graph of $1/k_{obs}$ against 1/[S] gives a straight line graph with slope equal to $K_M/k_2$ and intercept equal to $1/k_2$.[3,4] Figure 6 shows this plot obtained with α-chymotrypsin (30μM with p-Nitrophenolacetate concentrations ranging from 125μM to 4mM.

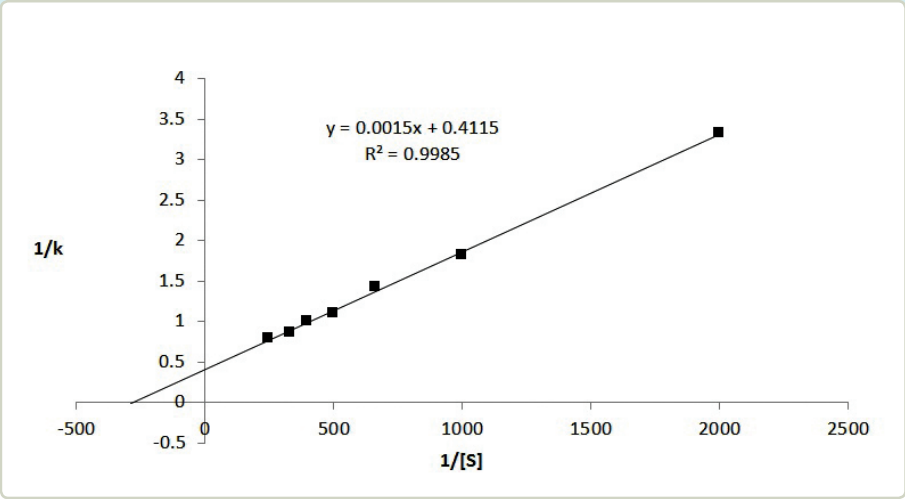


Figure 6. Lineweaver-Burk plot of data obtained with α-chymotrypsin. The fit was generated using linear regression.

| | Non-Linear Regression | Linear Regression |
|---|---|---|
| $k_2$ ($s^{-1}$) | 2.38 | 2.43 |
| $K_M$ ($10^{-3}$ M) | 3.40 | 3.65 |

Table 1. Parameters extracted from Figure 5

Although it is generally considered that non-linear regression is a more accurate method for analysis, in this case we obtain similar values using both fitting methods.[5]

Once the reaction has reached the steady state, the rate of reaction depends only on step C in Figure 3. This is because it is much slower than the preceding two steps and is thus rate-determining. We can determine the steady state rate of reaction from the linear part of the trace in Figure 4 because we can relate the absorbance change to the change in p-Nitrophenolate concentration according to Beer-Lambert Law. From the mechanism of this reaction, it is clear that the amplitude of the burst phase, i.e. point at which an extrapolation of the linear phase touches the y-axis, is approximately equal to the amount of active enzyme; this gives us a value for [Ea] from which we can determine $k_3$ via equation (5) (implied from Figure 3).[3] The value of $k_3$ was therefore assessed from each trace and the average value was calculated to be 0.016$s^{-1}$.

$$k_3 = \frac{d[Q]/dt}{[Ea]} \qquad (5)$$

## CONCLUSION

Applied Photophysics Ltd's SX20 stopped-flow spectrometer can be used to study the pre-steady state kinetics of enzymatic reactions. Here, kinetic parameters of the enzymatic hydrolysis of p-Nitrophenolacetate were obtained. Although this is a relatively slow reaction, SX20 stopped-flow spectrometers have the capacity to measure kinetics from 0.5ms after mixing.

## REFERENCES

**1.** Gutfreund, H. & Sturtevant, J.M. The mechanism of the reaction of chymotrypsin with p-nitrophenyl acetate. The Biochemical Journal 63, 656-61 (1956).

**2.** Hedstrom, L. Serine protease mechanism and specificity. Chemical Reviews 102, 4501-24 (2002).

**3.** Bender, M., Kezdy, F. & Wedler, F. α-Chymotrypsin : Enzyme Concentration and Kinetics. Journal of Chemical Education 44, 84-88 (1967).

**4.** Gutefreund Kinetics For The Life Sciences. (Cambridge University Press: Cambridge, 1995).

**5.** Leatherbarrow, R.J. Using linear and non-linear regression to fit biochemical data.

Trends in Biochemical Sciences 15, 455-8 (1990).

**AppliedPhotophysics**

*Ultrasensitive Spectroscopy for the Life Sciences*

4140Q010 V 1.1

SX20 is a trademark of Applied Photophysics Ltd.

All third party trademarks are the property of their respective owners.

© 2011 Applied Photophysics Ltd.— All rights reserved.

**Applied Photophysics Ltd,**
21, Mole Business Park, Leatherhead, Surrey, KT22 7BA, UK
Tel (UK): +44 1372 386 537
Tel (USA): 1-800 543 4130
Fax: +44 1372 386 477

Applied Photophysics was established in 1971 by
The Royal Institution of Great Britain

www.photophysics.com