MOSBRI
Molecular-Scale Biophysics
Research Infrastructure

SAPIENZA
UNIVERSITÀ DI ROMA

ESC4
Advanced kinetics approaches to
unravel protein structure and function

Complex enzyme inhibition systems (And simple inhibition systems rivisited)

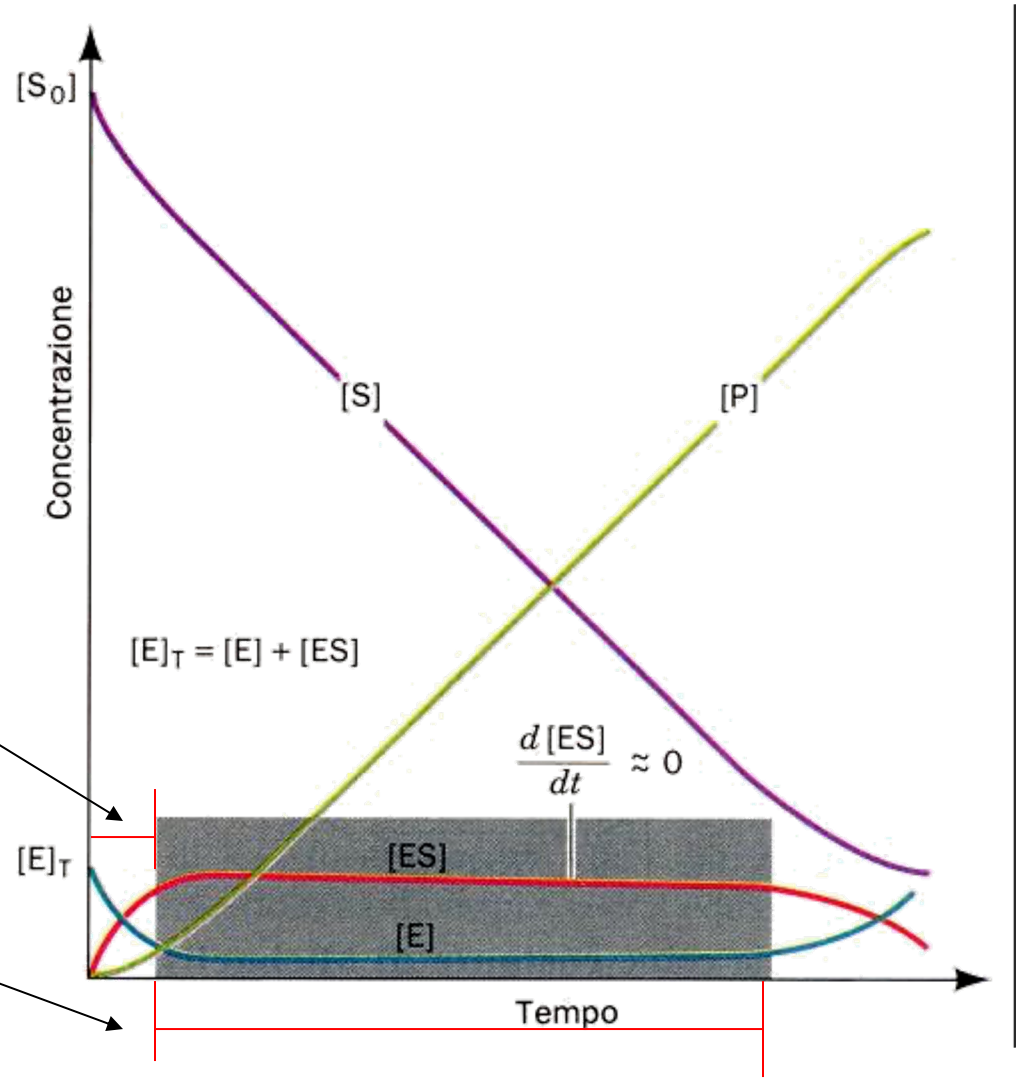
Prof. Roberto Contestabile

STEADY STATE ASSUMPTION

PHASES OF AN ENZYME CATALYSED REACTION:

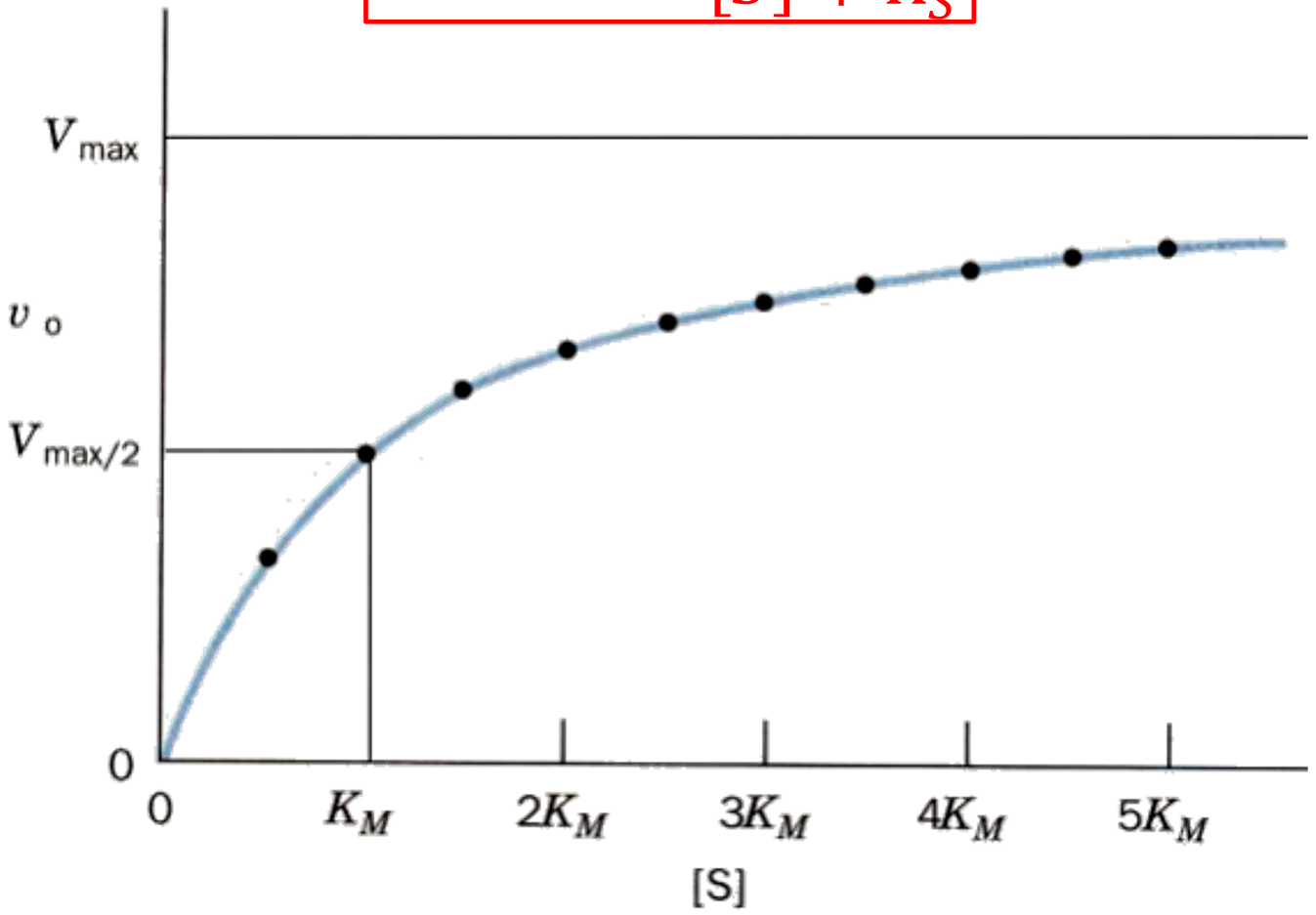
1) PRE-STATIONARY PHASE (PRE-STEADY STATE)

2) STATIONARY PHASE (STEADY STATE) (rapid or dynamic equilibrium)

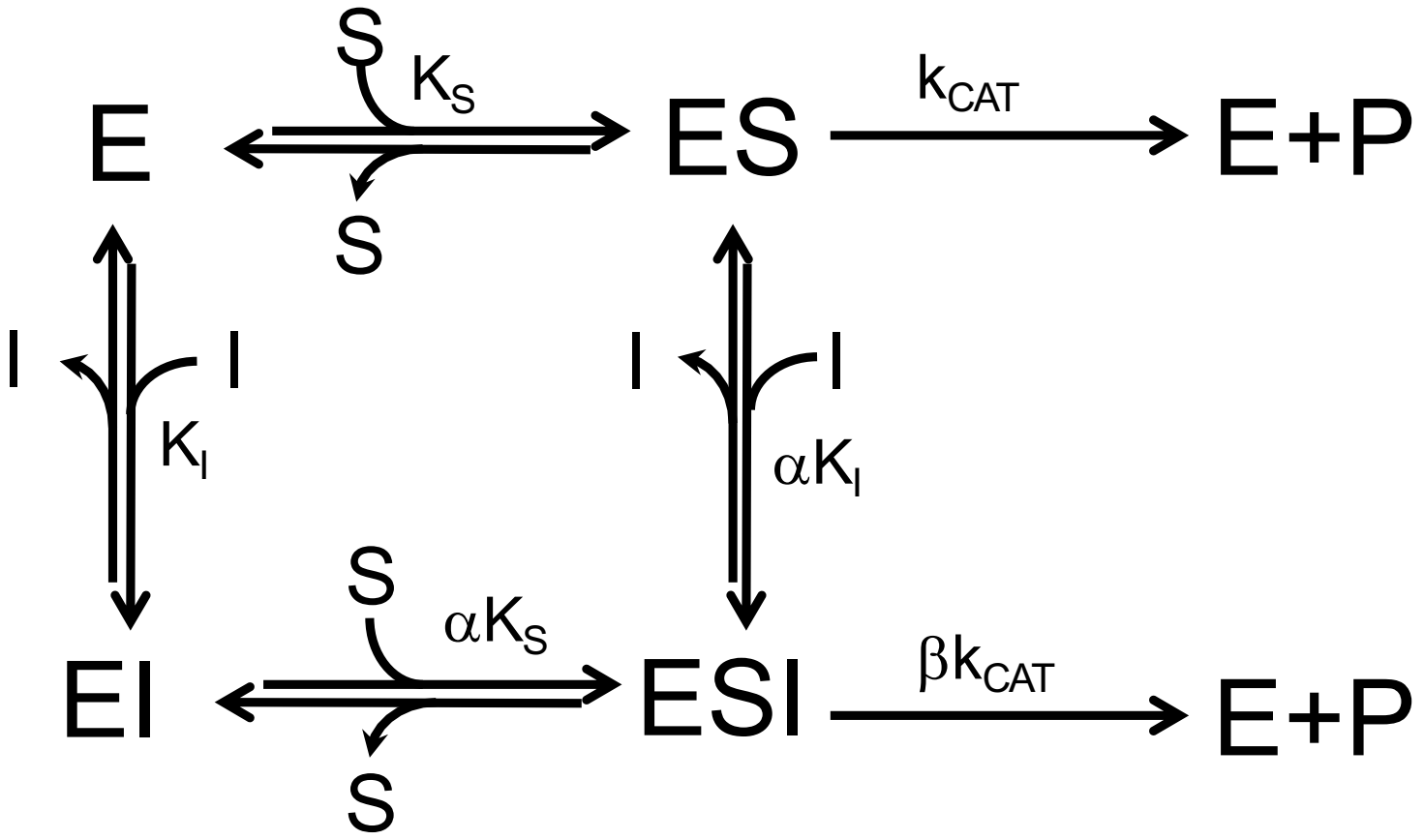


Michaelis – Menten Equation

$$v_0 = V_{max} \frac{[S]}{[S] + K_S}$$



Enzyme inhibition can be expressed in a general way



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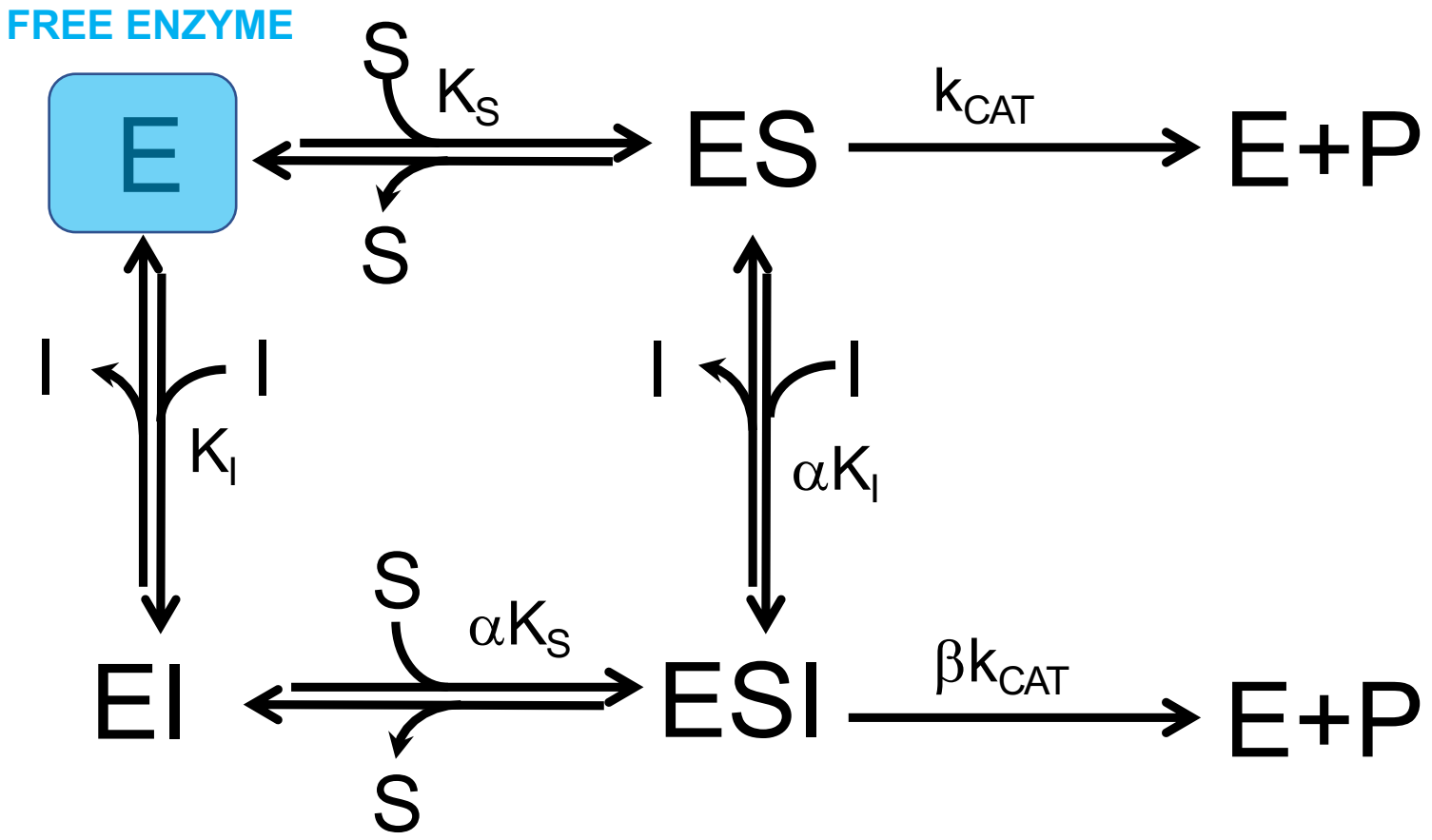

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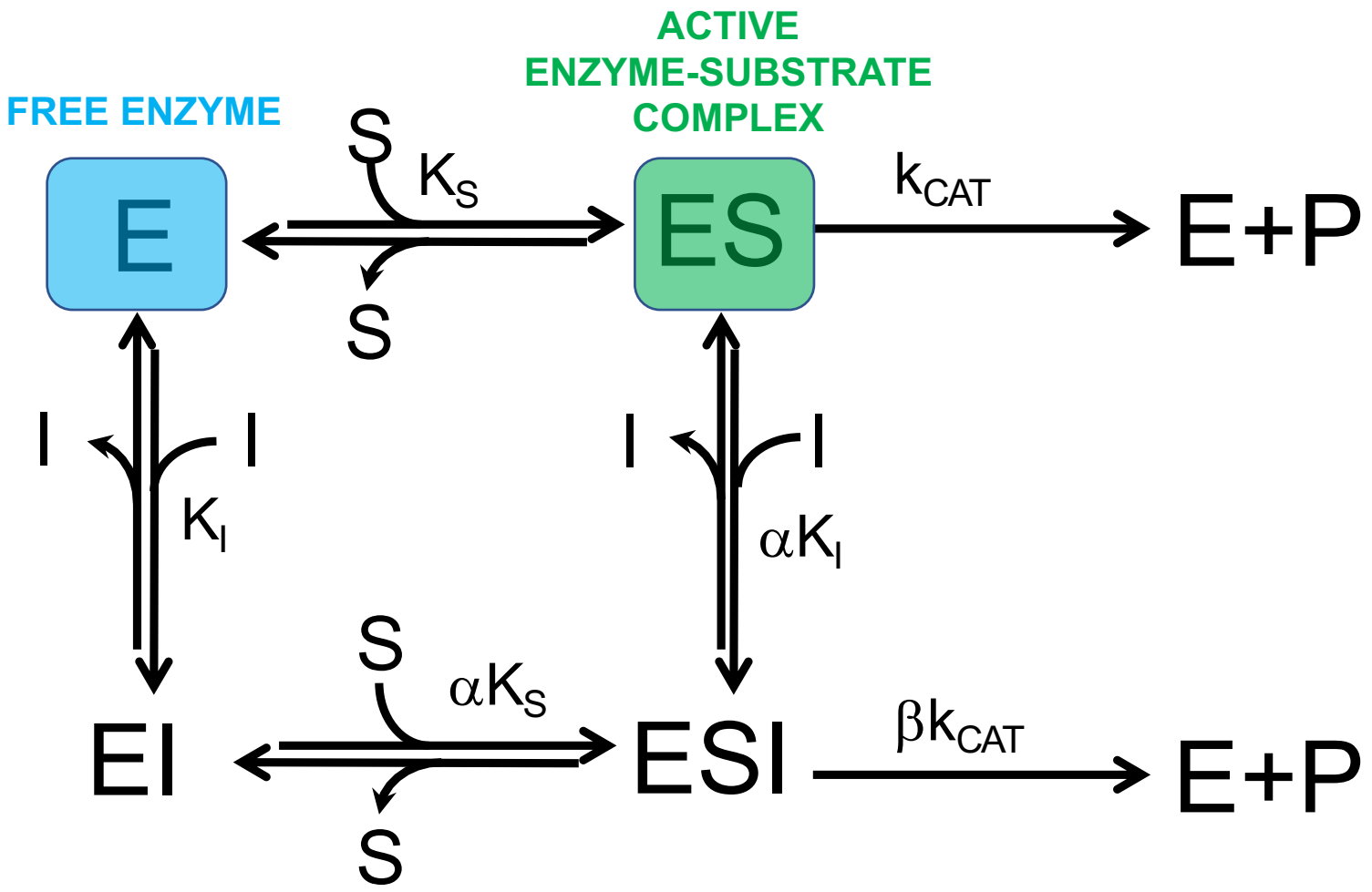
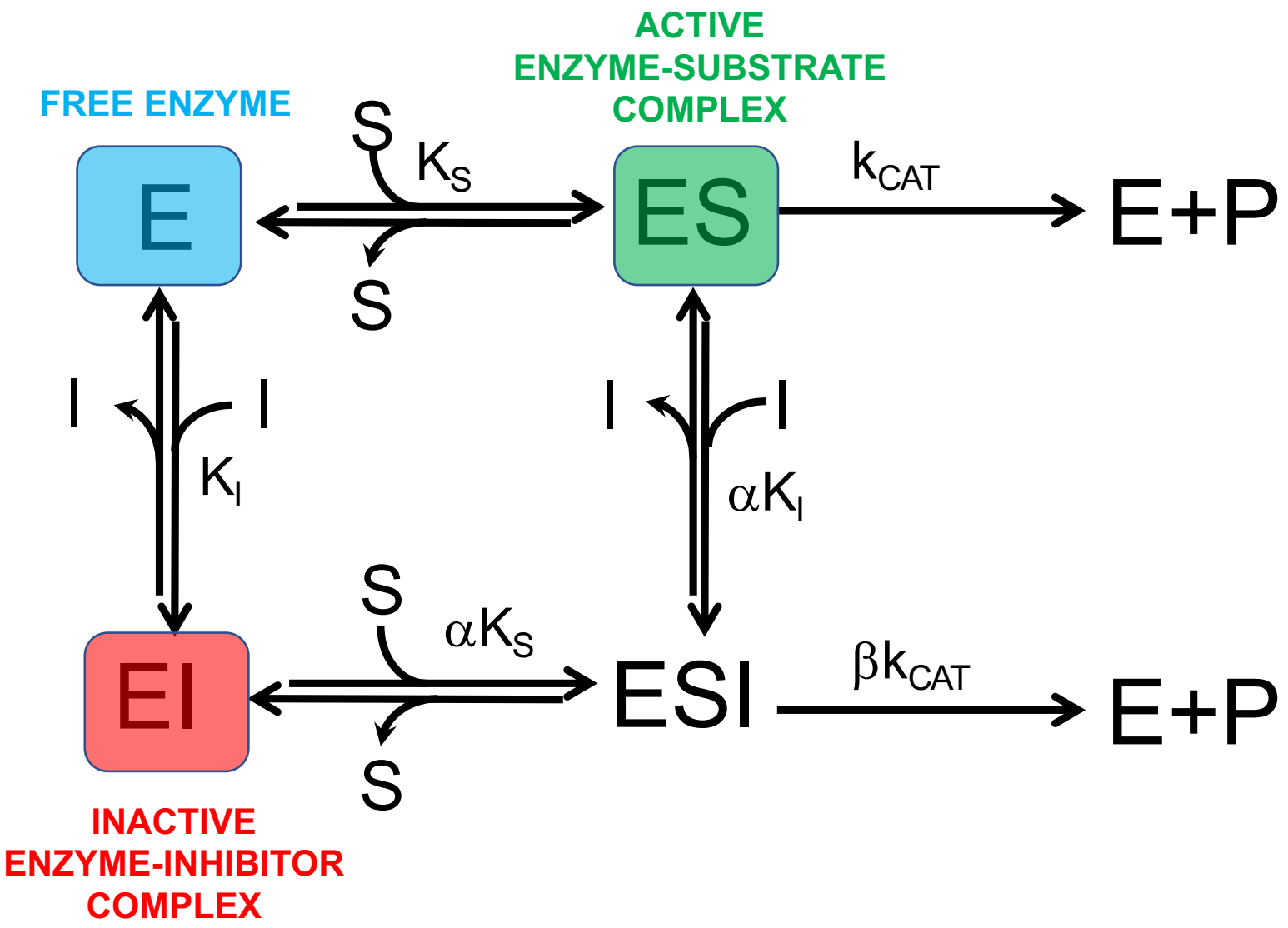
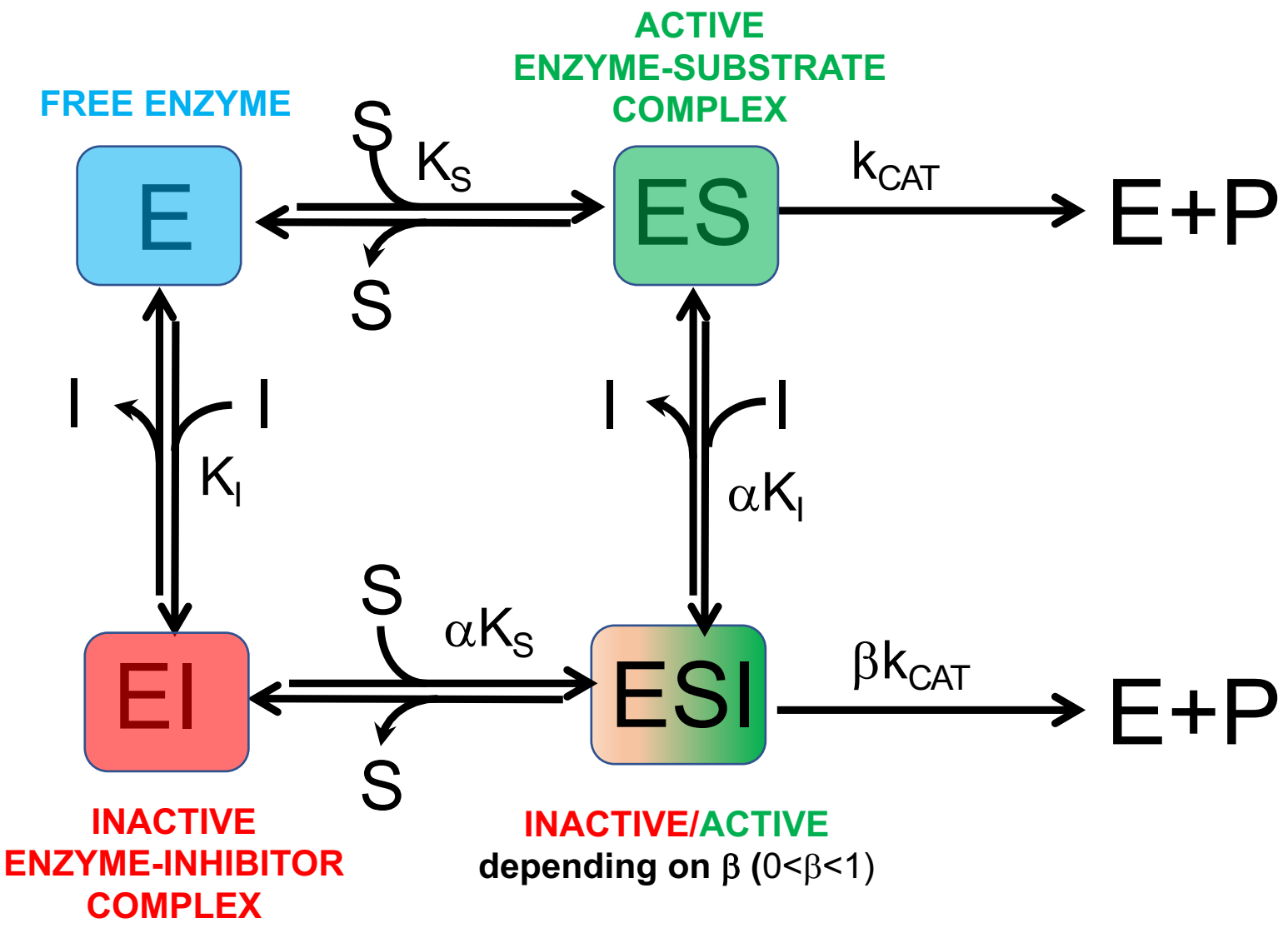


