ITC in Enzyme Characterization

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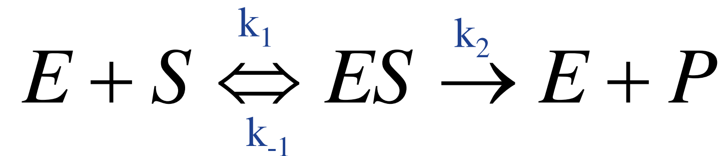
Enzyme Kinetics

K_M is analogous to K_d



$$K_M = \frac{k_2 + k_{-1}}{k_1}$$

When $k_{-1} >> k_2$

$$K_M = K_d$$

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Enzyme Kinetics

V_{max} is proportional to [enzyme]

$$V_{max}=k_{cat}[E]$$

Km is independent of [E]

Velocity of reaction is given by Michaelis-Menten equation

$$v = \frac{[S]}{K_M + [S]} k_{cat} [E]$$

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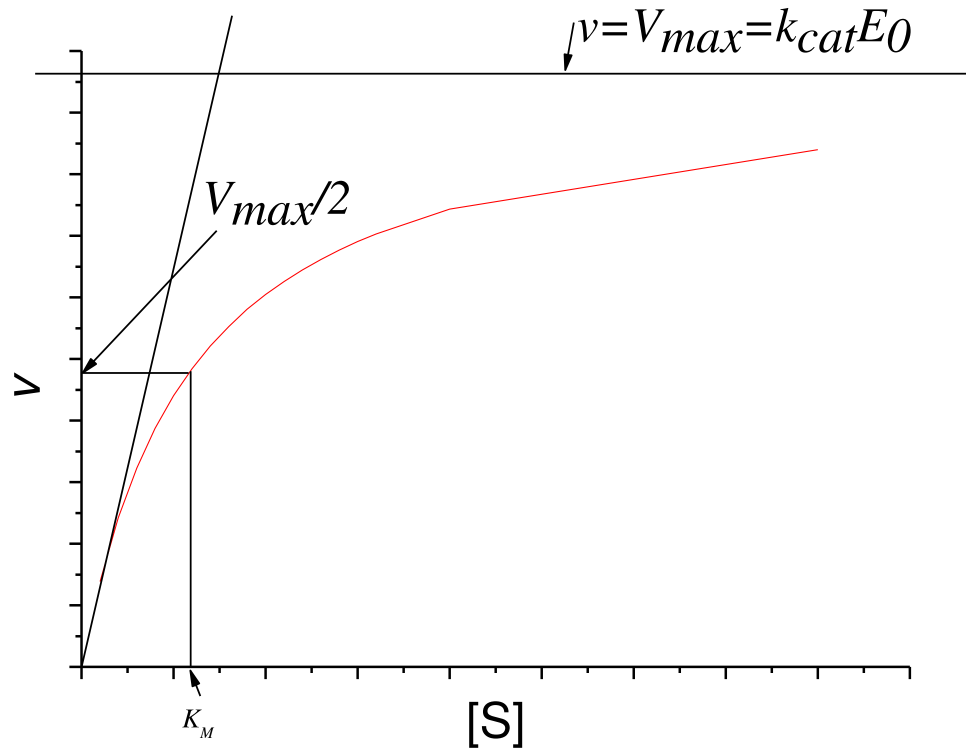
Classical Enzyme Kinetics



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Two ITC methods

Multiple substrate injections

- Low enzyme concentration
- Steady state conditions

Continuous assay

- Higher enzyme concentration
- Single injection of substrate

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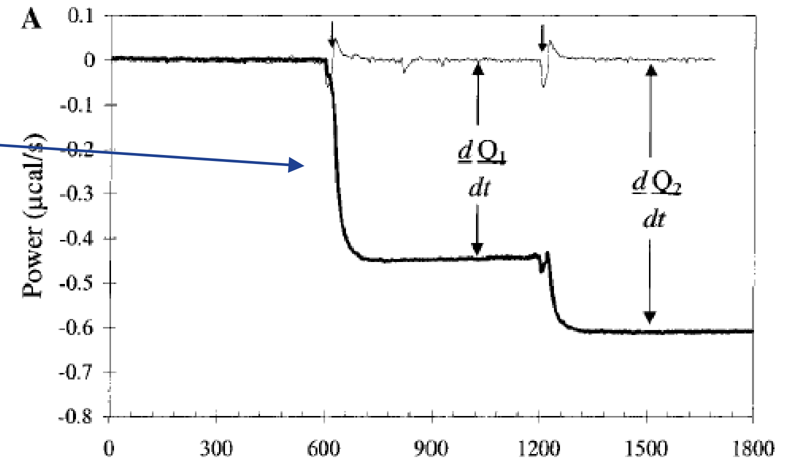
Enzyme Kinetics By ITC