Enzyme inhibition can be expressed in a general way

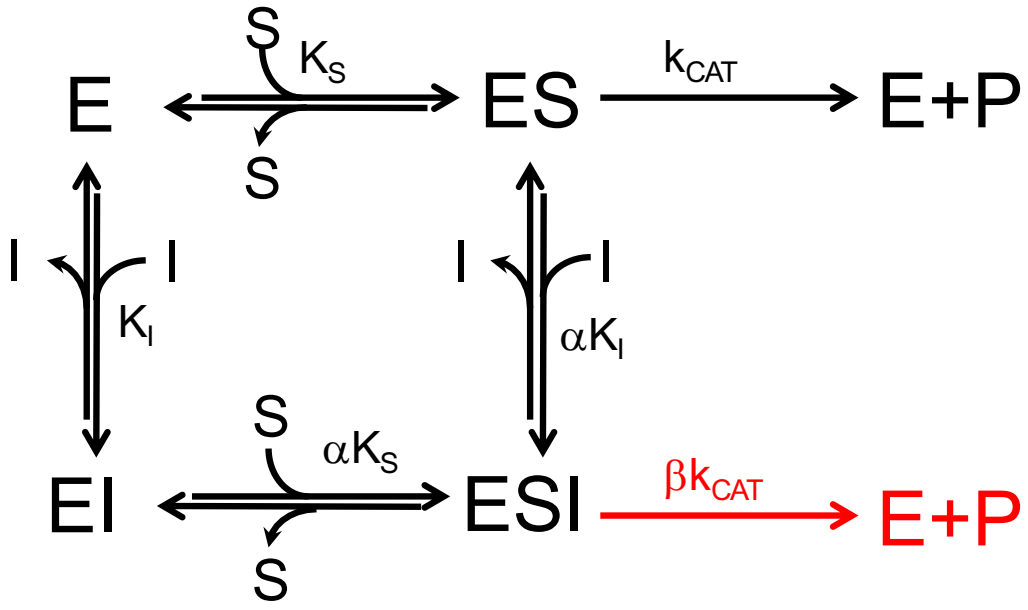


Enzyme inhibition can be expressed in a general way



"*SIMPLE*" OR "*LINEAR*" INHIBITION

WHEN $\beta = 0$

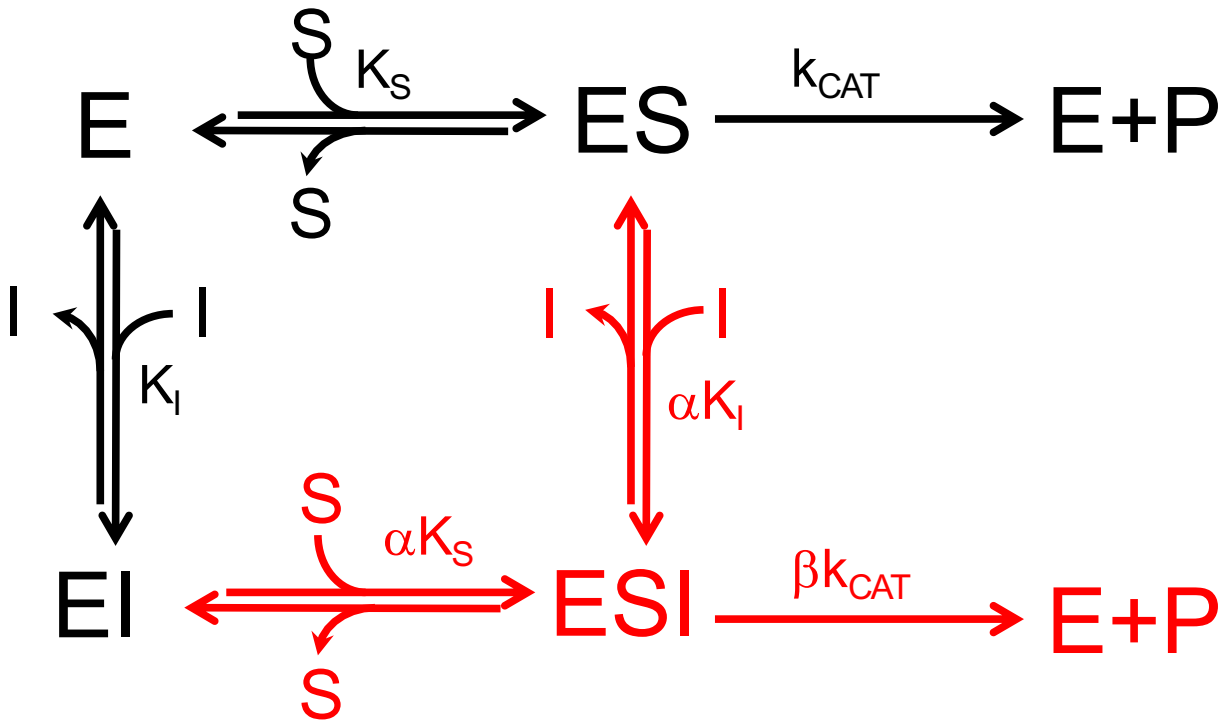


It is a ***complete inhibition*** since the enzyme has no catalitic activity when saturated with the inhibitor ($\beta = 0$).

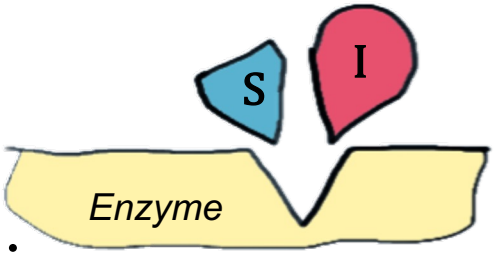
Linear in reference to the linear dependence of the apparent values of K_m/V_{MAX} and $1/V_{MAX}$ on the inhibitor concentration.

Depending on the value of α, we have different types of "simple" inhibition: ***competitive inhibition***, ***noncopetitive inhibition*** and ***mixed-type inhibition***

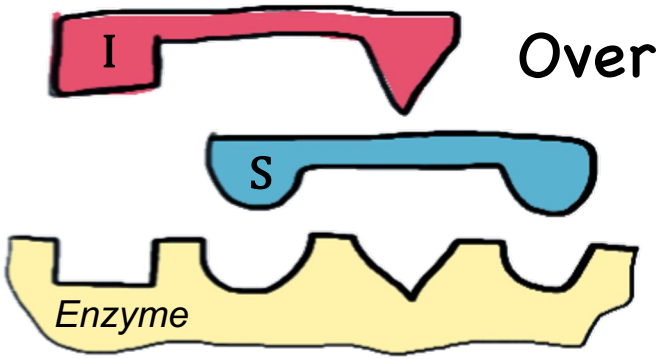
COMPETITIVE INHIBITION



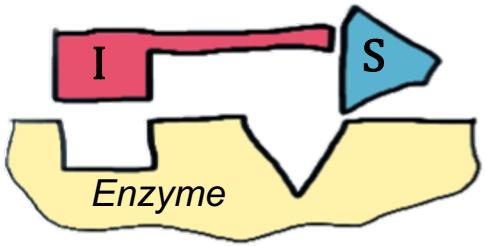
➢ When $\alpha=\infty$, we have **pure competitive inhibition**;



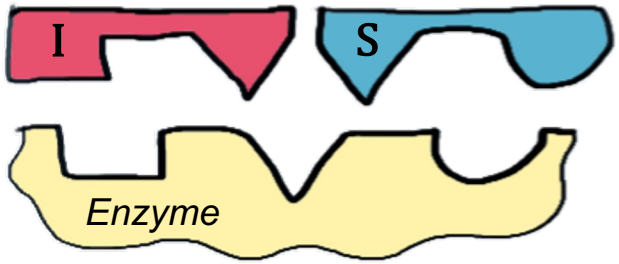
Classic



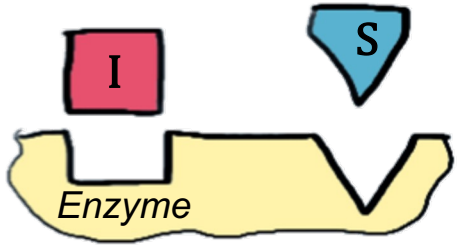
Overlapping

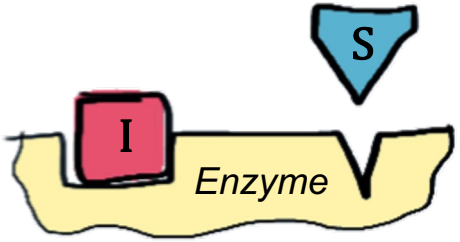


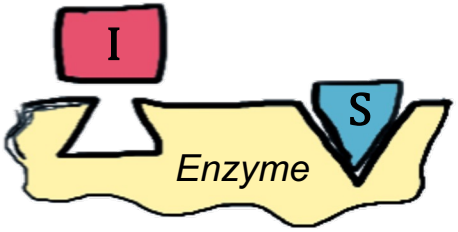
Steric hinderance



Common binding group

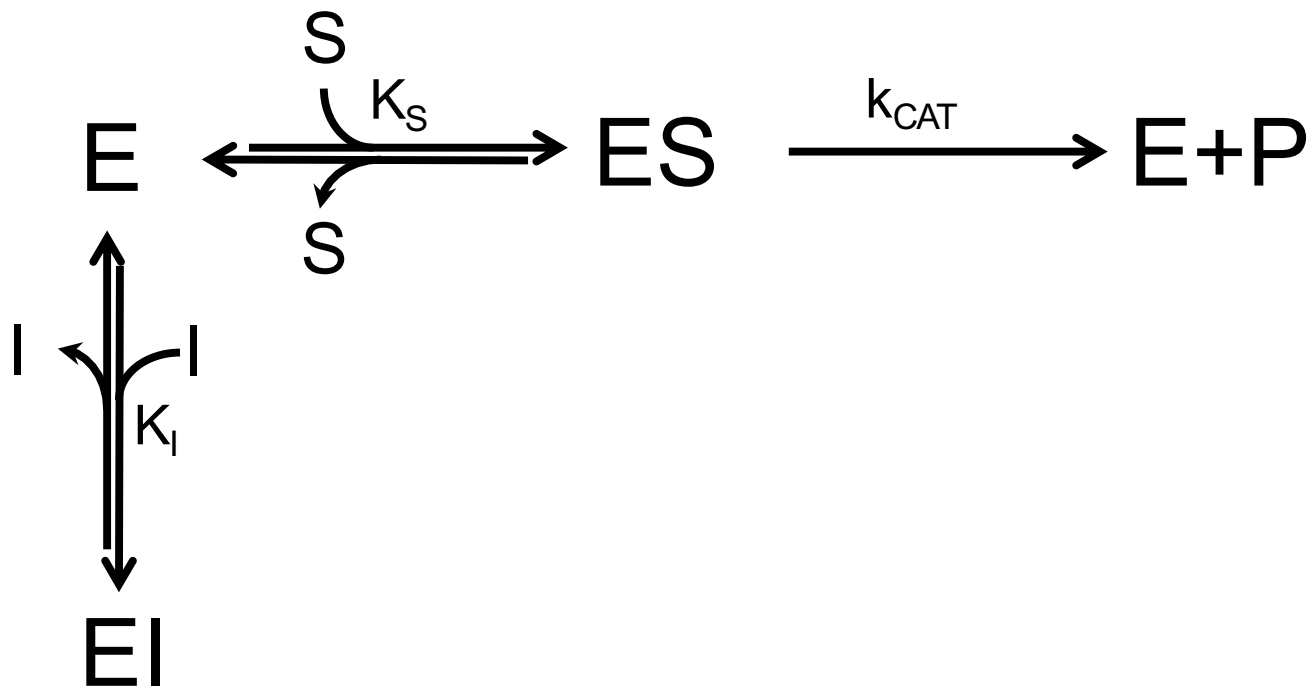






Allosteric inhibition

FEATURES OF COMPETITIVE INHIBITION

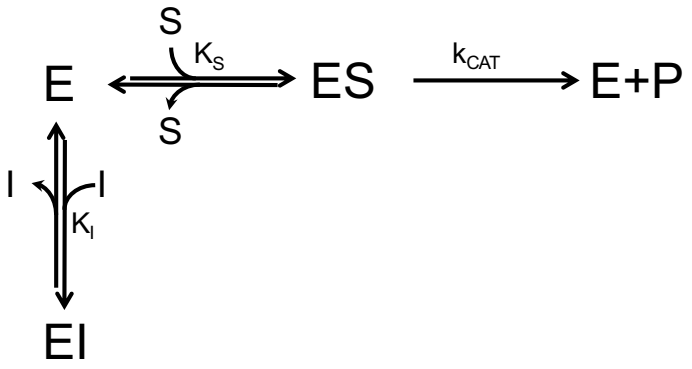


- apparent K_S (or K_M) depends on [I]
- V_{MAX} is independent of [I]

The *initial velocity equation* can be derived from the rapid equilibrium assuption.

$$v = k_{CAT}[ES]$$

$$K_S = \frac{[E][S]}{[ES]}$$



$$\frac{v}{[E]_t} = \frac{k_{CAT}[ES]}{[E] + [ES] + [EI]}$$

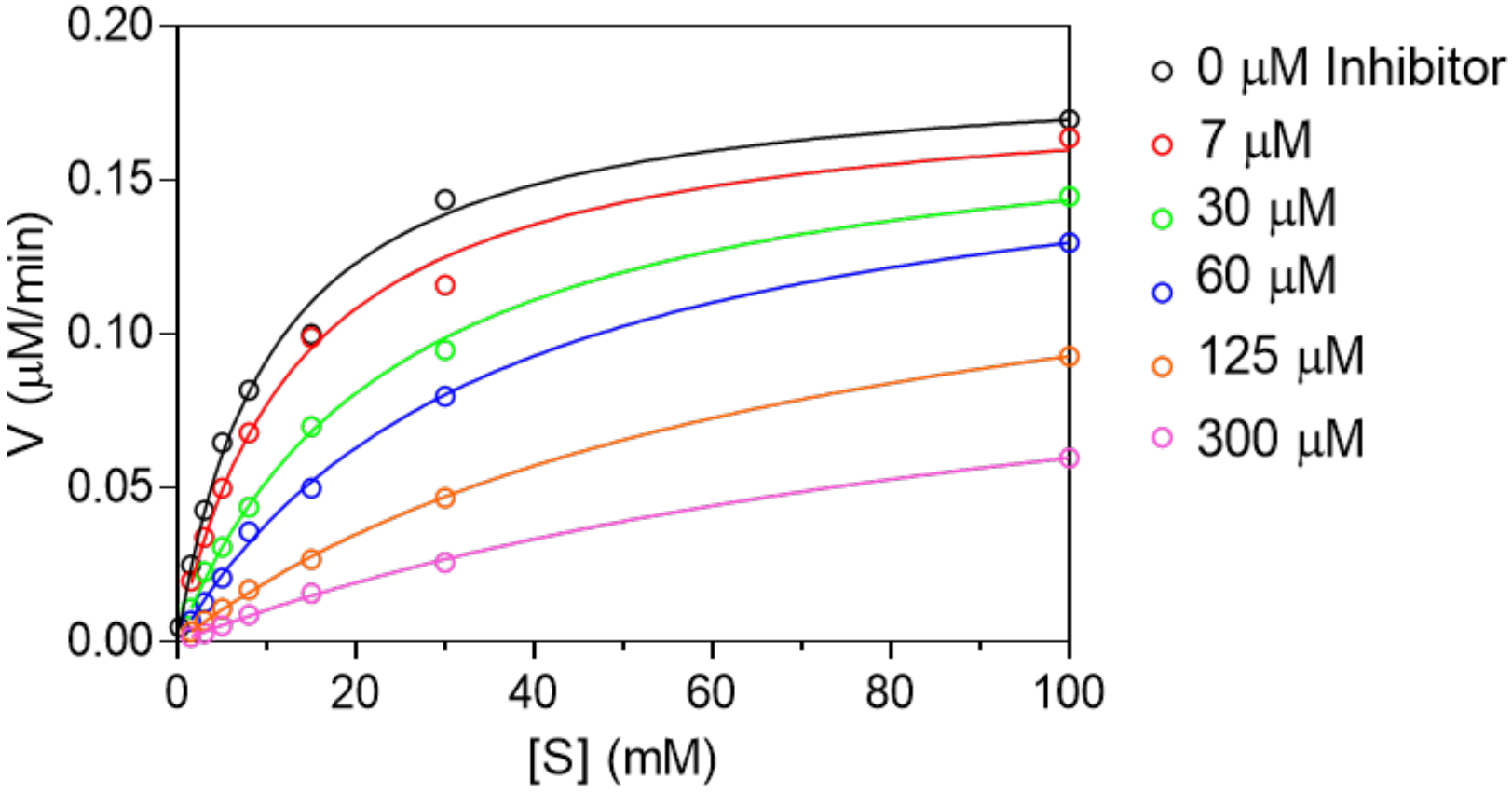
$$K_I = \frac{[E][I]}{[EI]}$$

$$\frac{v}{k_{CAT}[E]_t} = \frac{\frac{[S]}{K_S}}{1 + \frac{[S]}{K_S} + \frac{[I]}{K_I}}$$

$$\boldsymbol{v = V_{MAX}\frac{[S]}{\left(1 + \frac{[I]}{K_I}\right)K_S + [S]}}$$

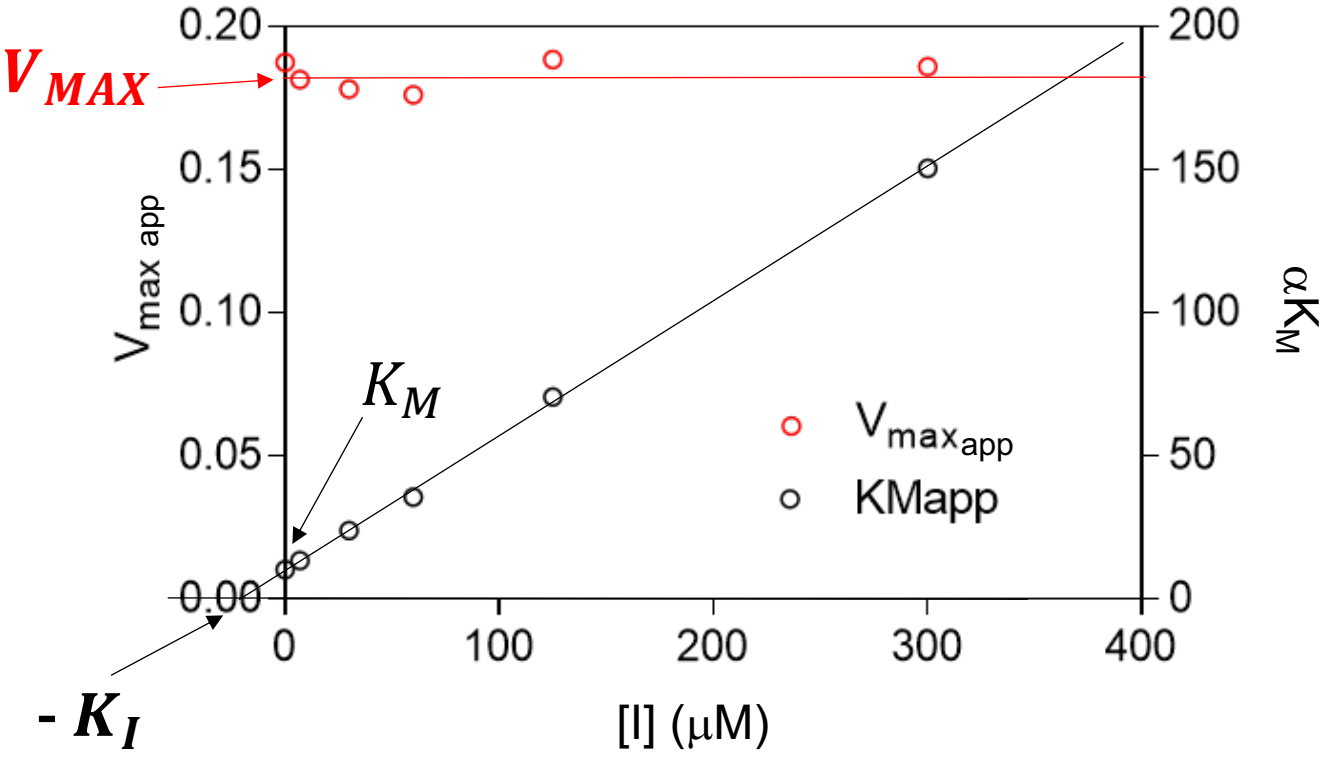
$$a = \left(1 + \frac{[I]}{K_I}\right) \qquad K_{M\,app} = aK_M \qquad K_{M\,app} = \left(1 + \frac{[I]}{K_I}\right)K_M$$

Understanding of inhibition model and determination of K_I by fitting the M&M equation to saturation curves



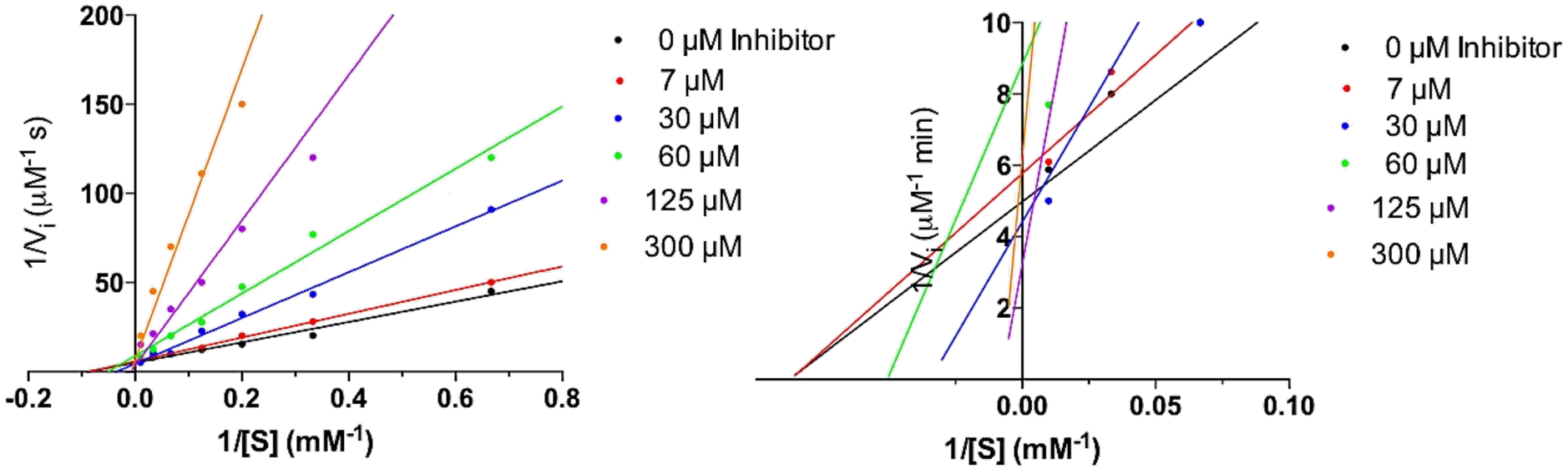
The fitting yields values of apparent K_M and V_{MAX}

PLOTS OF V_{MAXapp} AND K_{Mapp} *vs* [I] determined from fitting of saturation curves



$$aK_M = \left(1 + \frac{[I]}{K_I}\right) K_M \qquad aK_M = K_M + \frac{K_M}{K_I}[I]$$

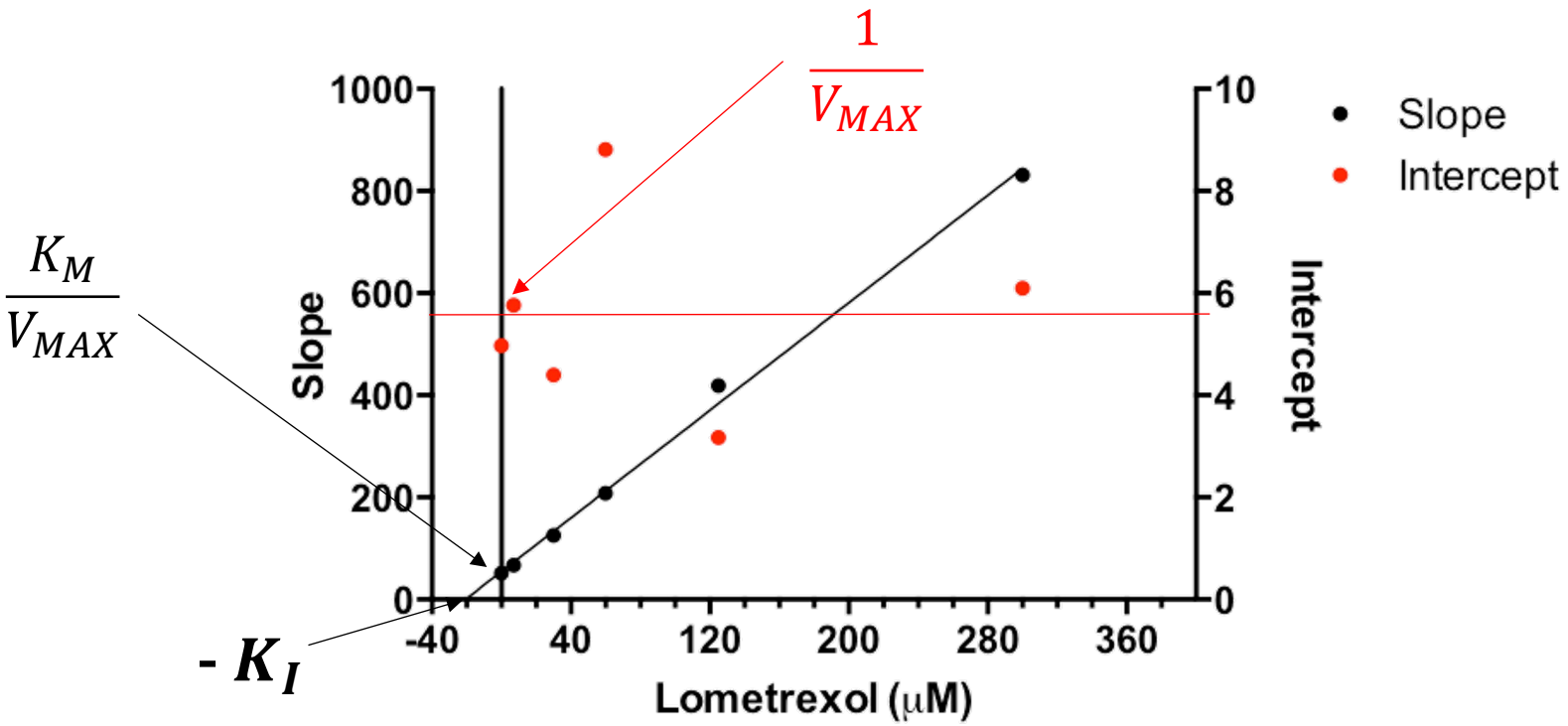
The outdated and inadequate use of the reciprocal plot



$$\frac{1}{v} = \frac{K_M}{V_{MAX}}\left(1 + \frac{[I]}{K_I}\right)\frac{1}{[S]} + \frac{1}{V_{MAX}}$$

$$\boldsymbol{slope = \frac{K_M}{V_{MAX}}\left(1 + \frac{[I]}{K_I}\right)} \qquad \boldsymbol{intercept\ on\ Y\ axis = \frac{1}{V_{MAX}}}$$

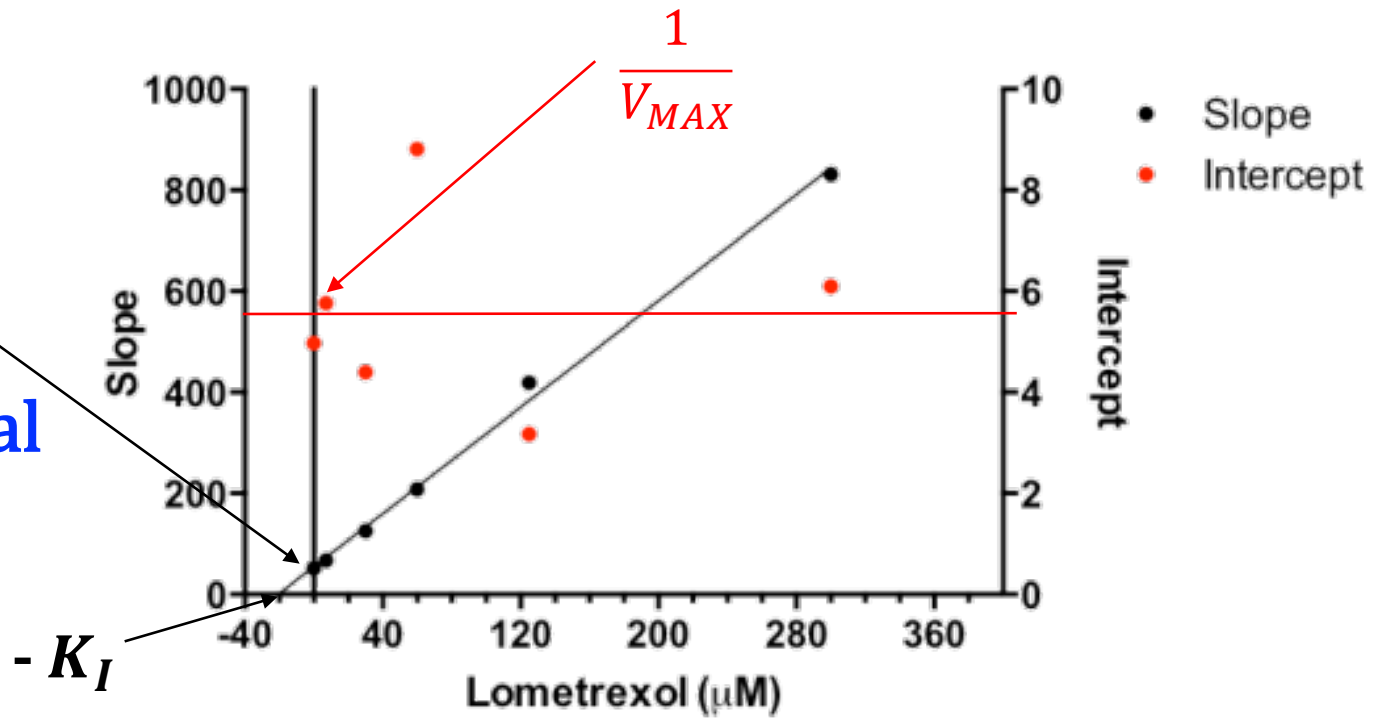
REPLOTS OF SLOPE AND Y-INTERCEPT



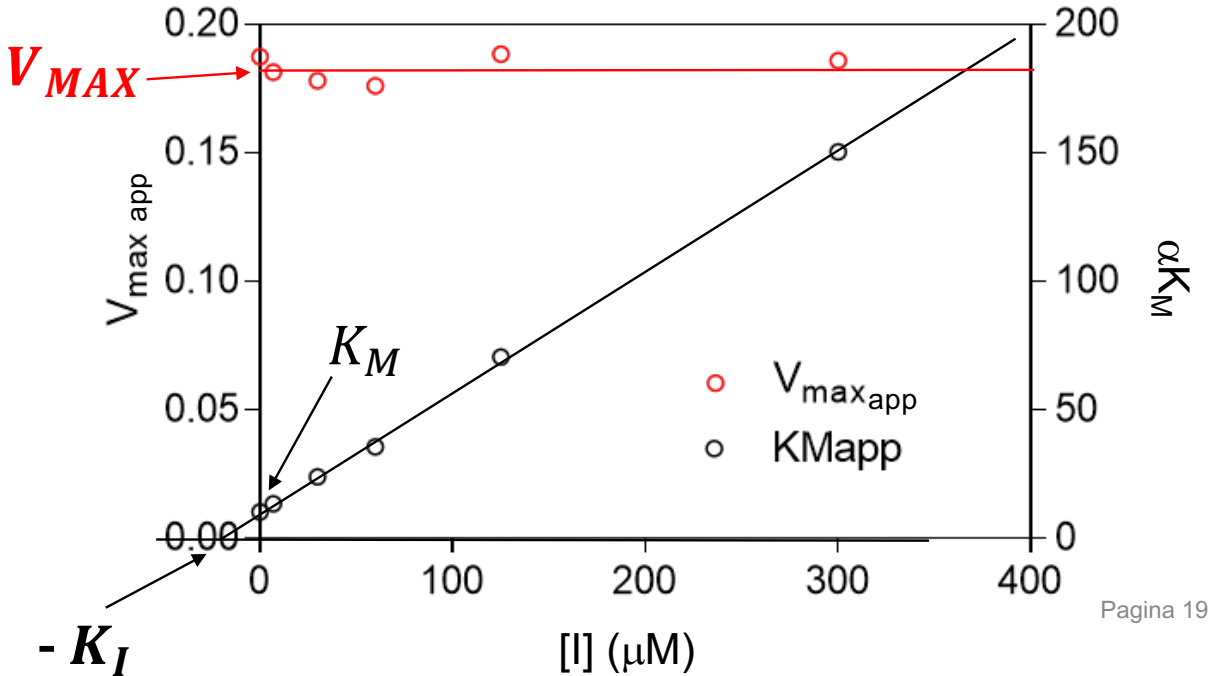
$$slope = \frac{K_M}{V_{MAX}}\left(1 + \frac{[I]}{K_I}\right)$$

$$intercept\ on\ Y\ axis = \frac{1}{V_{MAX}}$$

From reciprocal plot

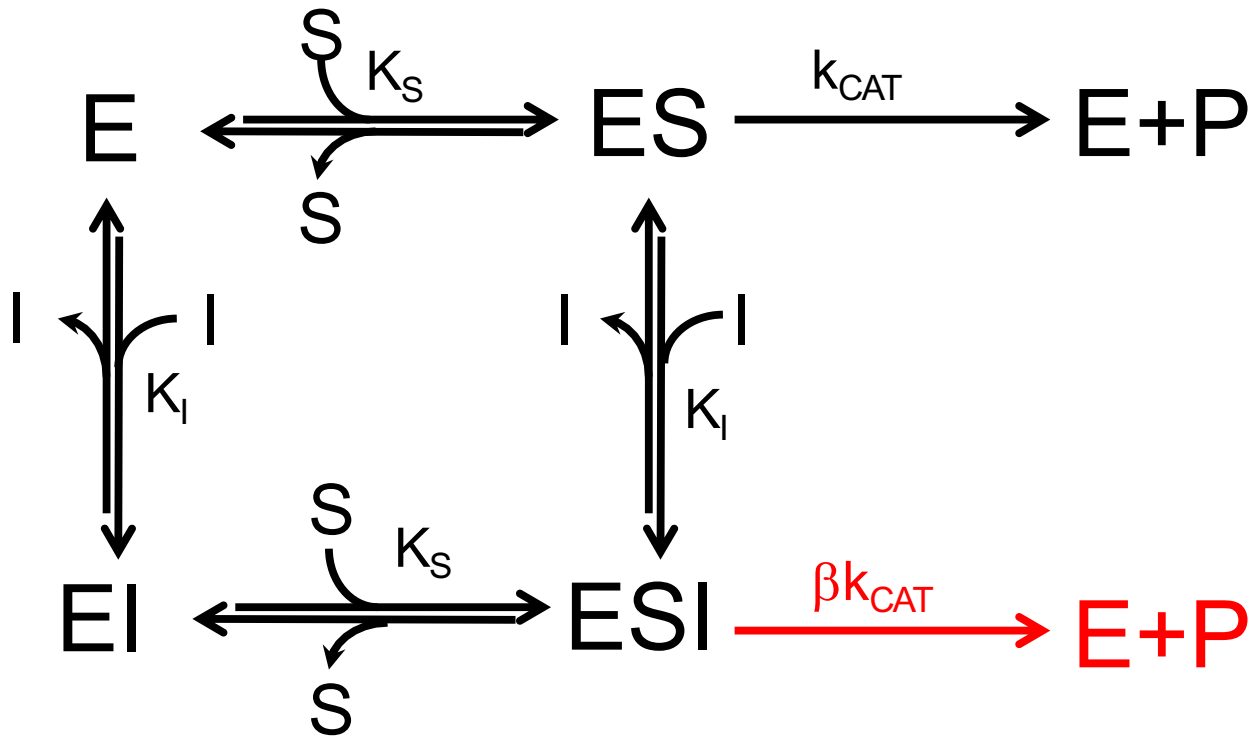


From fitting the M&M eq to saturation curves

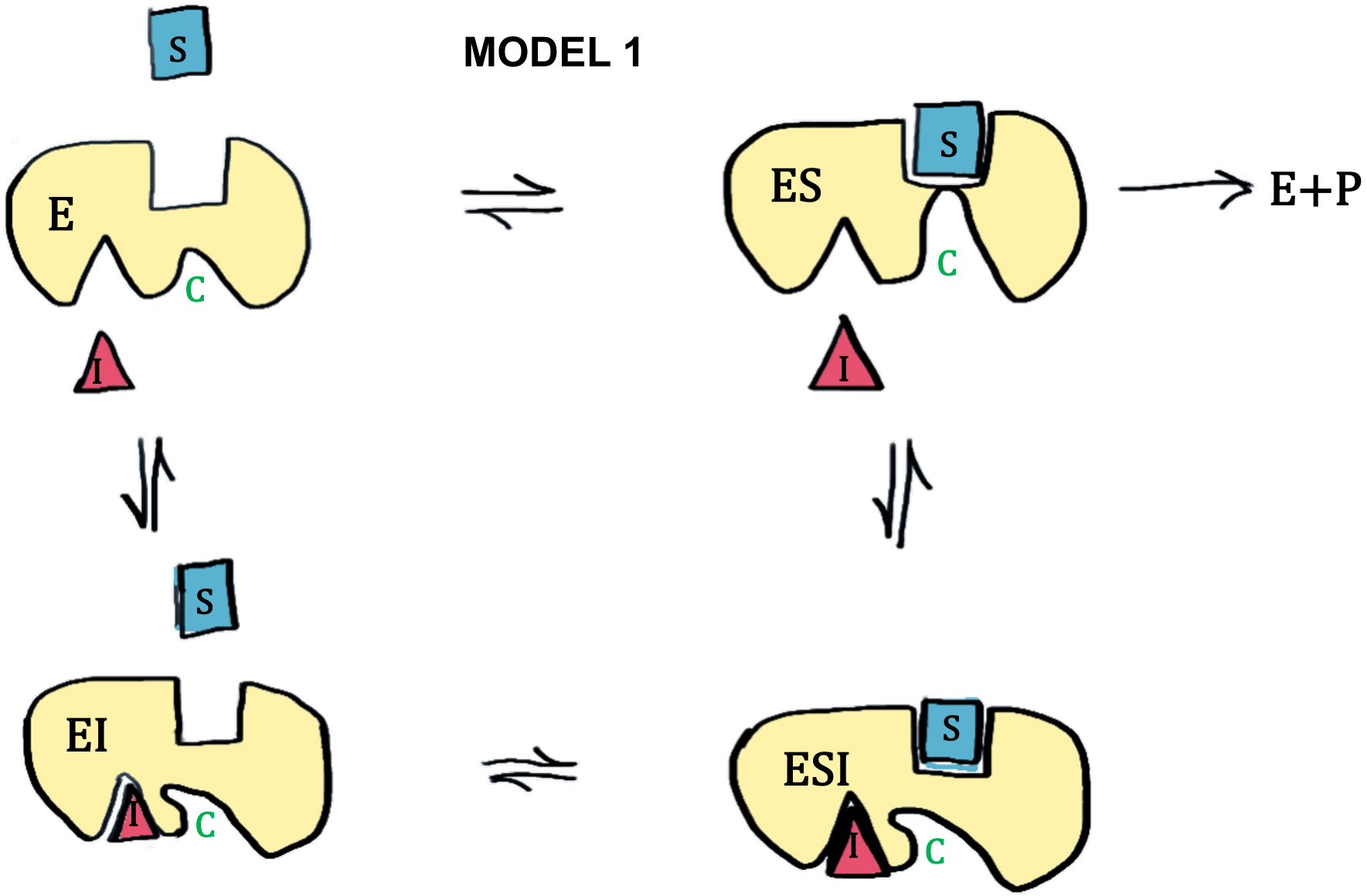


NONCOMPETITIVE INHIBITION

The inhibitor has no effect on substrate binding and vice-versa

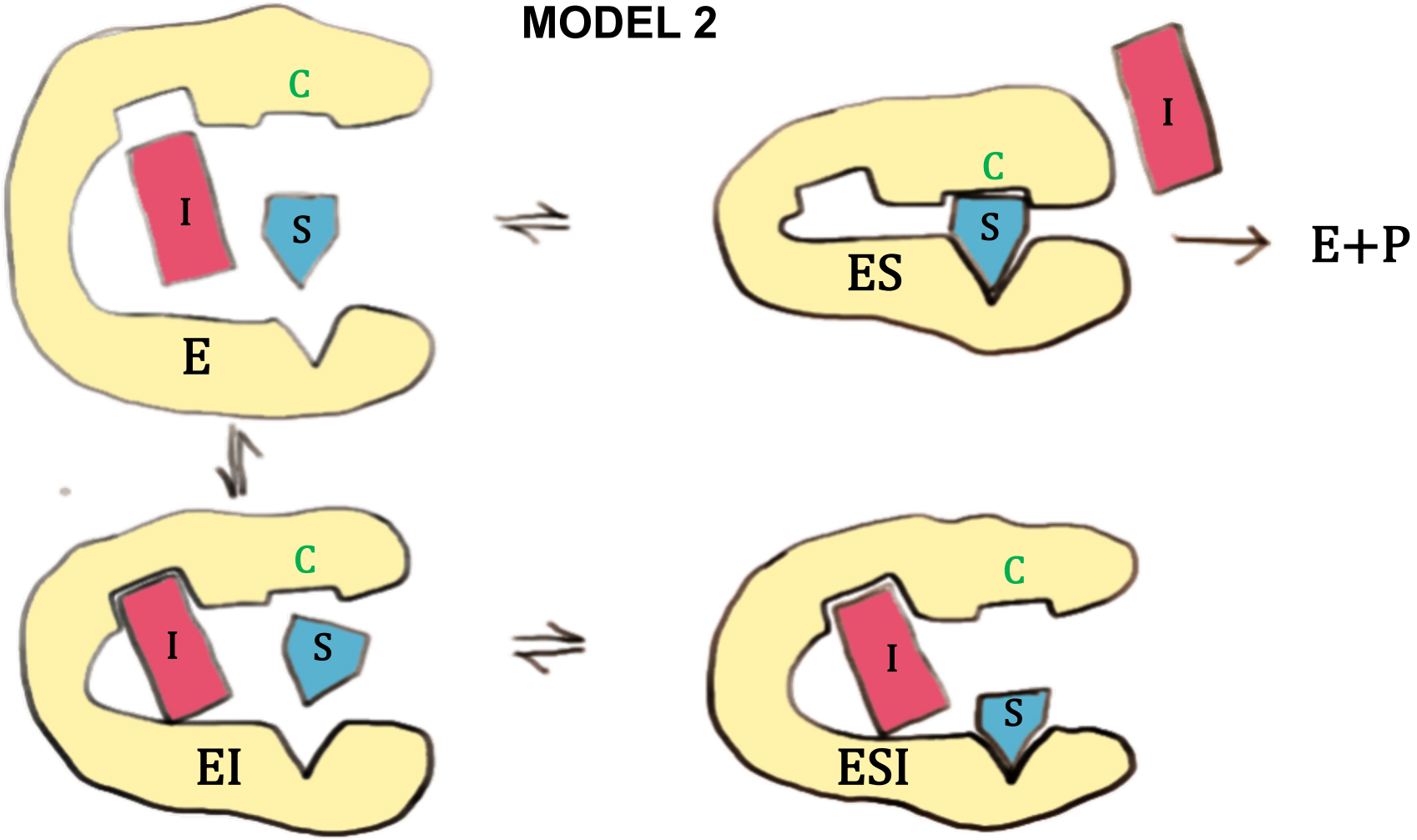


- When $\alpha=1$ and $\beta=0$, we have **pure noncompetitive inhibition**;



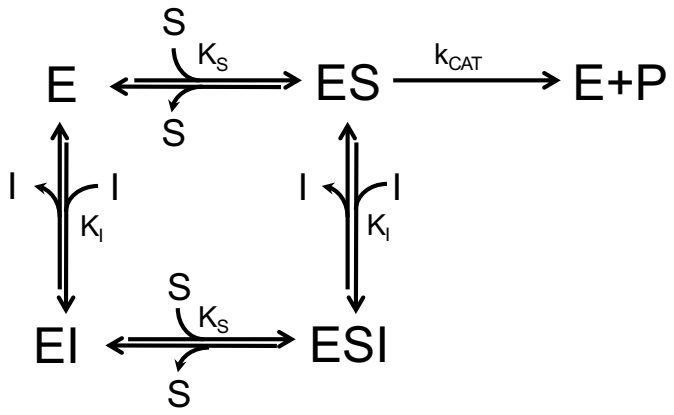
I interferes with the conformational change that alligns C but it has no effect on binding of S