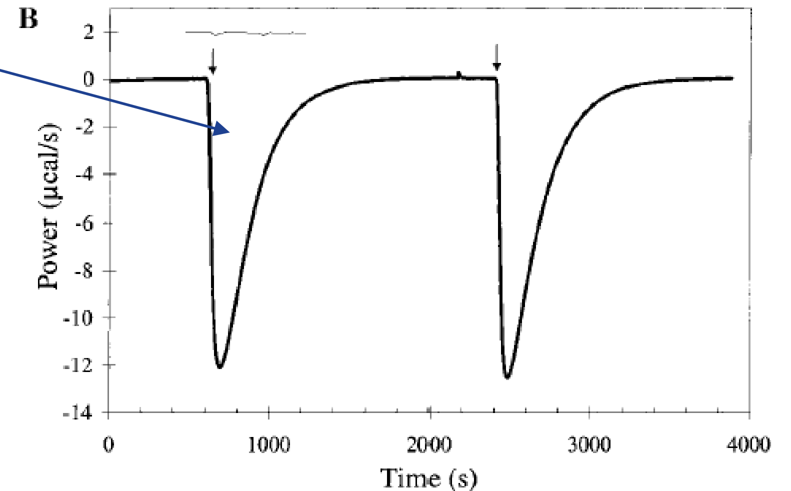
$$Power = \frac{dQ}{dt}$$

$$Q = n\Delta H_{app} = [P]_{total} V\Delta H_{app}$$

$$\frac{dQ}{dt} = \frac{d[P]}{dt} V\Delta H_{app}$$

$$Rate = \frac{d[P]}{dt} = \frac{1}{V\Delta H_{app}} \frac{dQ}{dt}$$





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Enzyme Kinetics

$$Rate = \frac{k_{cat}.[E_{total}].[S]}{K_m + [S]}$$

Rate (µmoles/minute)

0.3
0.2
0.1
0.0

0
1
2
3

PNPP (mM)

UV-Vis
ITC

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[Enzyme] and [Substrate]

Following each injection of substrate there should be no appreciable depletion of substrate (<5%) prior to the next injection.

However substrate concentration cannot be too high otherwise [S] will be above K_m after the first few injections.

If enzyme-substrate affinity is high then enzyme concentration needs to be low

However this relies upon there being a sufficiently large enthalpy to detect.