MODEL 2

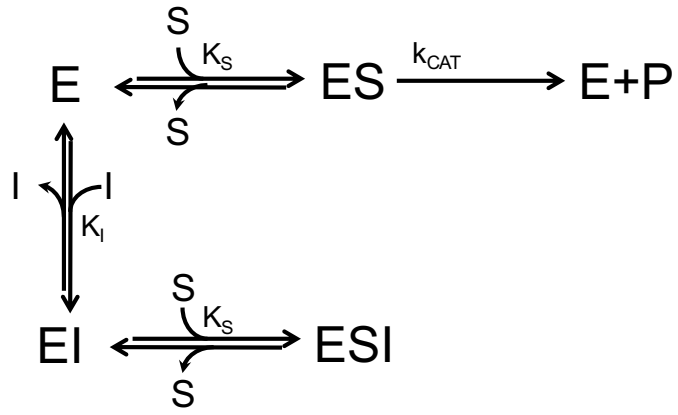


- I cannot bind to ES
- S binds to EI forming an inactive ESI complex

MODEL 1



MODEL 2

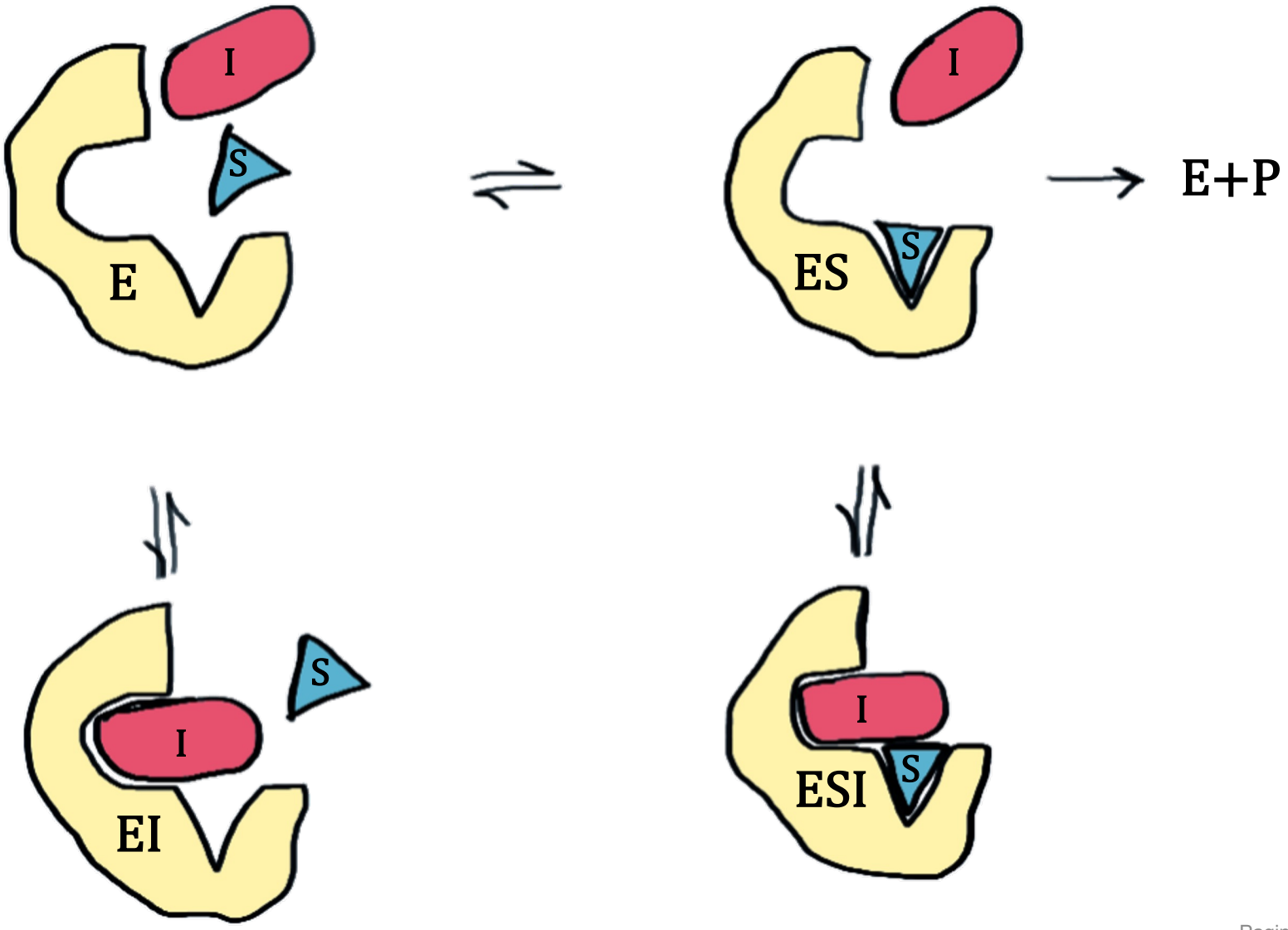


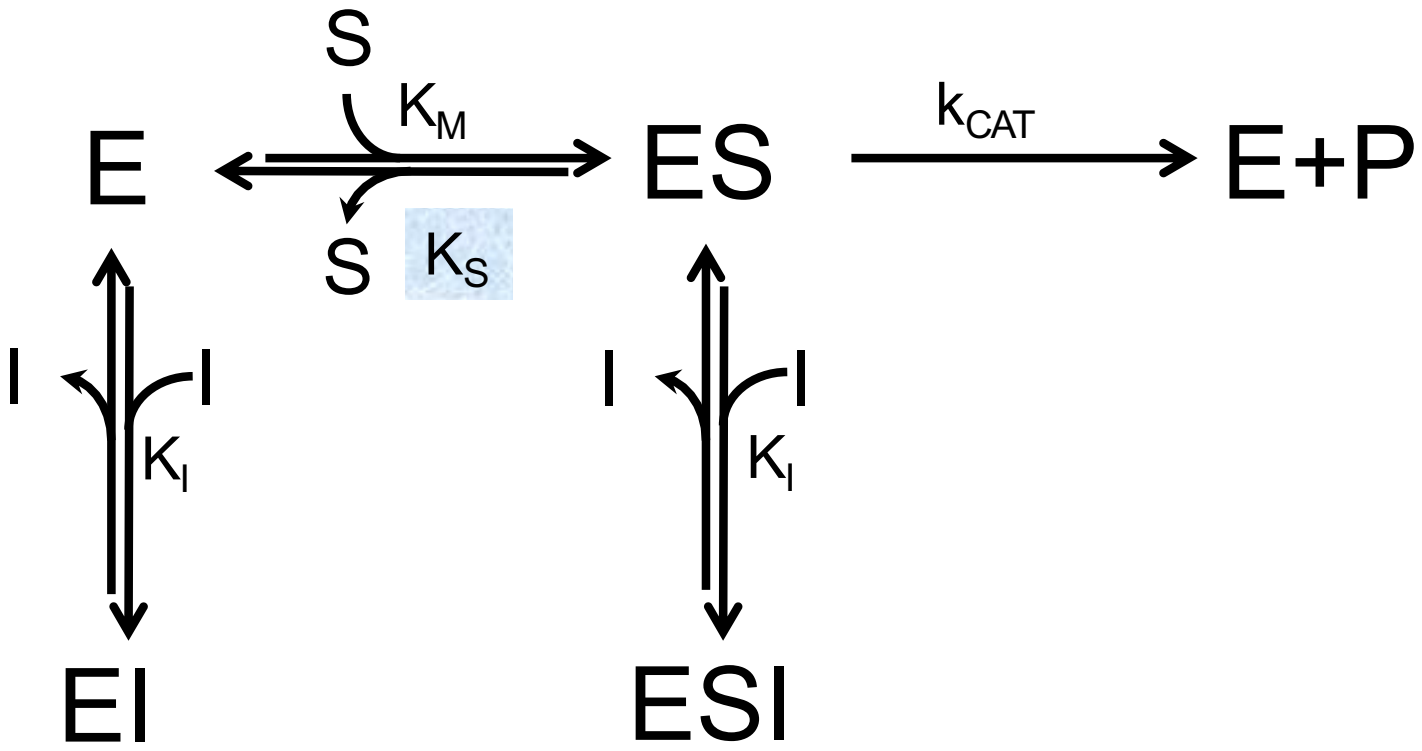
- The properties of models 1° and 2° are identical when the same four species are at rapid equilibrium

- **At any [I], an infinitely high [S] cannot drive all the enzyme to ES;** (a portion of the enzyme will remain as the non-productive **ESI** complex). Therefore, V_{max} in the presence of inhibitor will be lower;

- **The K_M (K_S) value will be unchanged**, since **E** and **EI** have equal affinity for **S**;

A 3rd MODEL is possible, in which **I** sterically hinders binding of **S**

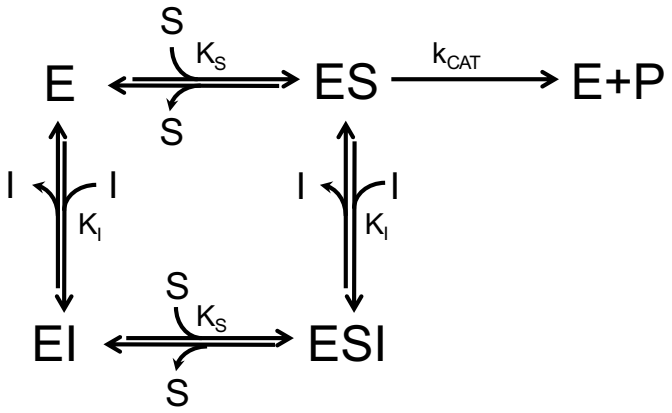




This is a situation that could be either at rapid equilibrium or at steady state and still give the same equation as model 1 and 2

The initial velocity equation can be derived from the rapid equilibrium assumption.

The equilibria are:



$$K_S = \frac{[E][S]}{[ES]} = \frac{[EI][S]}{[ESI]}$$

$$K_I = \frac{[E][I]}{[EI]} = \frac{[ES][I]}{[ESI]}$$

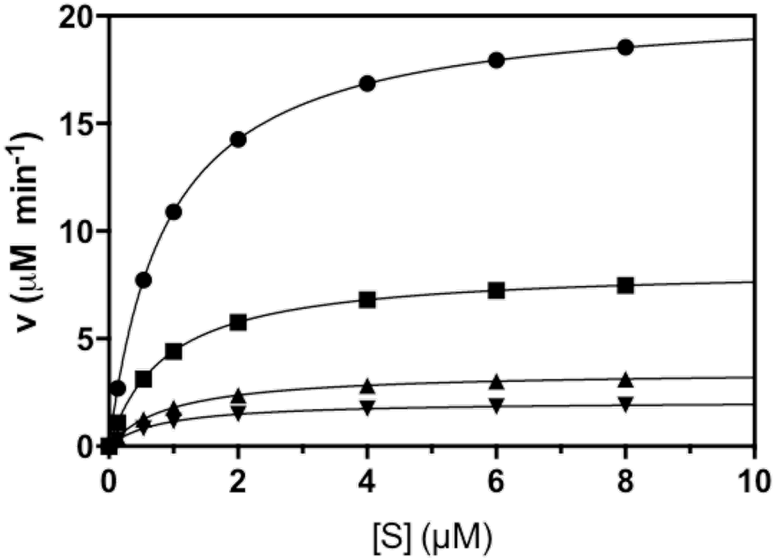
$$\frac{v}{k_{CAT}[E]_t} = \frac{\frac{[S]}{K_S}}{1 + \frac{[S]}{K_S} + \frac{[I]}{K_I} + \frac{[I][S]}{K_S K_i}}$$

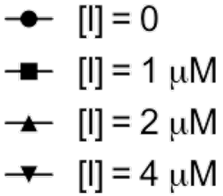
$$v = k_{CAT}[ES]$$

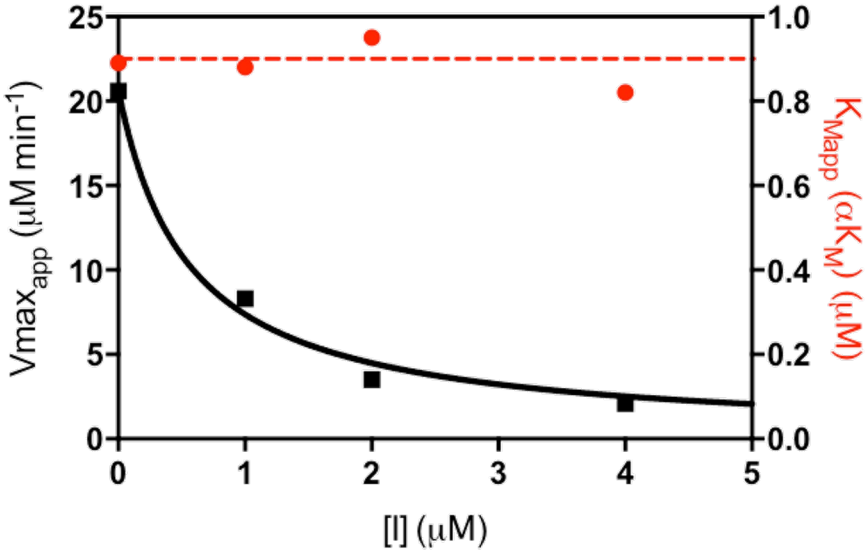
$$\boldsymbol{v = V_{MAX} \frac{[S]}{K_S\left(1 + \frac{[I]}{K_I}\right) + [S]\left(1 + \frac{[I]}{K_I}\right)}}$$

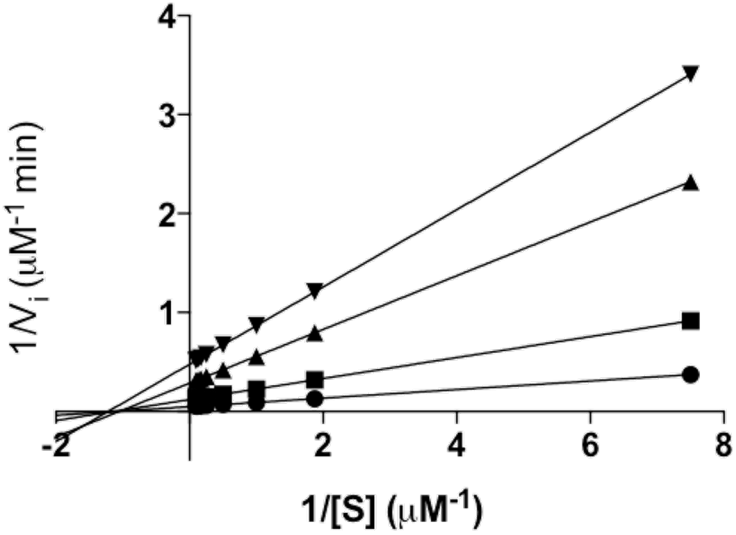
$$\frac{v}{[E]_t} = \frac{k_{CAT}[ES]}{[E] + [ES] + [EI] + [ESI]}$$

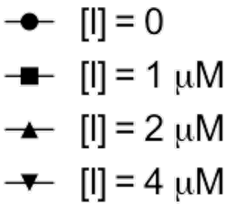
$$\boldsymbol{v = \frac{V_{MAX}}{\left(1 + \frac{[I]}{K_I}\right)} \frac{[S]}{(K_S + [S])}}$$


v (μM min-1)
[S] (μM)
0
5
10
15
20
0
2
4
6
8
10

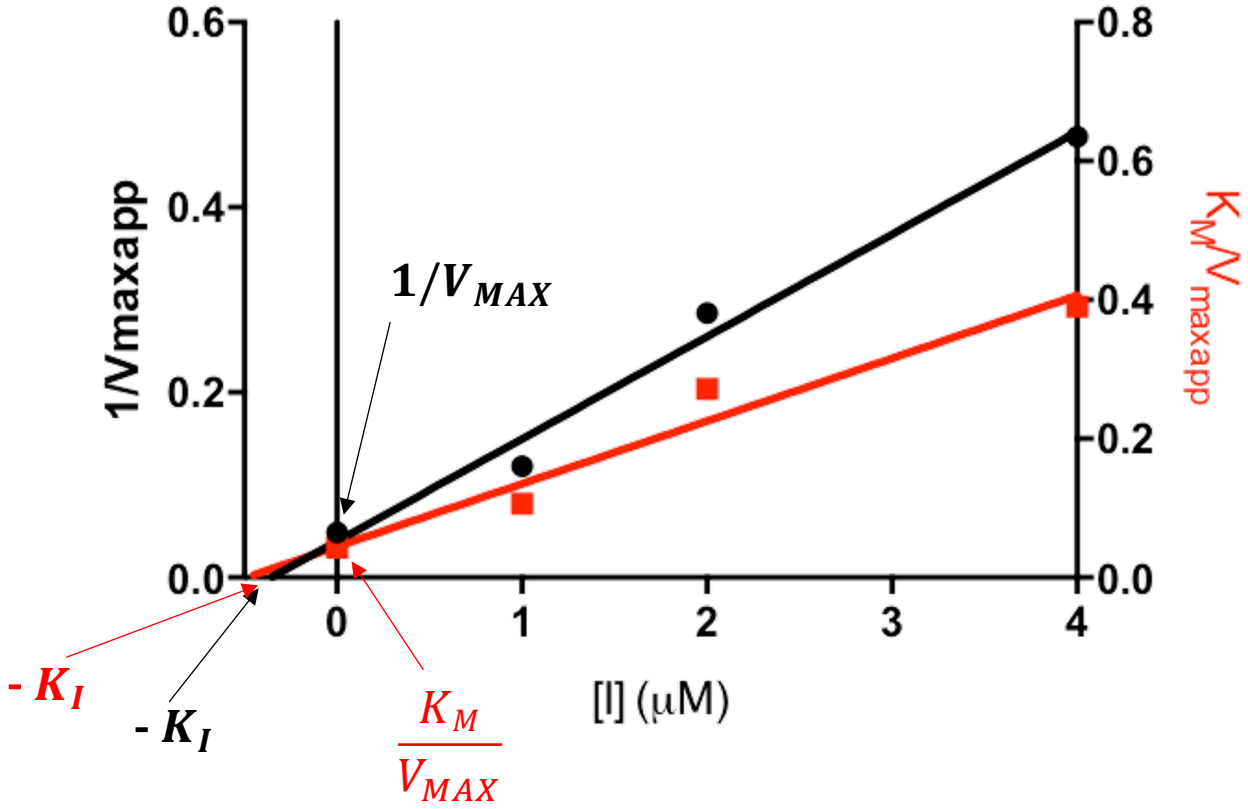

[I] = 0
[I] = 1 μM
[I] = 2 μM
[I] = 4 μM


Vmax_app (μM min-1)
K_Mapp (αK_M) (μM)
[I] (μM)
0
5
10
15
20
25
0.0
0.2
0.4
0.6
0.8
1.0
0
1
2
3
4
5


1/V_i (μM-1 min)
1/[S] (μM-1)
-2
1
2
3
4
2
4
6
8


[I] = 0
[I] = 1 μM
[I] = 2 μM
[I] = 4 μM

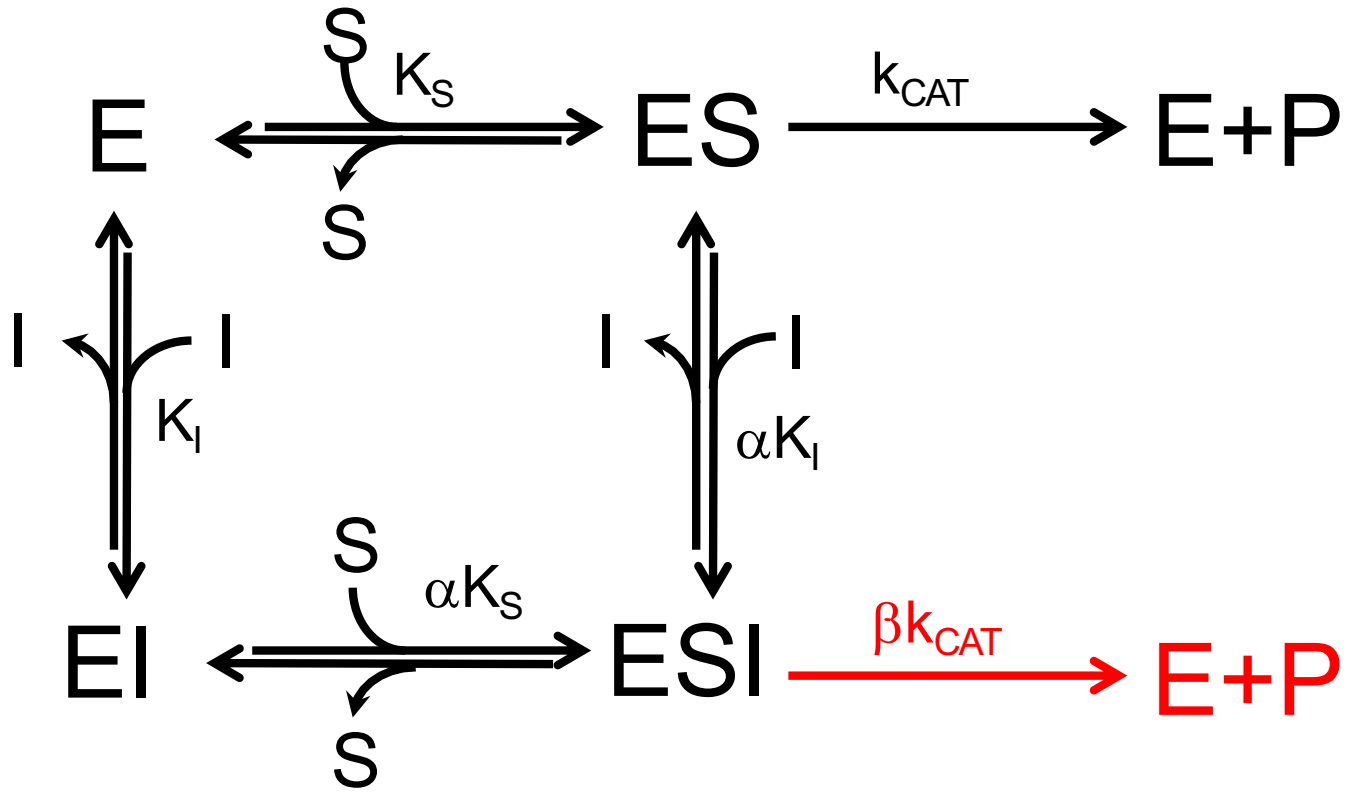
PLOTS OF INTERCEPTS ($1/V_{MAXapp}$) AND SLOPE (K_{mapp}/V_{MAX}) (obtained from fitting of hyperbolic saturation curves) vs [I]



$$\frac{1}{V_{MAXapp}} = \frac{1}{V_{MAX}K_I}[I] + \frac{1}{V_{MAX}}$$

$$\frac{K_M}{V_{MAXapp}} = \frac{K_M}{V_{MAX}K_I}[I] + \frac{K_M}{V_{MAX}}$$

LINEAR MIXED-TYPE INHIBITION



- When $1<\alpha<\infty$ and $\beta=0$, we have a ***linear <u>mixed-type</u> inhibition*** that may be considered as a ***mixture of pure competitive and pure noncompetitive inhibition***;
- I affects both V_{MAX} and K_M values

The initial velocity equation can be derived assuming rapid equilibrium conditions:

$$v = k_{CAT}[ES]$$

$$\frac{v}{[E]_t} = \frac{k_{CAT}[ES]}{[E] + [ES] + [EI] + [ESI]}$$

$$\frac{v}{k_{CAT}[E]_t} = \frac{\frac{[S]}{K_S}}{1 + \frac{[S]}{K_S} + \frac{[I]}{K_I} + \frac{[I][S]}{\alpha K_S K_i}}$$

$$K_S = \frac{[E][S]}{[ES]}; \quad \alpha K_S = \frac{[EI][S]}{[ESI]}$$

$$K_I = \frac{[E][I]}{[EI]}; \quad \alpha K_I = \frac{[ES][I]}{[ESI]}$$

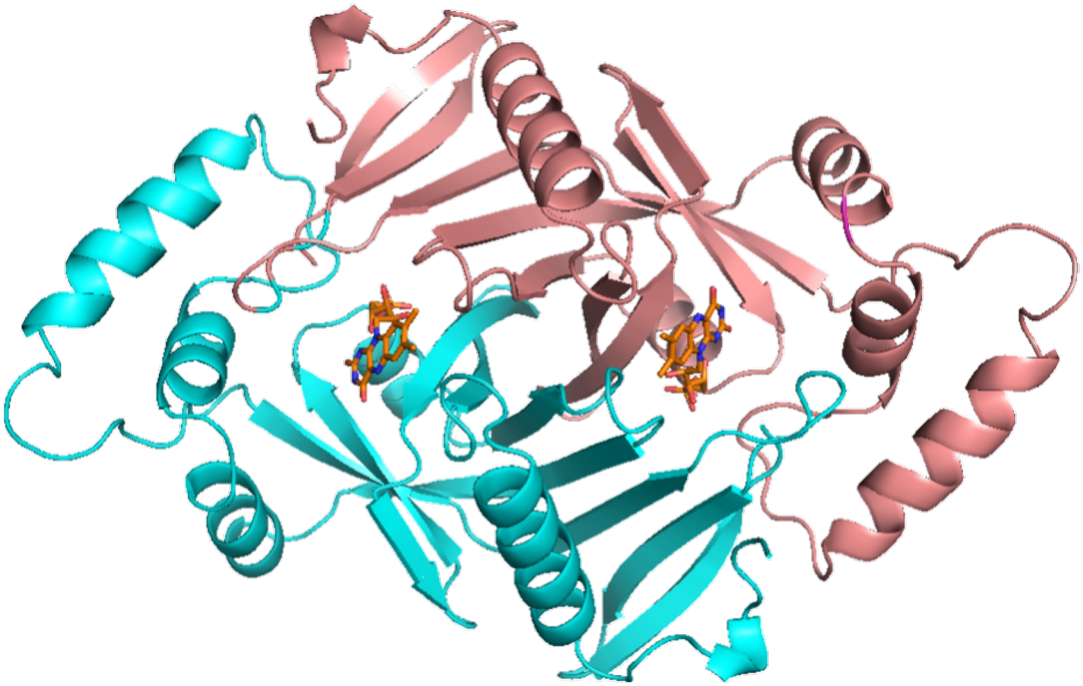
$$\boldsymbol{v = \frac{V_{MAX}}{\left(1 + \frac{[I]}{\alpha K_I}\right)} \frac{[S]}{K_S \frac{\left(1 + \frac{[I]}{K_I}\right)}{\left(1 + \frac{[I]}{\alpha K_I}\right)} + [S]}}$$

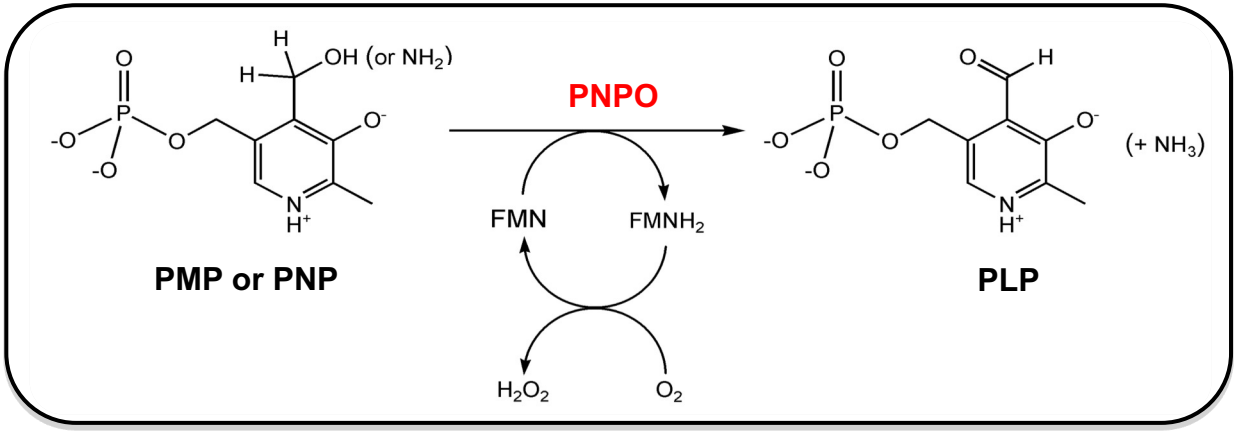
$$\boldsymbol{v = V_{MAX} \frac{[S]}{K_S\left(1 + \frac{[I]}{K_I}\right) + [S]\left(1 + \frac{[I]}{\alpha K_I}\right)}}$$

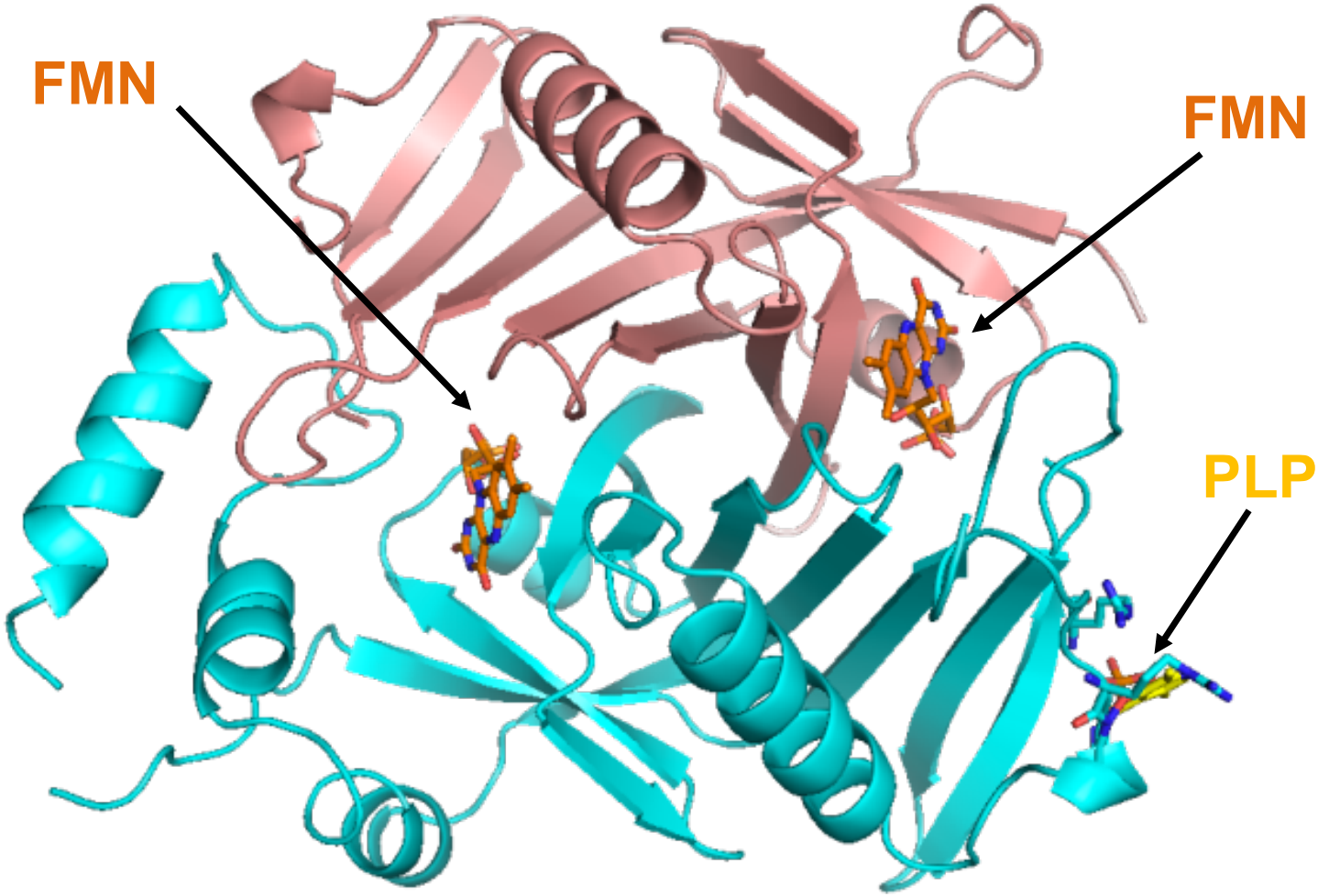
ALLOSTERIC INHIBITION CAN MANIFEST ITSELF AS LINEAR MIXED-TYPE INHIBITION

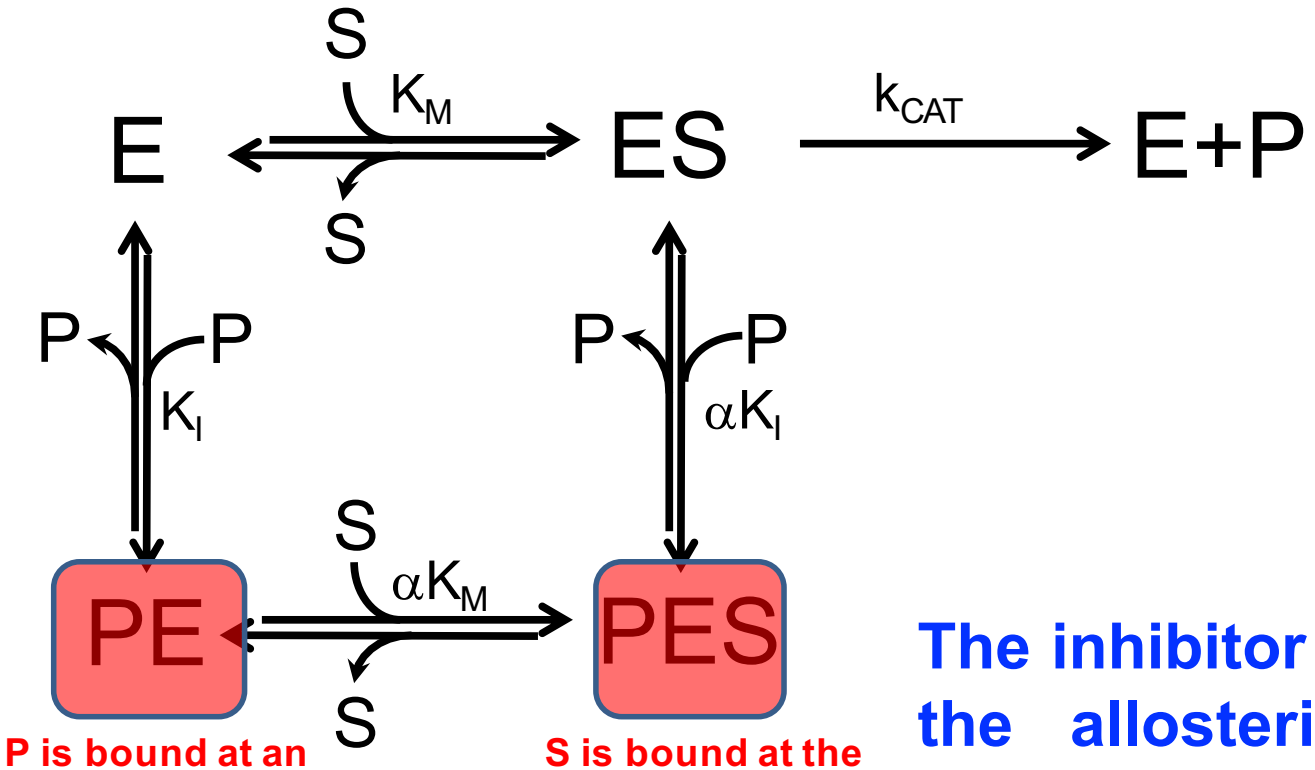
THE CASE OF PNP OXIDASE: ALLOSTERIC FEEDBACK INHIBITION

Pyridoxine 5’-phosphate oxidase (PNPO)





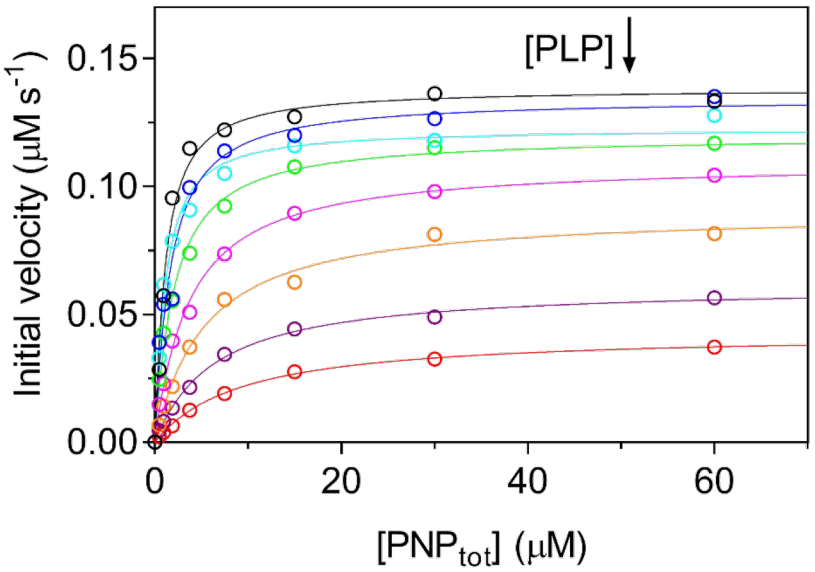

FMN
FMN
PLP



The inhibitor (P) only binds to the allosteric site. This is because the affinity for the allosteric site is much greater.

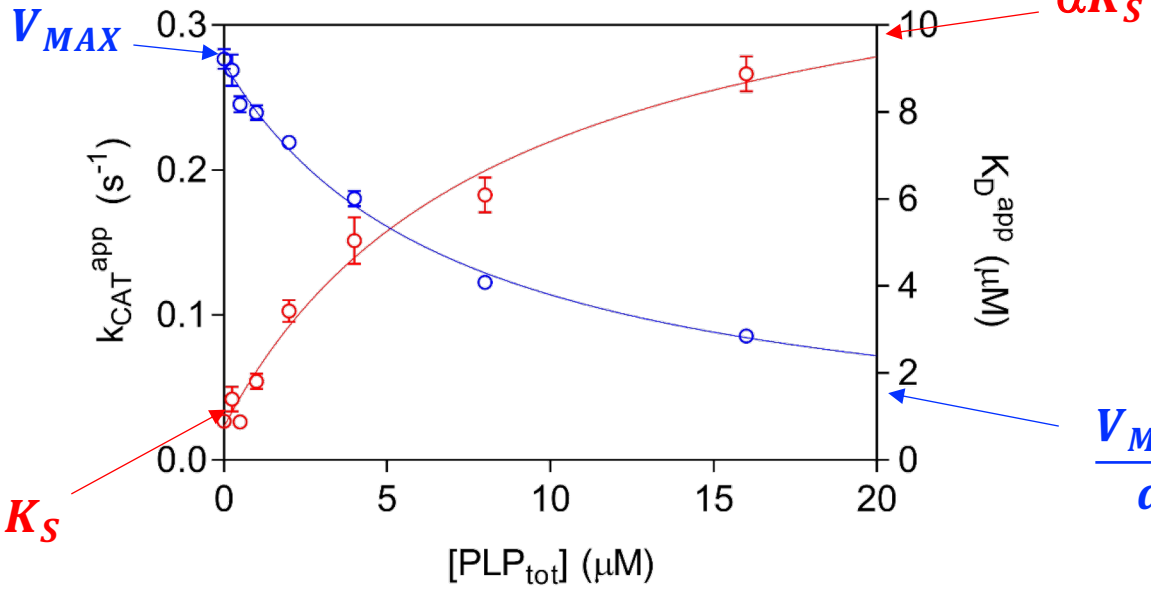
Once the inhibitor (P) has bound to the allosteric site its affinity for the active site is even lower, so that only the substrate can bind at the active site, forming the PES complex, which is inactive.

A

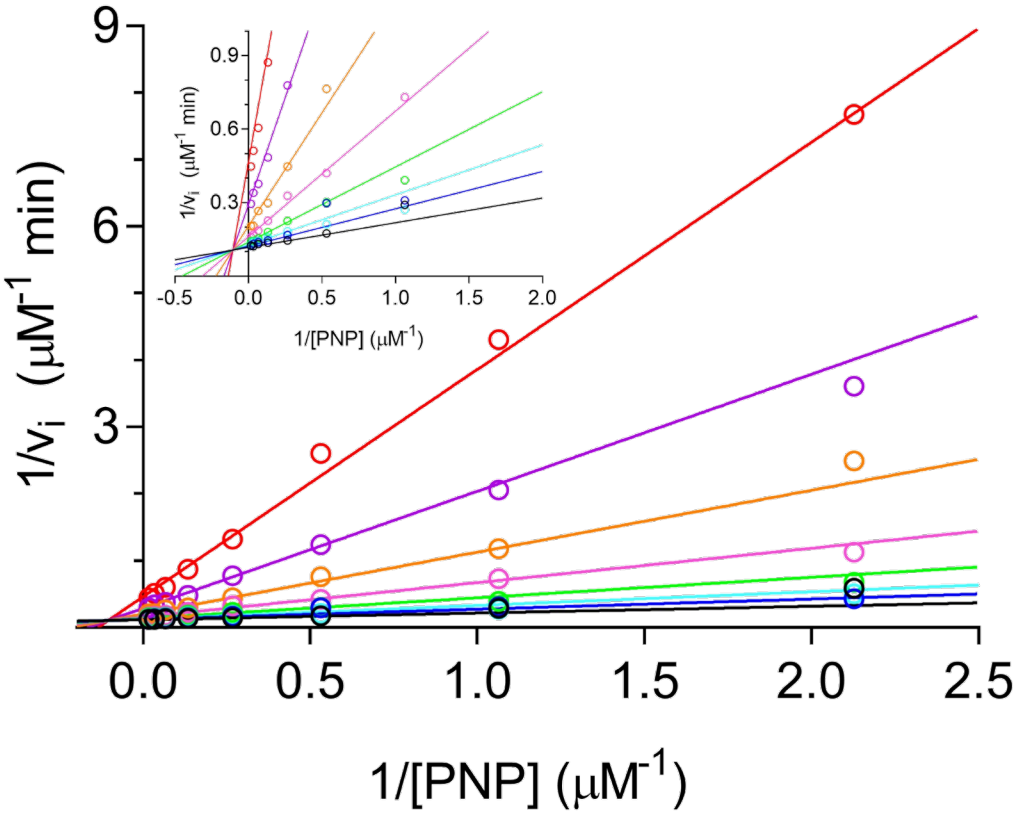


$$K_{Sapp} = K_S \frac{\left(1 + \frac{[I]}{K_I}\right)}{\left(1 + \frac{[I]}{\alpha K_I}\right)}$$

B

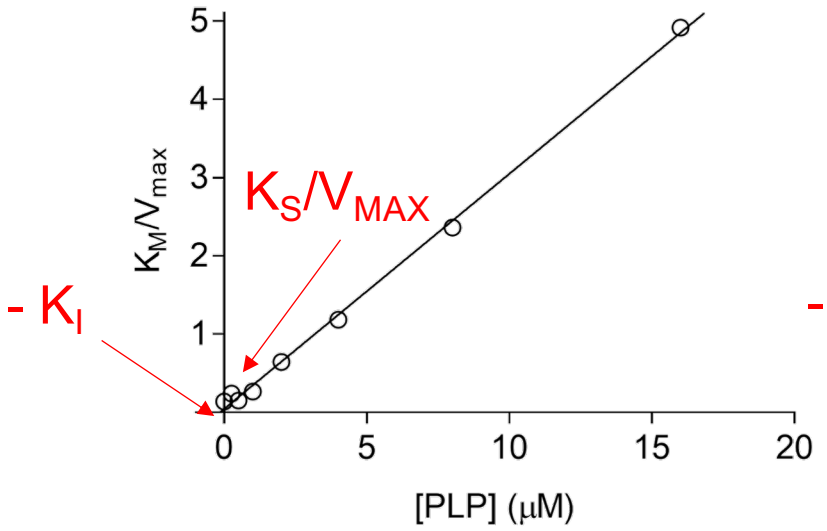


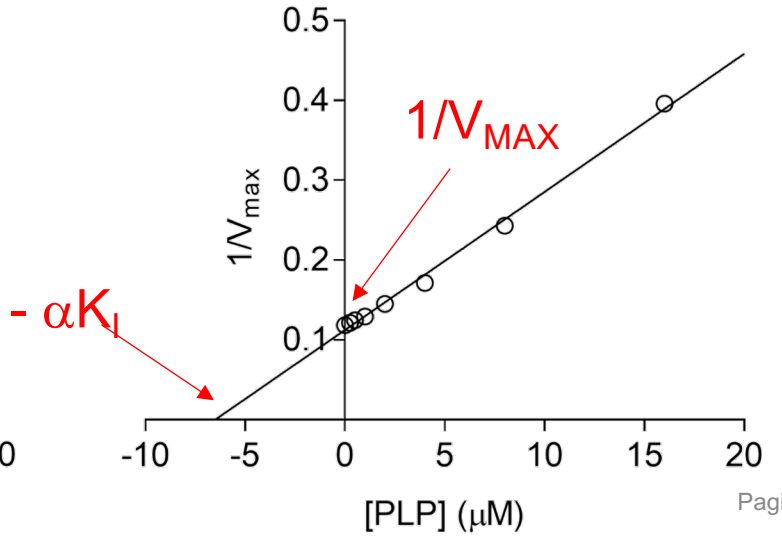
$$V_{MAXapp} = \frac{V_{MAX}}{\left(1 + \frac{[I]}{\alpha K_I}\right)}$$



$$\frac{K_{Sapp}}{V_{MAXapp}} = K_S \frac{\left(1 + \frac{[I]}{K_I}\right)}{V_{MAX}}$$

$$\frac{1}{V_{MAXapp}} = \frac{\left(1 + \frac{[I]}{\alpha K_I}\right)}{V_{MAX}}$$



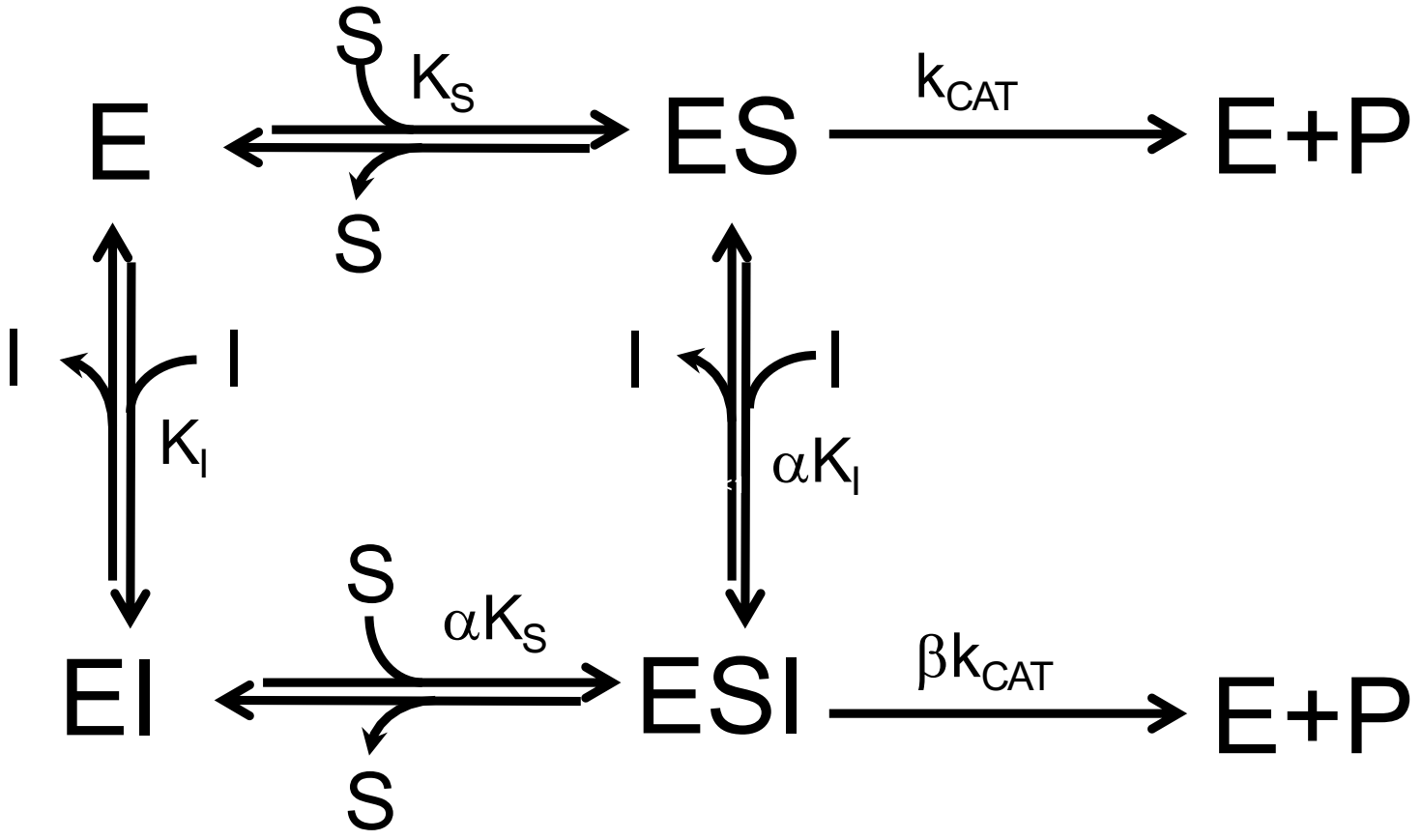


COMPLEX INHIBITION SYSTEMS in which $0<\beta<1$

Also called

"PARTIAL" INHIBITION SYSTEMS

In this cases $\beta \neq 0$



The inhibition is PARTIAL since even at an infinitely high [I] the velocity is not zero.