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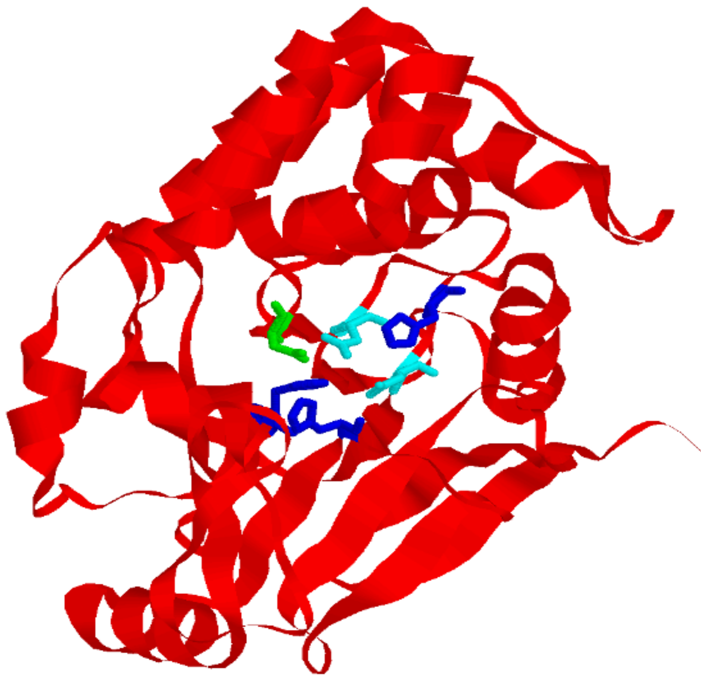
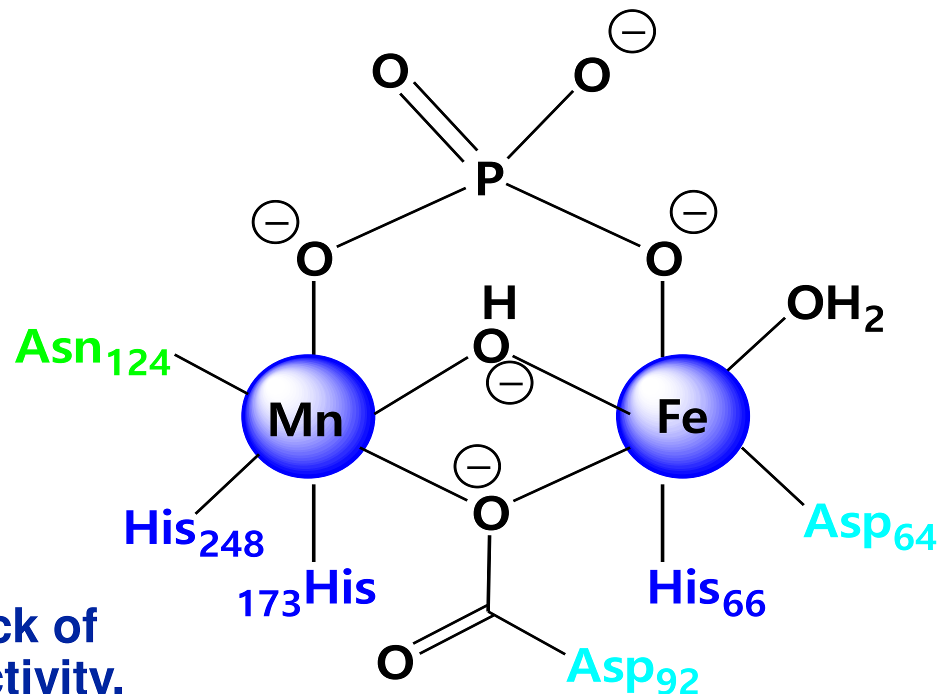
PP1-γ Phosphatase



Phosphatases catalyse the hydrolytic removal of a phosphate group. In this study we examine a serine/threonine phosphatase (PP1-γ).



This enzyme catalyses the direct attack of water and it requires metal ions for activity.

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Enzyme Kinetics

Raw calorimetric data for the measurement of the reaction rate for the hydrolysis of PNPP by PP1-γ.

Power (μcal/sec)

24
22
20
18
16
14
12
10
8
6

0 5 10 15 20 25 30 35 40 45

Time (minutes)

[Substrate] lower than K_M

[Substrate] higher than K_M

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Enzyme Kinetics

ITC	UV
K_m = 1.2(±0.2) mM	K_m = 0.9(±0.2) mM
k_{cat} = 0.6 (±0.1) s^{-1}	k_{cat} = 0.5 (±0.1) s^{-1}
V_{max} = 0.43 μM min^{-1}	V_{max} = 0.39 μM min^{-1}

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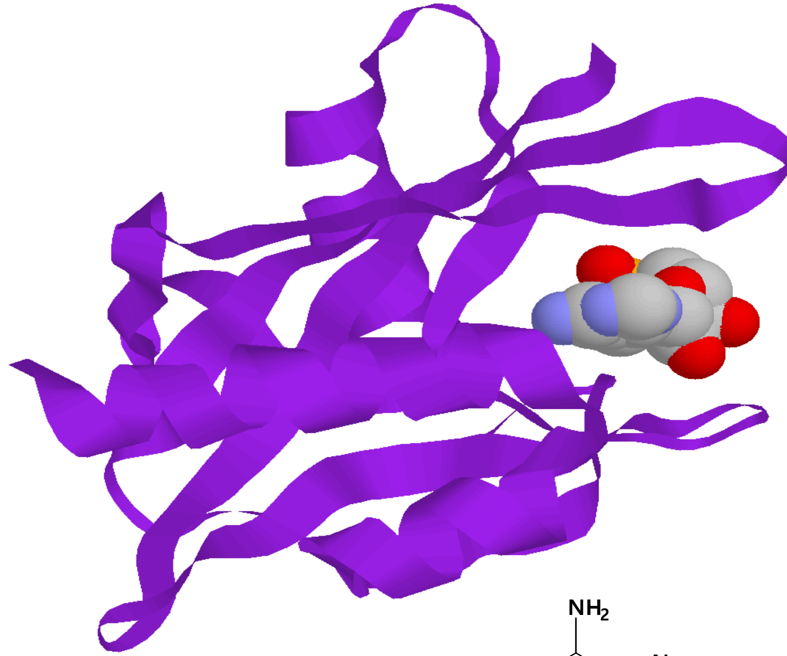
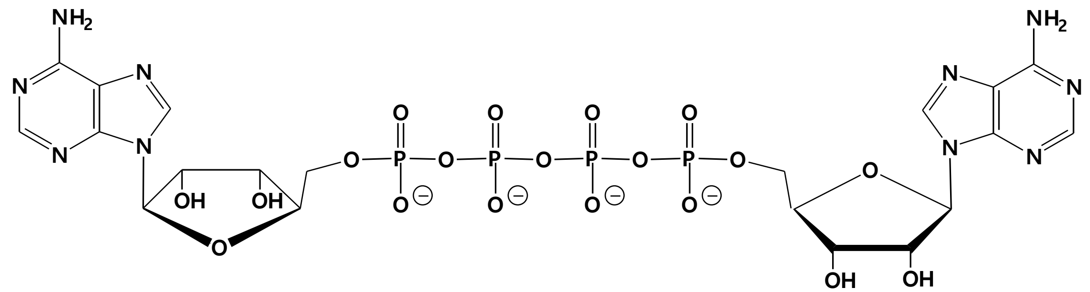
Enzyme Kinetics



Ap4A Hydrolase



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Enzyme Kinetics

Raw calorimetric data for the hydrolysis of 1 mM P^1,P^4-di(adenosine-5´)tetraphosphate by 2 nM human Ap_4A hydrolase at 37 °C

Power (µcal/sec)

9.10
9.05
9.00
8.95
8.90
8.85
8.80

0 5 10 15 20 25 30 35 40 45 50 55 60

Time (minutes)

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Enzyme Kinetics

Power (µcal/sec)

9.0
8.5
8.0
7.5

0 10 20 30 40 50 60 70 80 90

Time (minutes)

Determination of ΔH_{app} for the hydrolysis of 1 mM P^1,P^4-di(adenosine-5') tetraphosphate by100 nM AP4A hydrolase at 37 ^{o}C

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Enzyme Kinetics

5
4
3
2
1
0

Rate (s^{-1})

0.000 0.005 0.010 0.015 0.020 0.025 0.030

[P^1,P^4-diadenosine-5')tetraphosphate] mM

Calorimetric determination of enzyme kinetic parameters for the hydrolysis of 1 mM P^1,P^4-di(adenosine-5') tetraphosphate by 2 nM AP_4A hydrolase at 37 °C.

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Enzyme Kinetics

Rate (s^{-1})

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01

0.00 0.02 0.04 0.06 0.08 0.10

Calorimetric determination of enzyme kinetic parameters for the hydrolysis of 4 mM P^1,P^5-di(adenosine-5')pentaphosphate by 2 nM AP_4A hydrolase at 37 °C.