A general initial velocity equation can be derived assuming rapid equilibrium conditions:

$$v = k_{CAT}[ES] + \beta k_{CAT}[ESI]$$

$$\frac{v}{[E]_t} = \frac{k_{CAT}[ES] + \beta k_{CAT}[ESI]}{[E] + [ES] + [EI] + [ESI]}$$

$$\frac{v}{[E]_t} = \frac{k_{CAT}\frac{[S]}{K_S}[E] + \beta k_{CAT}\frac{[I][S]}{\alpha K_S K_I}[E]}{[E] + \frac{[S]}{K_S}[E] + \frac{[I]}{K_I}[E] + \frac{[I][S]}{\alpha K_S K_I}[E]}$$

$$\frac{v}{V_{MAX}} = \frac{\frac{[S]}{K_S} + \beta\frac{[I][S]}{\alpha K_S K_I}}{1 + \frac{[S]}{K_S} + \frac{[I]}{K_I} + \frac{[I][S]}{\alpha K_S K_I}}$$

$$K_S = \frac{[E][S]}{[ES]}; \quad \alpha K_S = \frac{[EI][S]}{[ESI]}$$

$$K_I = \frac{[E][I]}{[EI]}; \quad \alpha K_I = \frac{[ES][I]}{[ESI]}$$

$$\boldsymbol{v = V_{MAX}\frac{[S]}{K_S\frac{\left(1+\frac{[I]}{K_I}\right)}{\left(1+\frac{\beta[I]}{\alpha K_I}\right)} + [S]\frac{\left(1+\frac{[I]}{\alpha K_I}\right)}{\left(1+\frac{\beta[I]}{\alpha K_I}\right)}}}$$

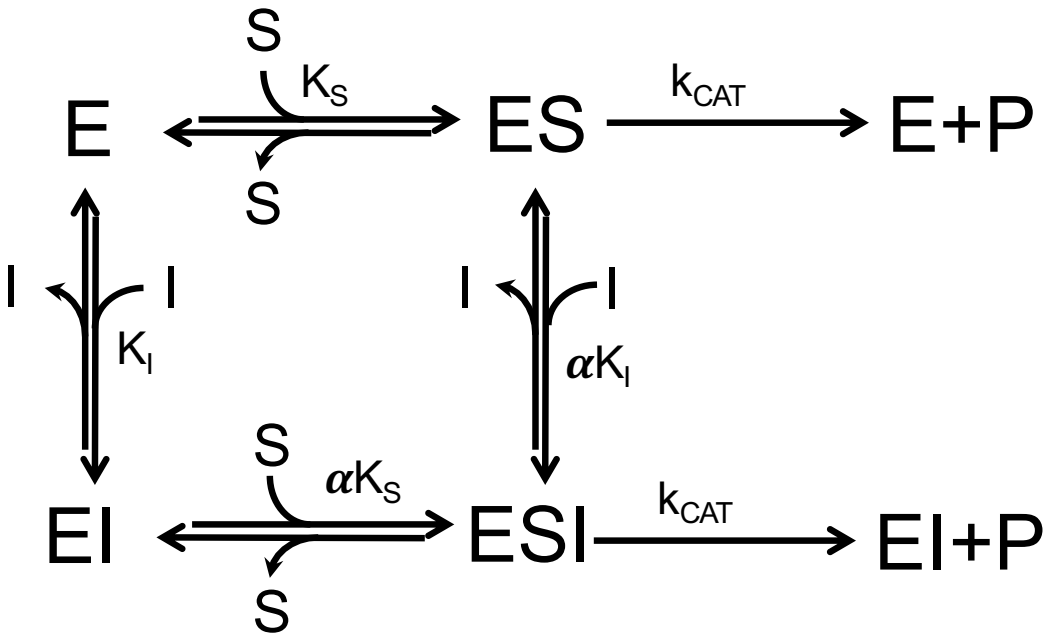
$$V_{MAXapp} = V_{MAX}\frac{\left(1+\frac{\beta[I]}{\alpha K_I}\right)}{\left(1+\frac{[I]}{\alpha K_I}\right)} \qquad K_{Sapp} = K_S\frac{\left(1+\frac{[I]}{K_I}\right)}{\left(1+\frac{[I]}{\alpha K_I}\right)}$$

The inhibition is PARTIAL since at an infinitely high [I], the velocity equation reduces to:

$$v = \beta V_{MAX}\frac{[S]}{\alpha K_S + [S]}$$

PARTIAL COMPETITIVE INHIBITION

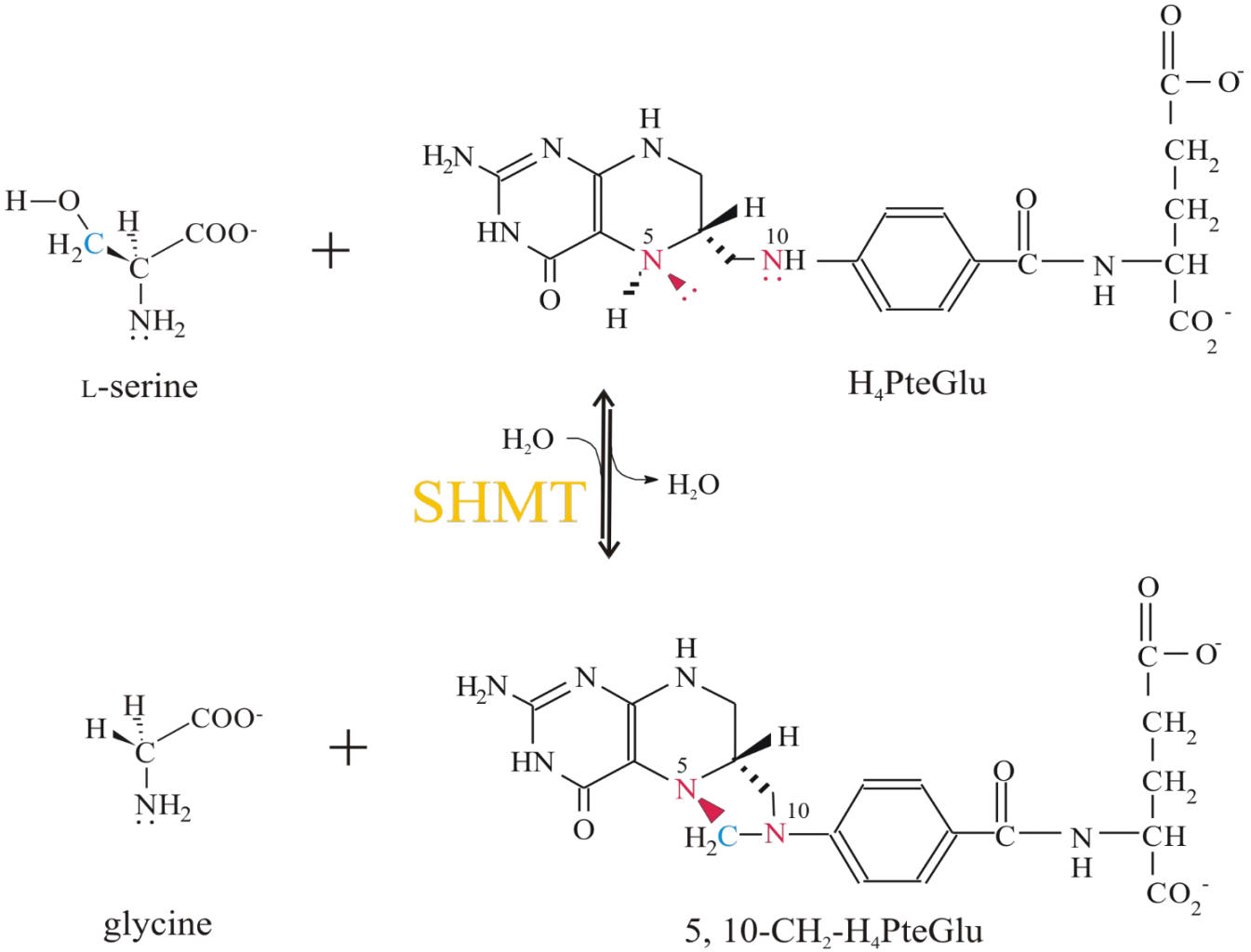
$(1<\alpha<\infty \text{ and } \beta=1)$



- **ES** and **ESI** complexes both yield product with the same k_{CAT};
- **V_{MAX} is unchanged but K_{Sapp} varies from K_S to a limit of αK_S** as [I] is increased from 0 to infinite
- ***v* cannot be driven to 0 by increasing [I].**

The moonlighting RNA-binding activity of cytosolic serine hydroxymethyltransferase contributes to control compartmentalization of serine metabolism.

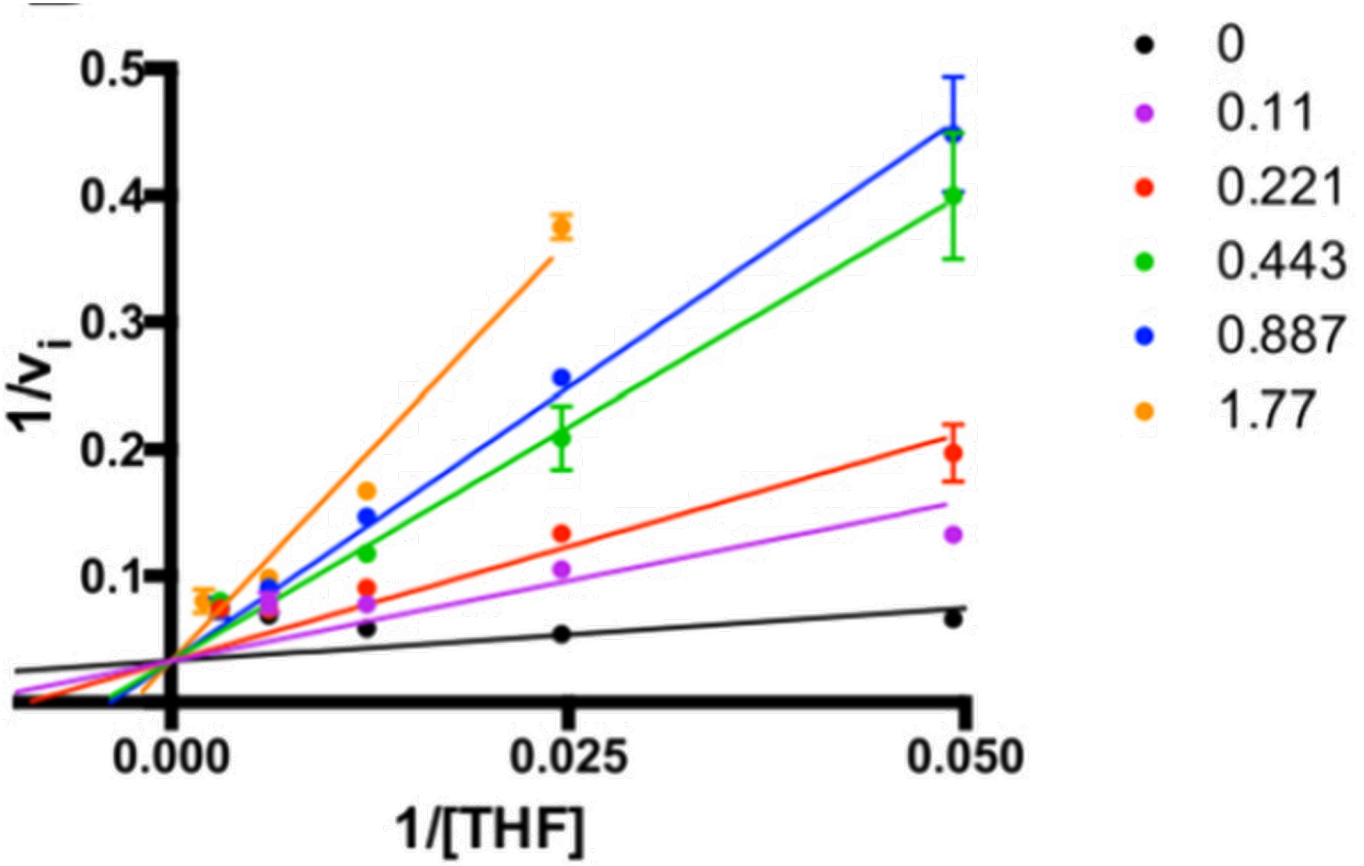
Guiducci et al. (2019) Nucleic Acids Research, 2019, Vol. 47, No. 8



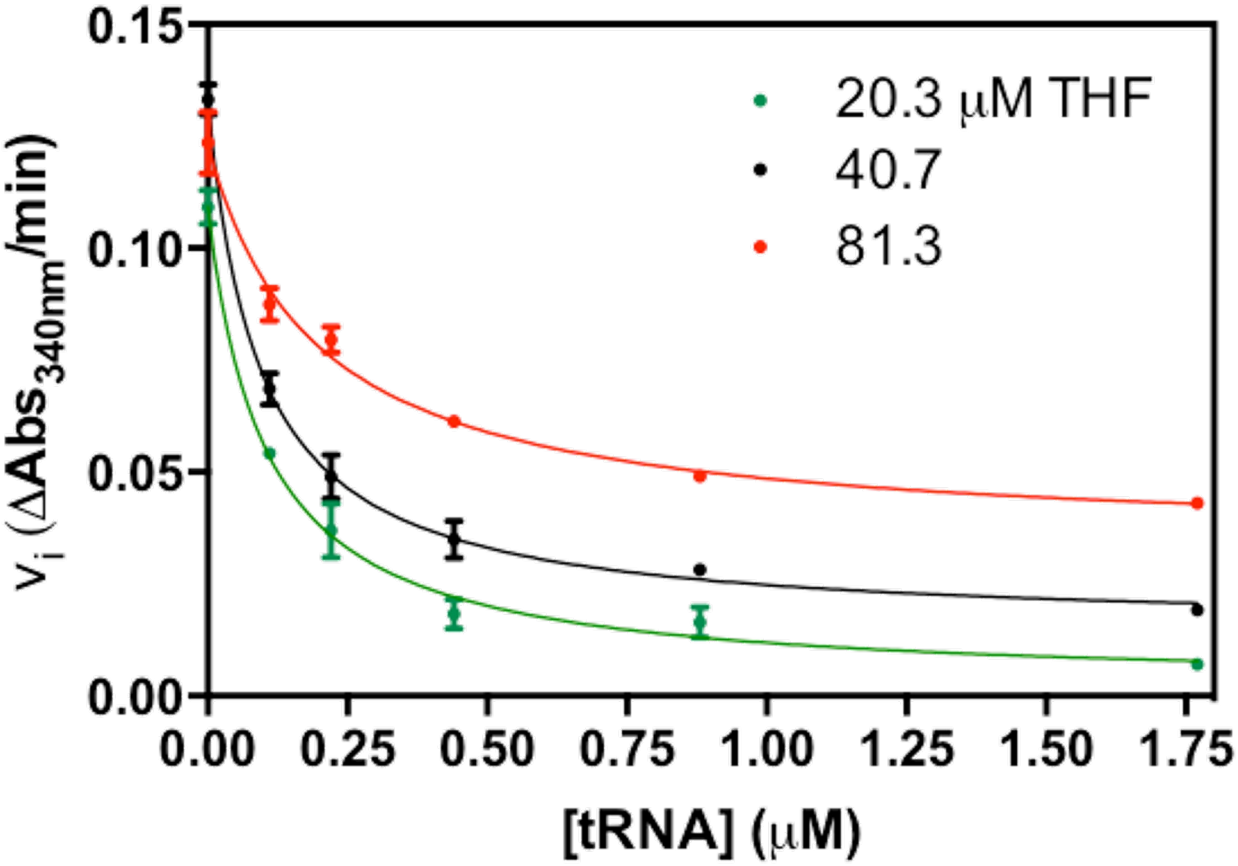
At saturating **[L-Ser]** concentration, no free enzyme is present and the enzyme mainly exists as the **E·L-Ser** complex

This system can be treated as a one-substrate system

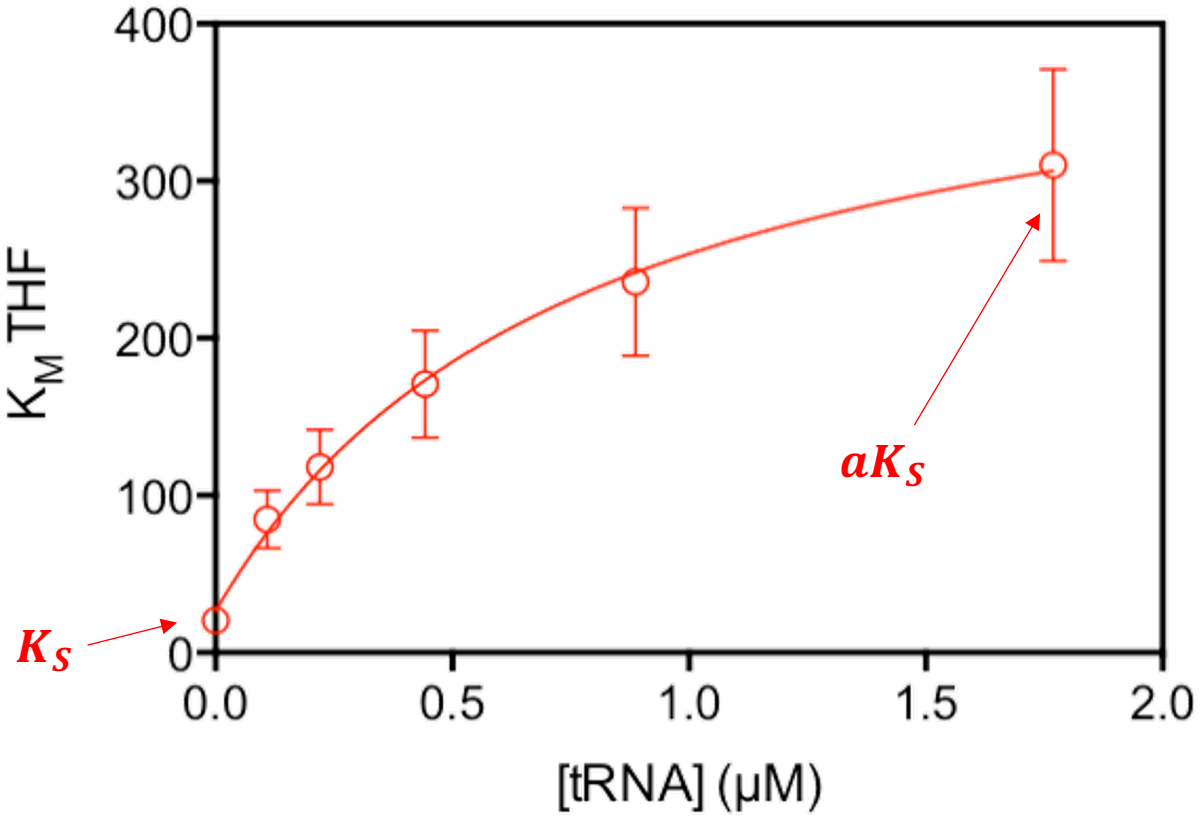
Fixed saturating [L-Ser] 10 mM



- V_{MAX} is constant as in pure competitive inhibition



- At constant [THF] (constant [S]), as [I] is increased to infinity, the velocity of the reaction cannot be driven to zero



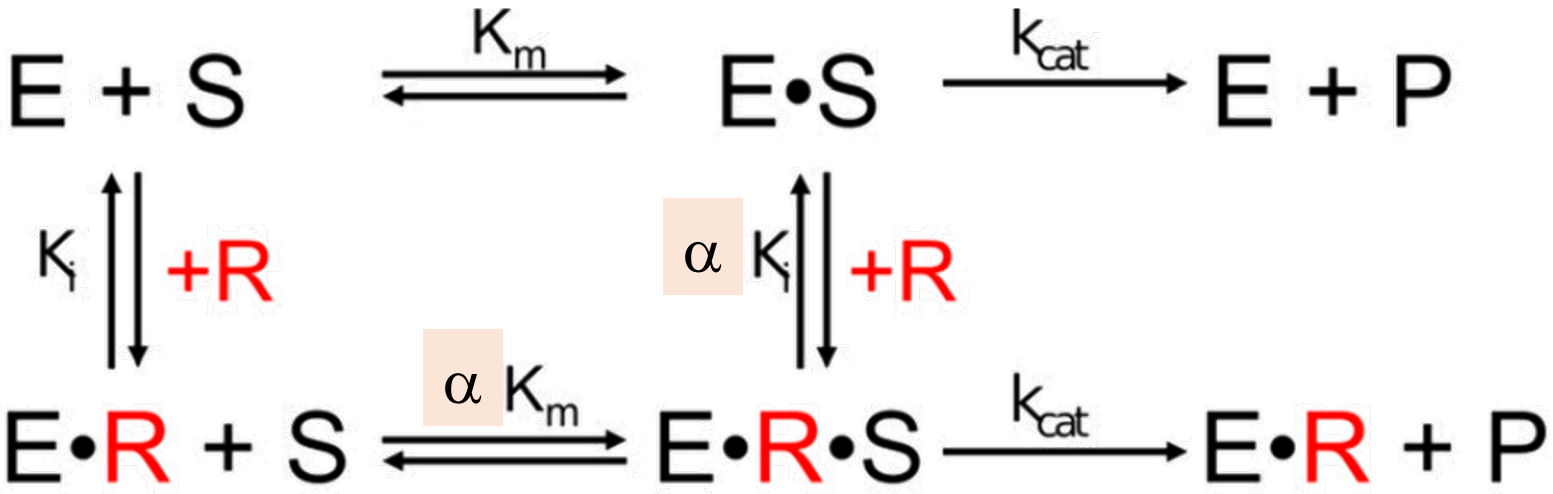
K_S = 26.2 +/- 6.4 μM

αK_S = 401.8 +/- 22.1 μM

α = 15.1

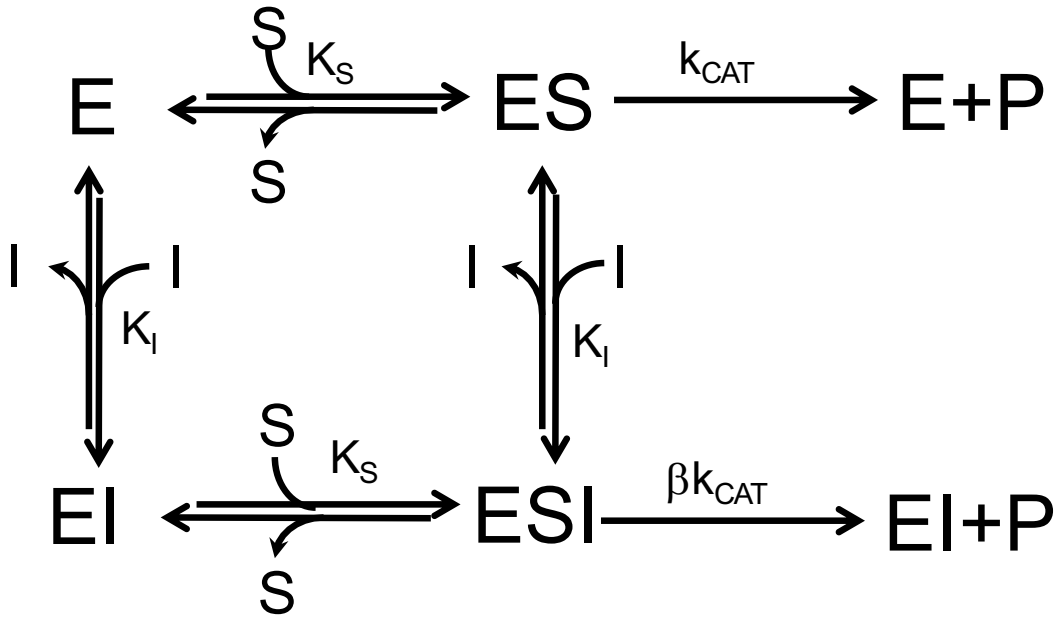
- MOREOVER, as [I] is increased from 0 to infinite, K_{sapp} increases from K_S to a limit of αK_S (hyperbolic inhibition)

$$K_{Sapp} = K_S \frac{\left(1 + \frac{[I]}{K_I}\right)}{\left(1 + \frac{[I]}{\alpha K_I}\right)}$$