[P^1,P^5-diadenosine-5')pentaphosphate] mM

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Enzyme Kinetics

Hydrolysis of diadenosine polyphosphates by Ap_4A Hydrolase

	P1,P4-di(adenosine-5') tetraphosphate	P1,P5-di(adenosine-5') pentaphosphate	ATP
K_m (μM)	2.68±0.80	34.5±1.8	*nd*
k_{cat} (s^{-1})	2.76±0.03	0.05±0.01	*nd*
V_{max} (nM s^{-1})	5.52	1.0	*nd*
ΔH_{app} (kcal mol^{-1})	-19.24±0.27	-16.38±0.74	-8.6±0.2

Enzyme Kinetics

Alternatively……

CFB (µcal/s)

12,0
11,8
11,6
11,4
11,2

$\Delta CFB = V_{max}$

0
5
10
15

Time (min)

Lonhienne *et al* Biochimica et Biophysica Acta 1545 (2001) 349-356

Enzyme Kinetics

12,5
12,0
11,5
11,0

CFB (µcal/s)

0 40 80 120 160

Time (min)

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Continuous Injection Method

Trypsin catalysed hydrolysis of BAEE and inhibition by benzamidine

9 nM Trypsin

9 nM Trypsin
+ 95 μM benzamidine

Final concentration of BAEE = 164 μM

μcal/sec

9
8
7
6
5
4
3

0 5 10 15 20 25 30

Time (min)

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Continuous Injection Method

From this raw data the **rate** at any time can be obtained using:

$$Rate = \frac{d[P]}{dt} = \frac{1}{V.\Delta H_{app}} \frac{dQ}{dt}$$

The amount of substrate left at any given time after the initial injection can be determined from the integral of the heat evolved:

$$S_t = S_{Total} - P_{(t)} = S_{Total} - \frac{\int_{t=0}^{t} Q_{(t)}}{\Delta H.V}$$

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Continuous Injection Method

0.00035
0.00030
0.00025
0.00020
0.00015
0.00010
0.00005
0.00000
-0.00005

Rate (millimoles/l/sec)

0.00 0.05 0.10 0.15

[S] (mM)

9 nM trypsin + 164 μM BAEE

9 nM trypsin + 164 μM BAEE + 0.1 μM benzamidine

K_m = 5.6 μM
k_{cat} = 36.2 s^{-1}
K_i = 16 μM

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Enzyme kinetics

Universal assay

No need to couple assay to colour change reaction

No labelling required

Modifications to substrate and/or enzyme do not effect assay

Turbid solutions can be used

Pico-femtomoles of protein required

Quick

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Comparison of calorimetric and other assay data

Enzyme	Calorimetric K_m	Calorimetric V_{max}	Literature Values K_m	Literature Values V_{max}
E.C. 1.5.1.3 (DHFR) [a]				
Substrate = DHF	1.2 µM	6 s^{-1}	6 µM	3 s^{-1} [i]
E.C. 2.7.1.1 (yeast hexokinase)[b]				
Substrate = glucose	72 µM	270 s^{-1}	100 µM	450 s^{-1} [ii]
E.C.3.3.2.6 *B. cereus* Penicillinase I[c]	120 µM	3600 s^{-1}	50 µM	2800 s^{-1} [iii]
E.C. 3.4.21.4 (Trypsin)[d]	4 µM	15 s^{-1}	5 µM	22 s^{-1} [iv]
E.C. 3.4.21.16 (HIV protease)[e]				
Substrate = KARVnLF(NO_2)EAnL	5 - 300 µM	10 s^{-1}	15 µM	45 s^{-1} [33]
Substrate = VSQNYPIVQ	[NaCl] dependent			
E.C. 3.5.1.5 (*H. pylori* urease)[f]	0.79 mM	1400 s^{-1}	0.17 mM	2700 s^{-1} [v]
E.C. 4.1.1.7 (*F. heparinum* heparinase)[g]	1.8 µM	0.059 s^{-1}	10.2 µM	92 s^{-1} [vi]
E.C. 4.1.1.39 (Rubisco)[h]				
Substrate = ribulose bis phosphate	0.15 mM	1.95 s^{-1}	0.053 mM	1.76 s^{-1} [vii]
E.C. 4.1.3.18 (Acetolactate synthase)[i]	4.8 mM	11 s^{-1}	5.5 mM	5.3 s^{-1} [viii]
E.C. 5.99 (GroEL)[j]	3 µM		5 µM	
	n = 2.9	0.052 s^{-1}	n = 2.5	0.08 s^{-1} [22]
E.C. 6.4.1.1 (Pyruvate carboxylase)[k]				
Substrate = ATP	85 µM		58 µM	
Substrate = pyruvate	105 µM		440 µM	[ix]

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Todd and Gomez, Anal. Biochem. 276, 179-187 (2001)