E + S
K_m
E•S
k_{cat}
E + P
K_i
+R
α K_i
+R
α K_m
E•R + S
E•R•S
k_{cat}
E•R + P

PARTIAL NONCOMPETITIVE INHIBITION

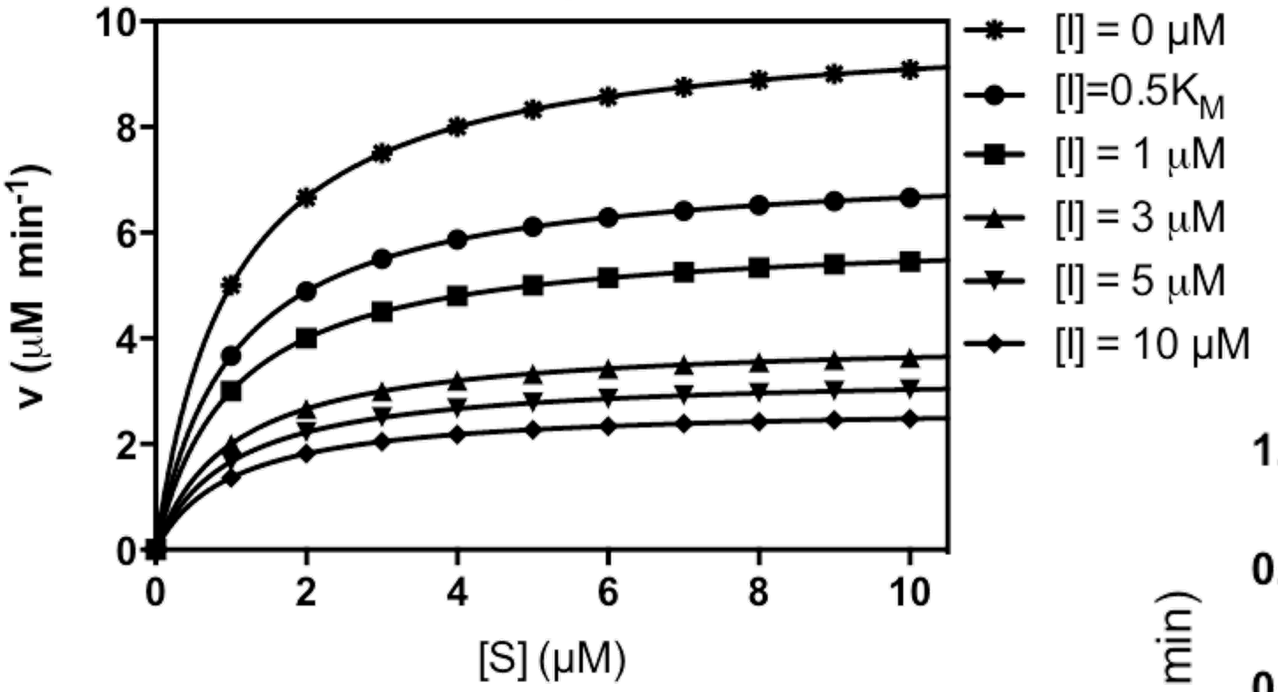
(α=1 and 0<β<1)

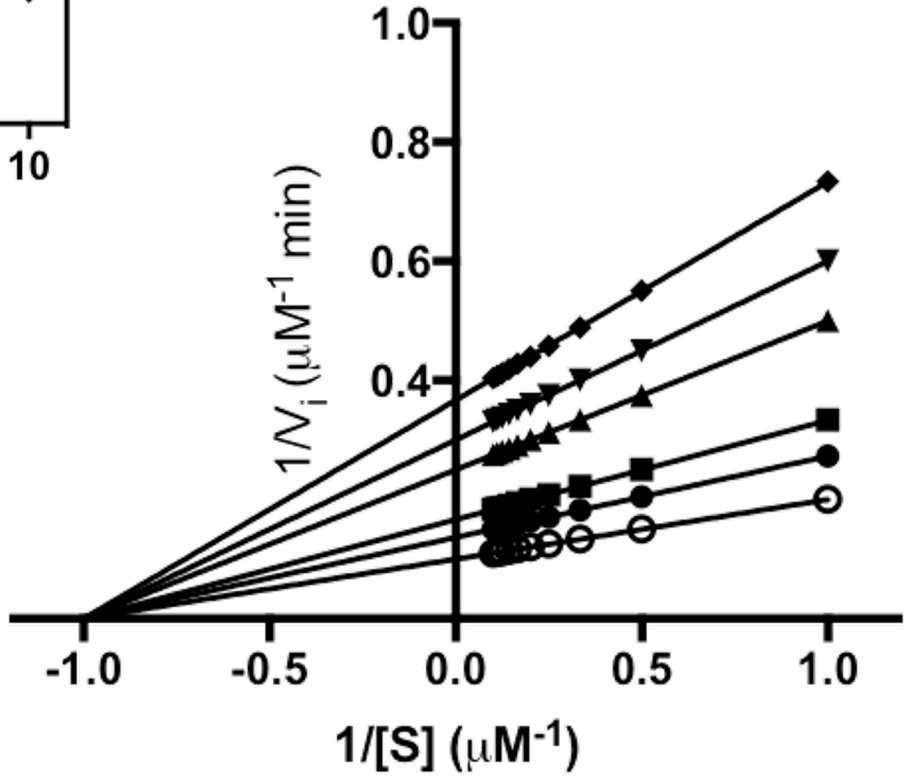


- ES and ESI complexes yield product with different k_{CAT} (0<β< 1);
- V_{MAX} decreases in the presence of I. K_S remains the same.

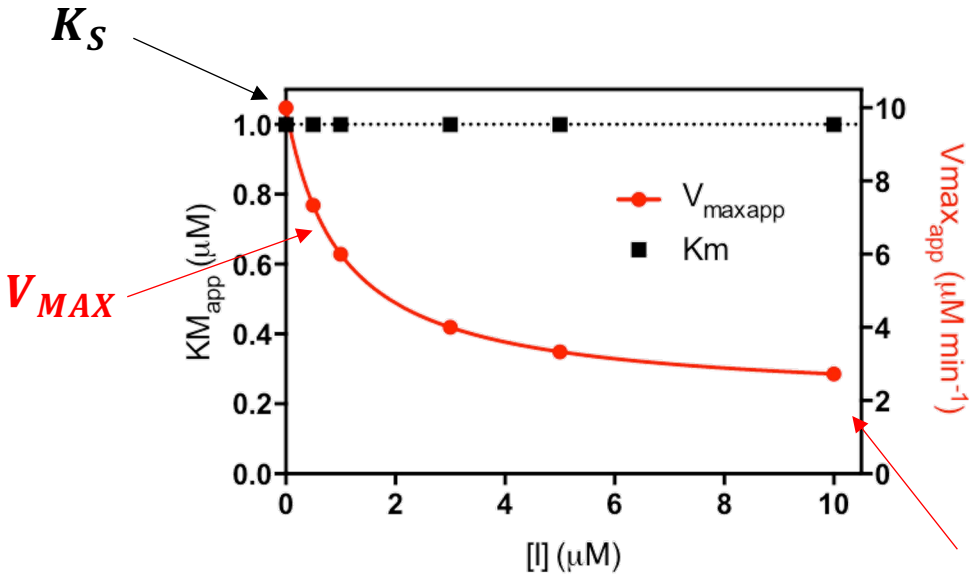
K_S=1M K_I=1M

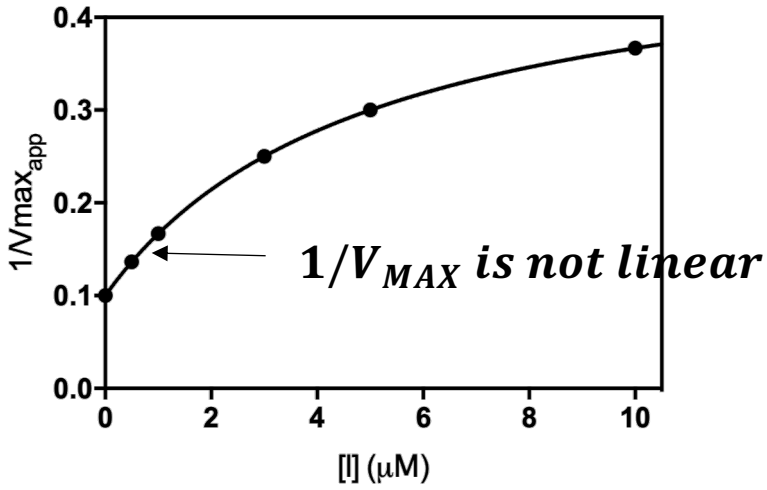
α=1 β=0.2





PLOTS OF V_{MAX} AND K_{Mapp} vs [I] determined from fitting of saturation curves

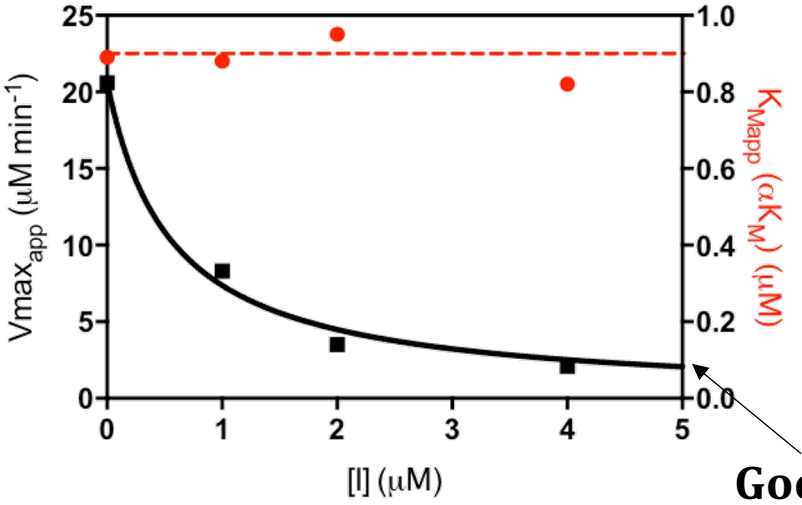




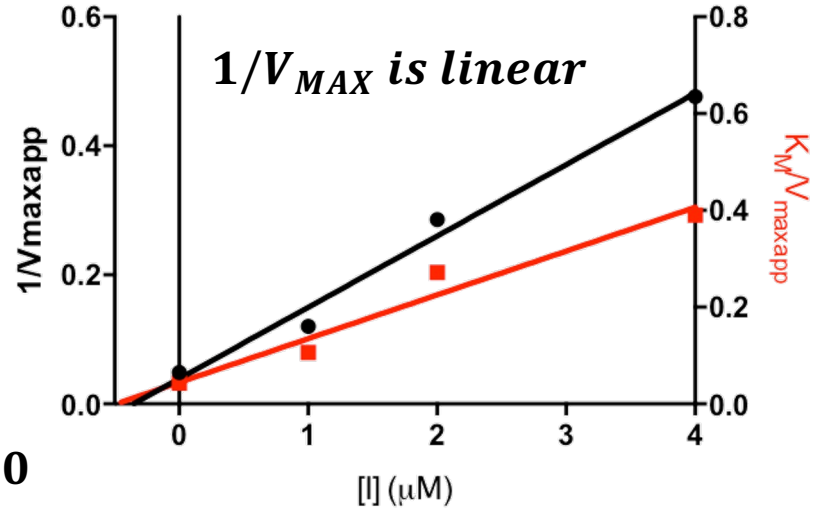
$$V_{MAXapp} = V_{MAX} \frac{\left(1 + \frac{\beta[I]}{K_I}\right)}{\left(1 + \frac{[I]}{K_I}\right)}$$

$$\frac{1}{V_{MAXapp}} = \frac{1}{V_{MAX}} \frac{\left(1 + \frac{[I]}{K_I}\right)}{\left(1 + \frac{\beta[I]}{K_I}\right)}$$

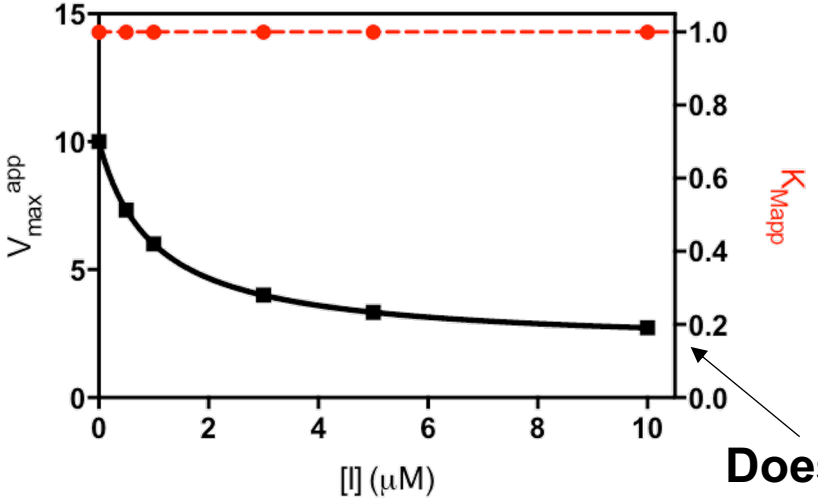
PURE NONCOMPETITIVE INHIBITION



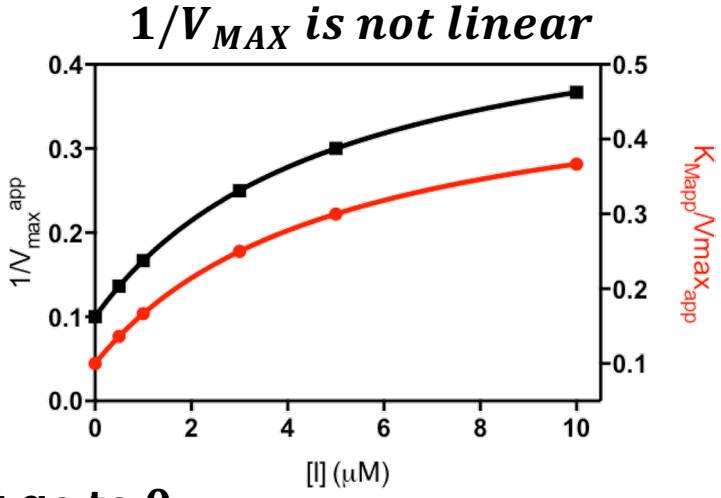
Goes to 0



PARTIAL NONCOMPETITIVE INHIBITION

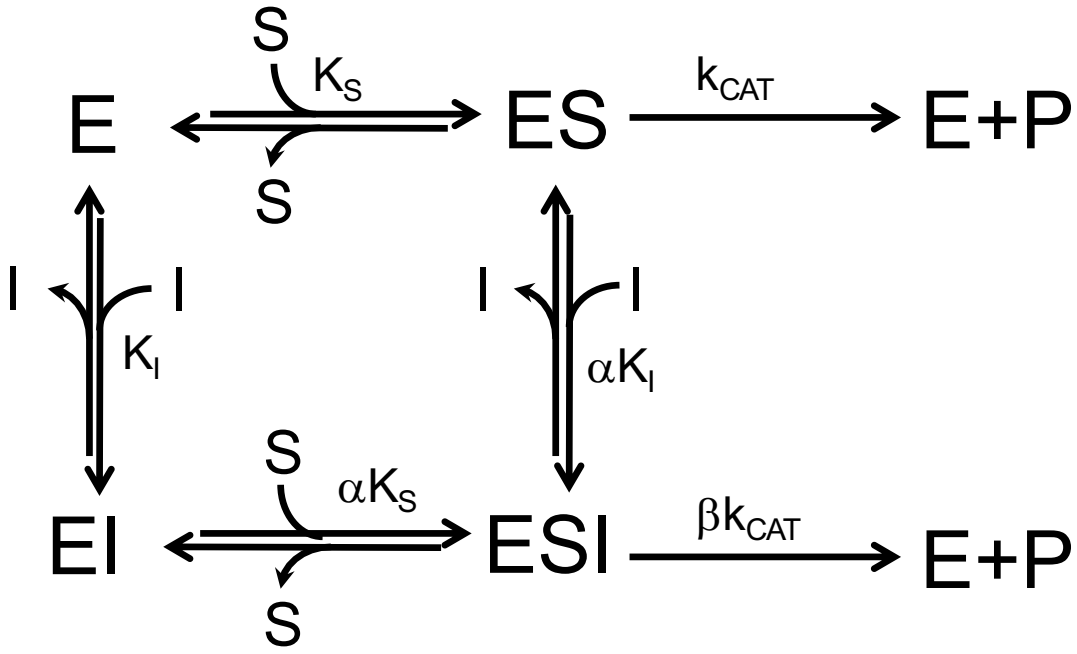


Does not go to 0

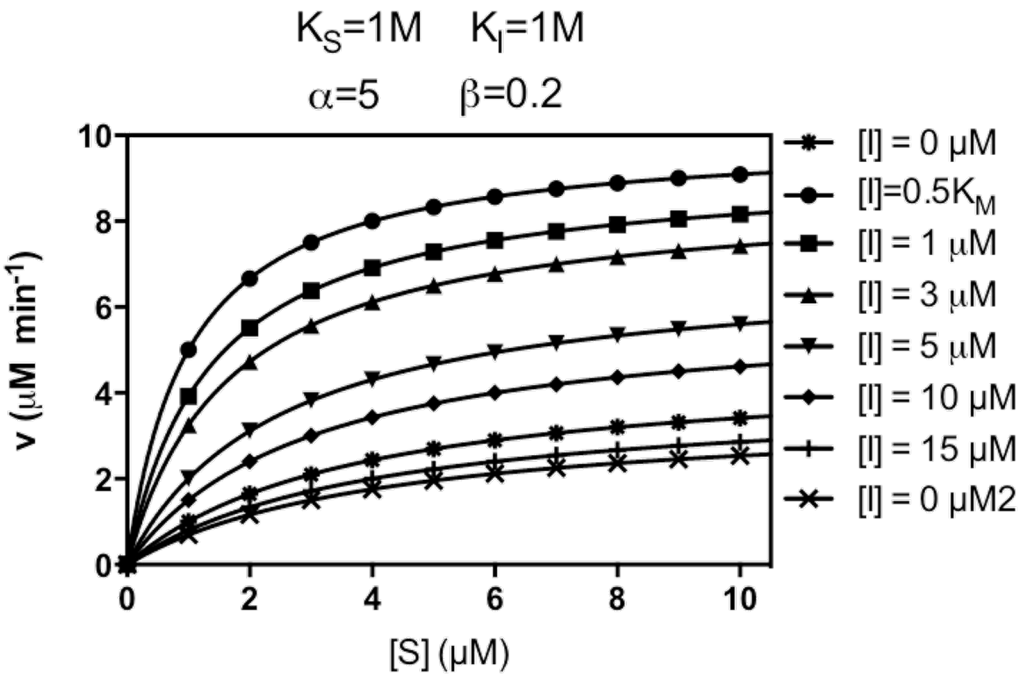


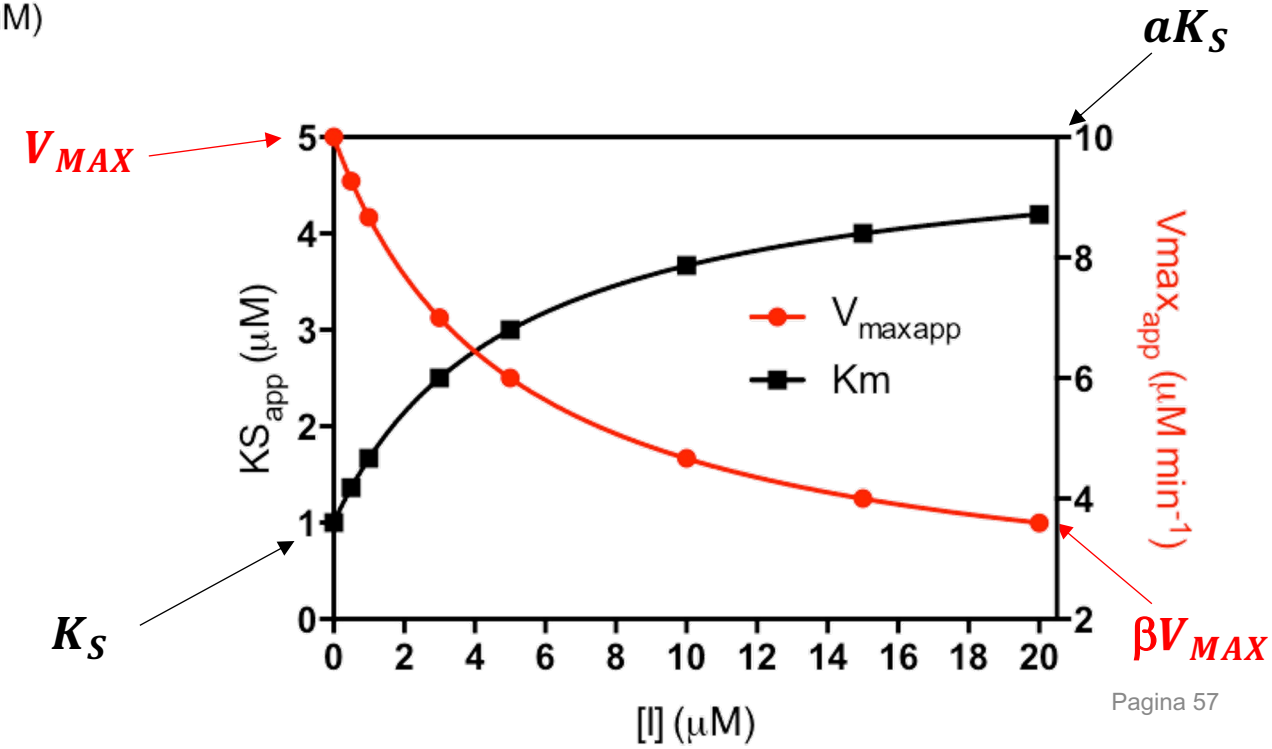
HYPERBOLIC MIXED-TYPE INHIBITION

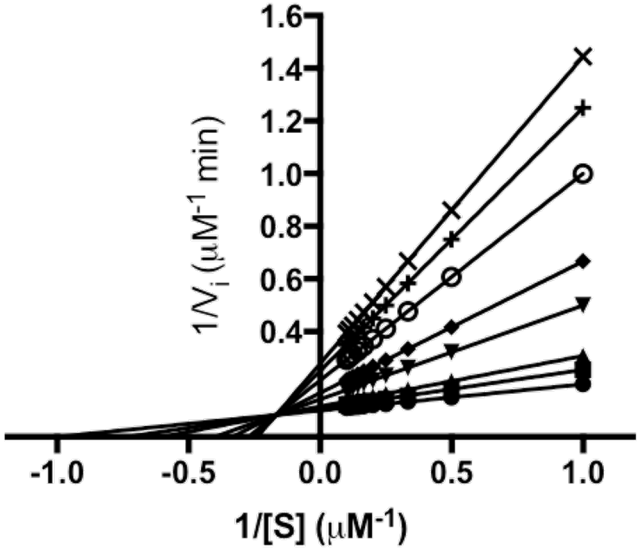
($1<\alpha<\infty$; $0<\beta<1$)

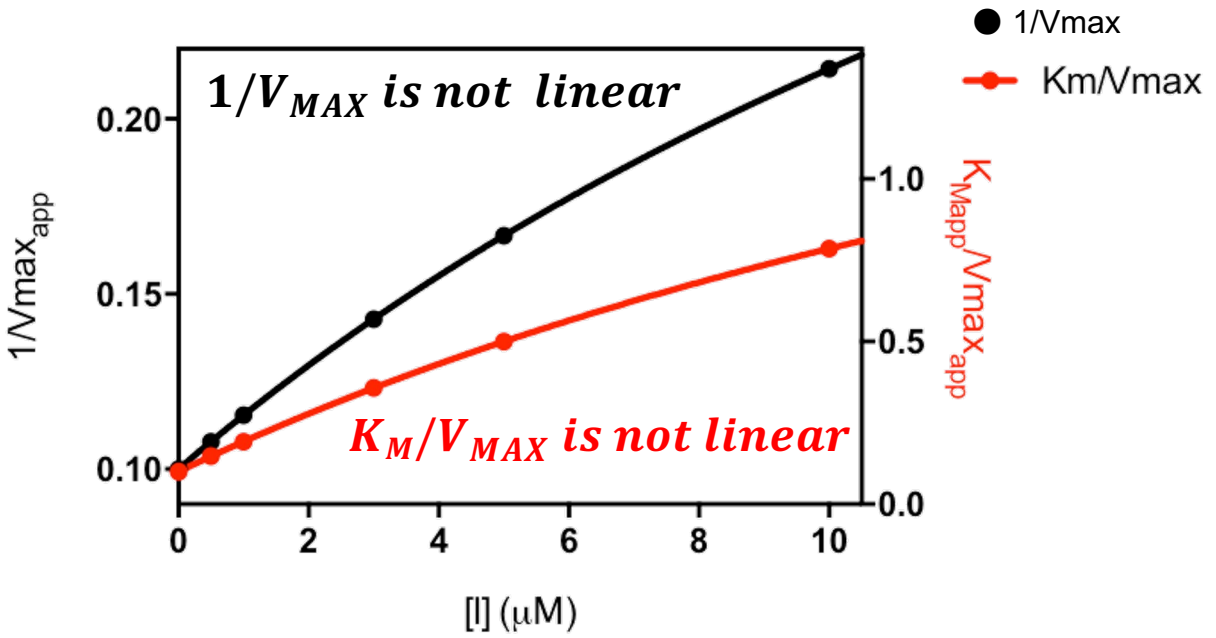


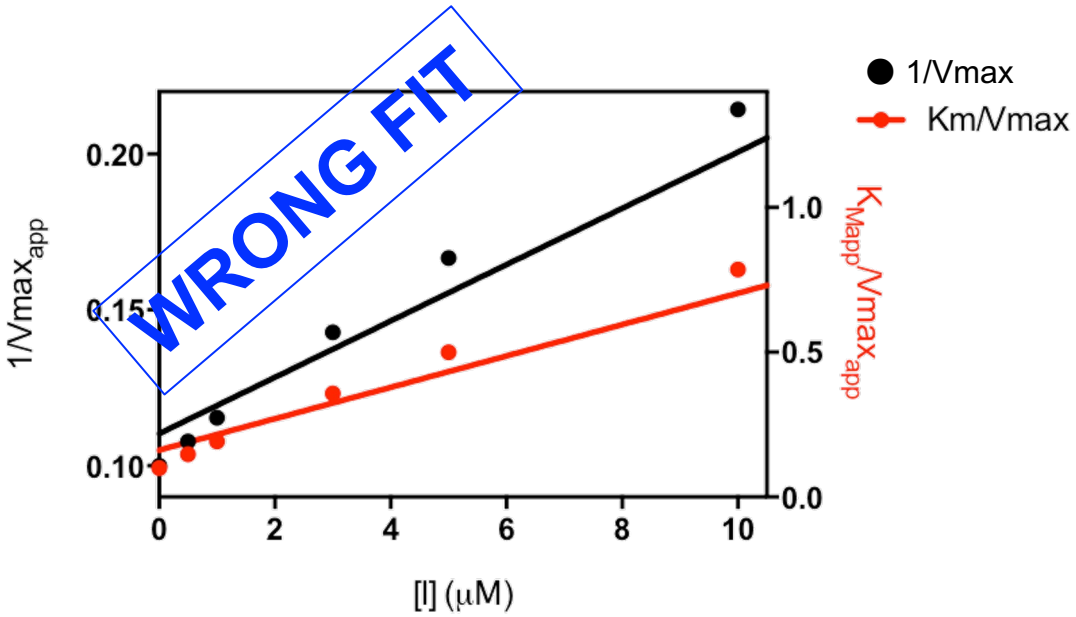
- The binding of one ligand has effect on the binding of the other;
- V_{MAX} decreases hyperbolically in the presence of I. K_{Sapp} increases hyperbolically from K_S to αK_S. v cannot be driven to 0 by increasing [I].


K_S=1M K_I=1M
α=5 β=0.2
v (μM min-1)
[S] (μM)
[I] = 0 μM
[I]=0.5K_M
[I] = 1 μM
[I] = 3 μM
[I] = 5 μM
[I] = 10 μM
[I] = 15 μM
[I] = 0 μM2


aK_S
V_{MAX}
K_S
βV_{MAX}
KS$_{app}$ (μM)
Vmax$_{app}$ (μM min^{-1})
V_{maxapp}
Km
[I] (μM)


1.6
1.4
1.2
1.0
0.8
0.6
0.4
1/Vi (μM-1 min)
-1.0
-0.5
0.0
0.5
1.0
1/[S] (μM-1)


1/Vmax
Km/Vmax
1/VMAX is not linear
KM/VMAX is not linear
0.20
0.15
0.10
1/Vmaxapp
1.0
0.5
0.0
KMapp/Vmaxapp
0
2
4
6
8
10
[I] (μM)

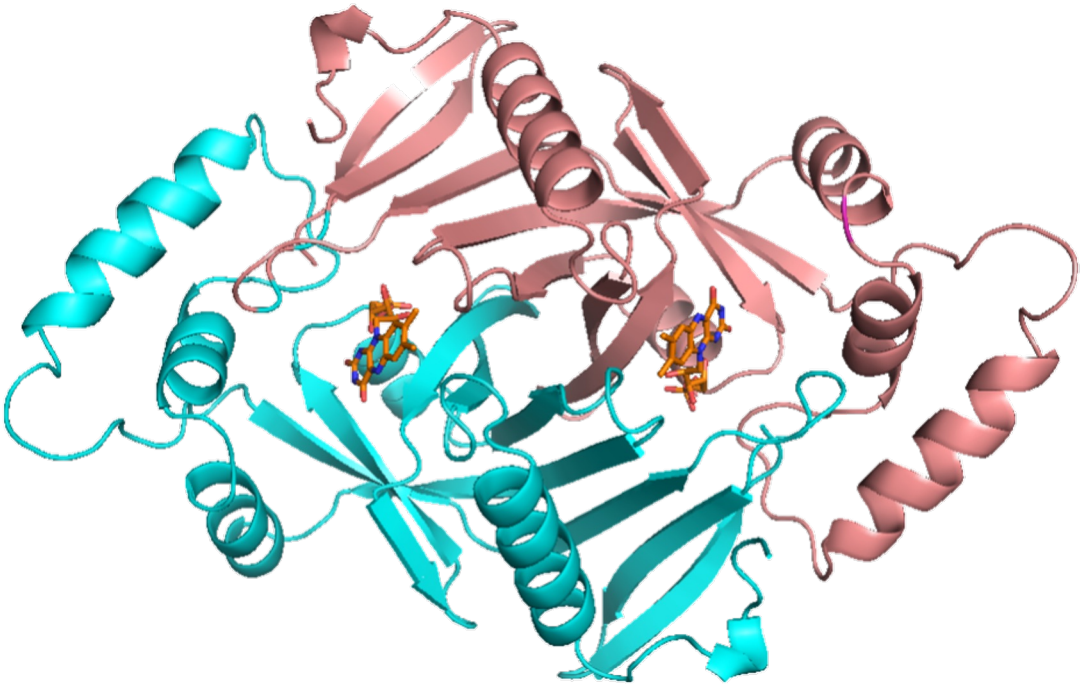

WRONG FIT
1/Vmax
Km/Vmax
0.20
0.15
0.10
1/Vmaxapp
1.0
0.5
0.0
KMapp/Vmaxapp
0
2
4
6
8
10
[I] (μM)

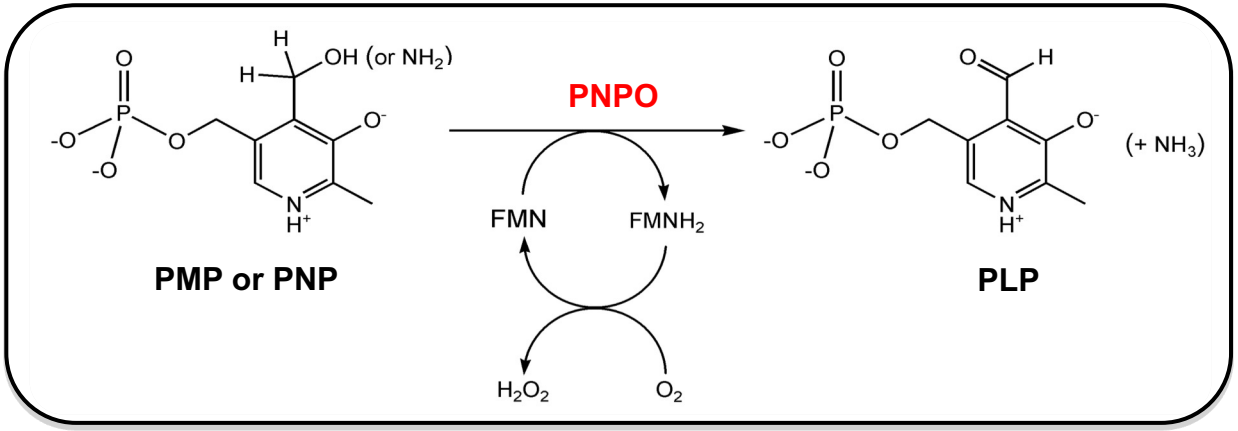
THE PECULIAR CASE OF THE "PARABOLIC INHIBITION"

When 2 molecules if inhibitor bind to the enzyme

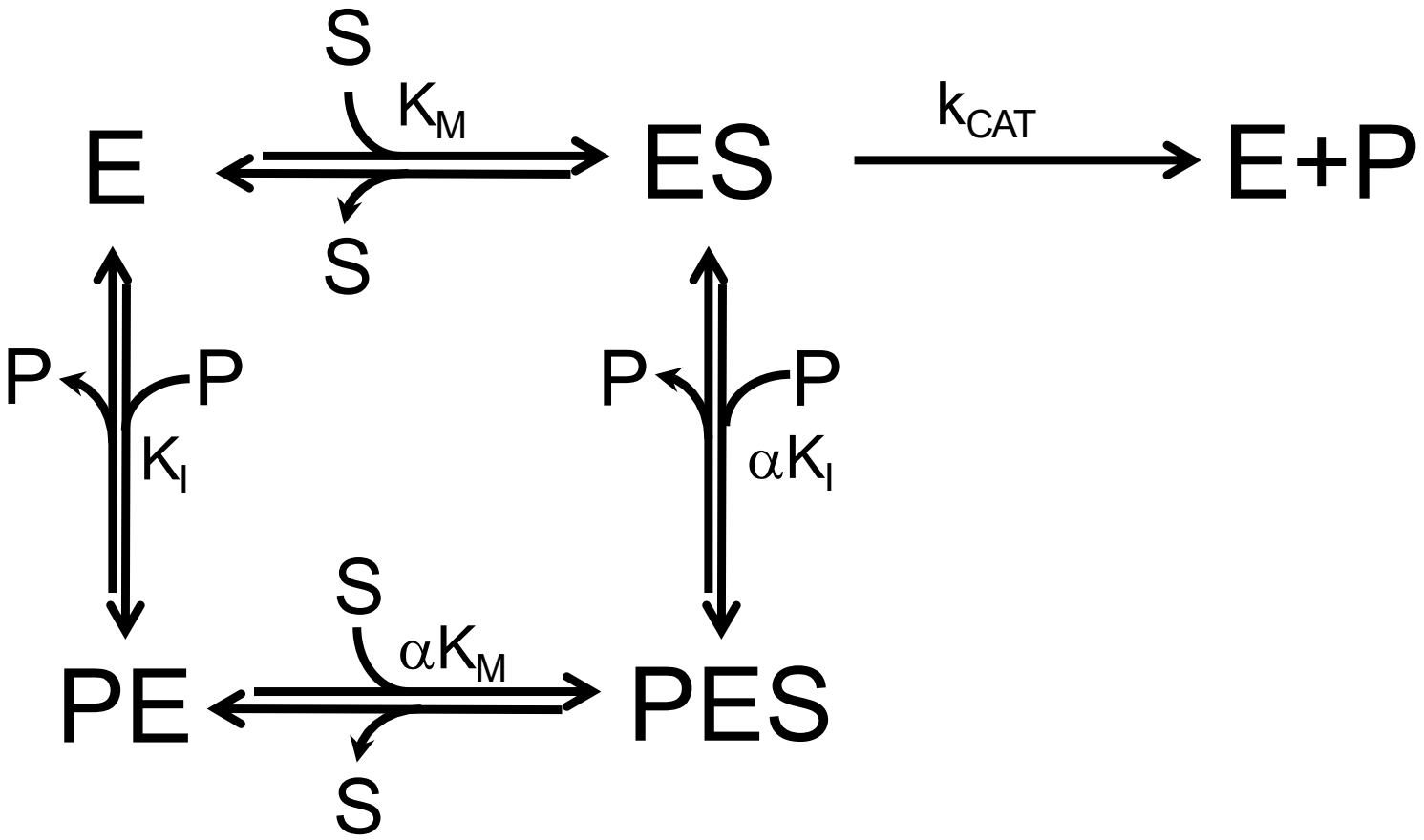
SITE-SPECIFIC MUTAGENESIS EXPERIMENTS ON THE ALLOSTERIC SITE OF *E. coli* PNPOx

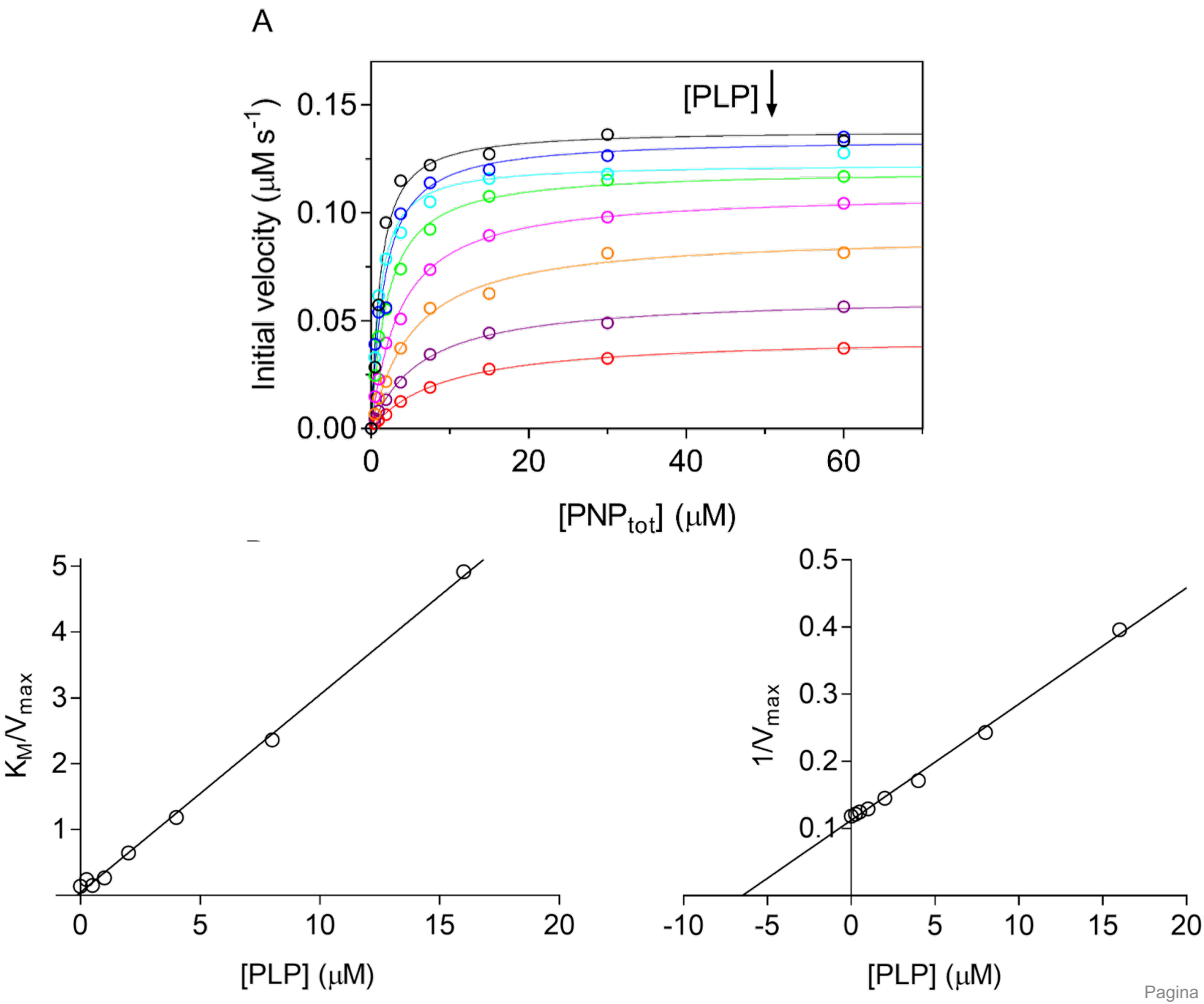
Pyridoxine 5’-phosphate oxidase (PNPO)

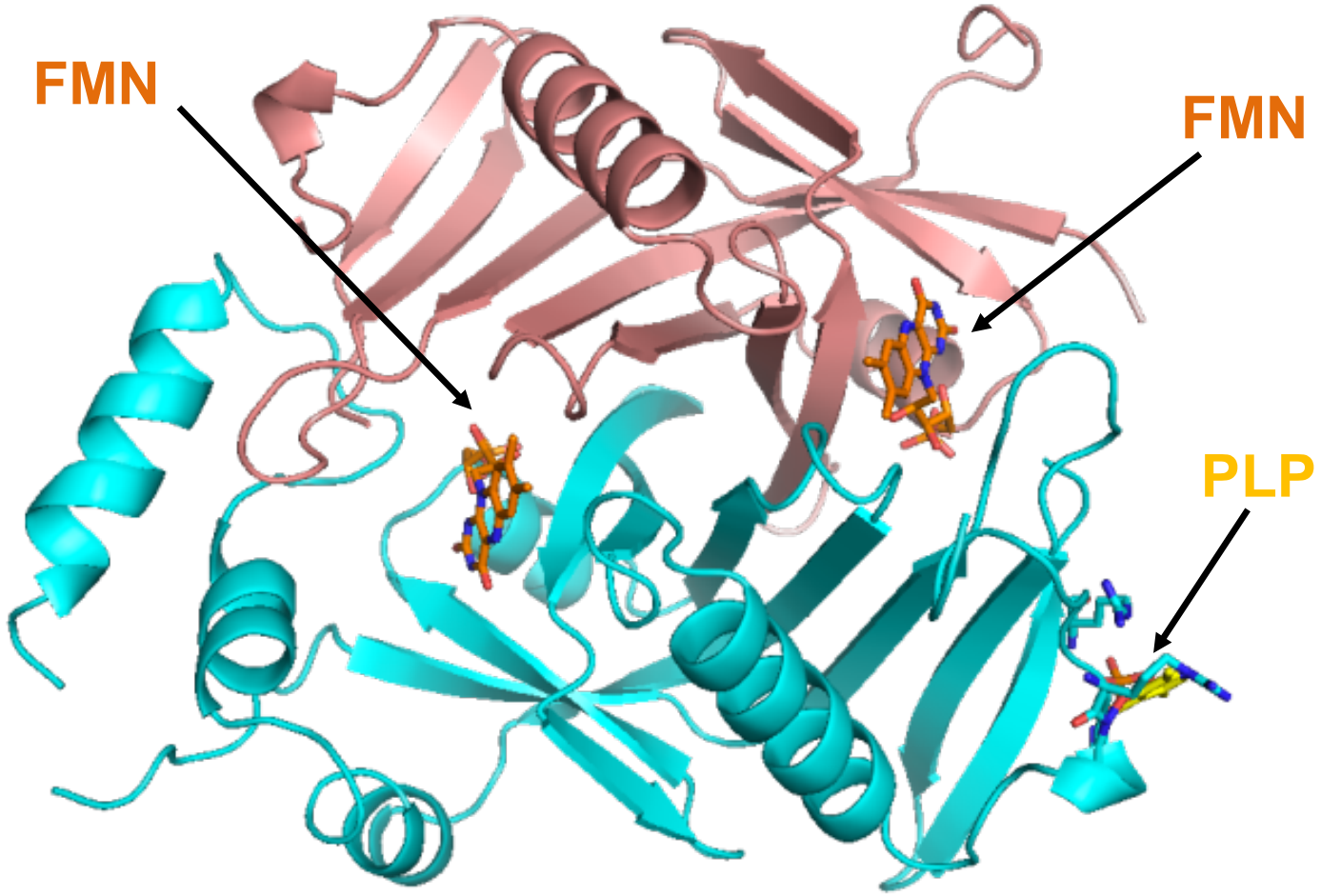




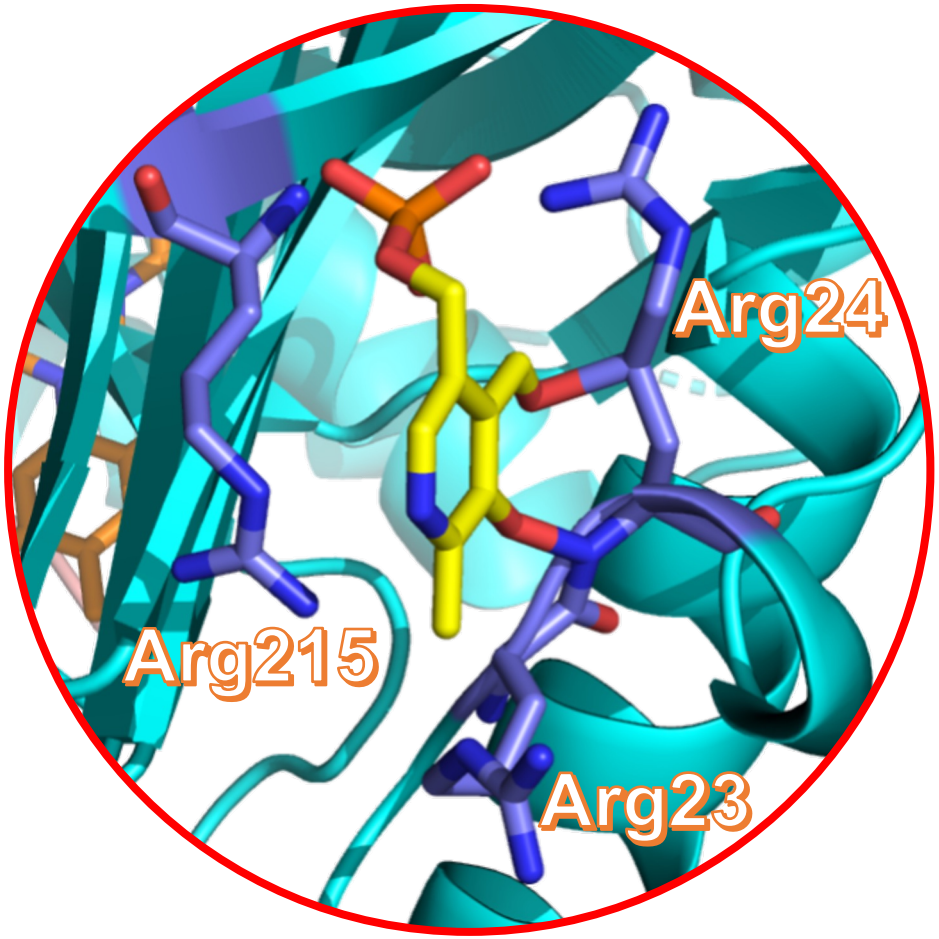
IN THE WILD-TYPE PNPO ALLOSTERIC FEEDBACK INHIBITION MANIFEST ITSELF AS LINEAR MIXED-TYPE INHIBITION




A
[PLP]
Initial velocity (μM s-1)
0.15
0.10
0.05
0.00
0
20
40
60
[PNPtot] (μM)
KM/Vmax
5
4
3
2
1
0
5
10
15
20
[PLP] (μM)
1/Vmax
0.5
0.4
0.3
0.2
0.1
-10
-5
0
5
10
15
20
[PLP] (μM)

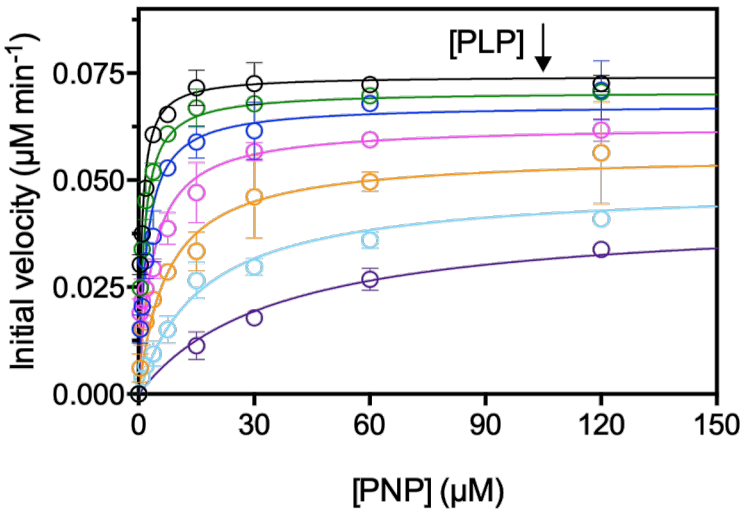

FMN
FMN
PLP

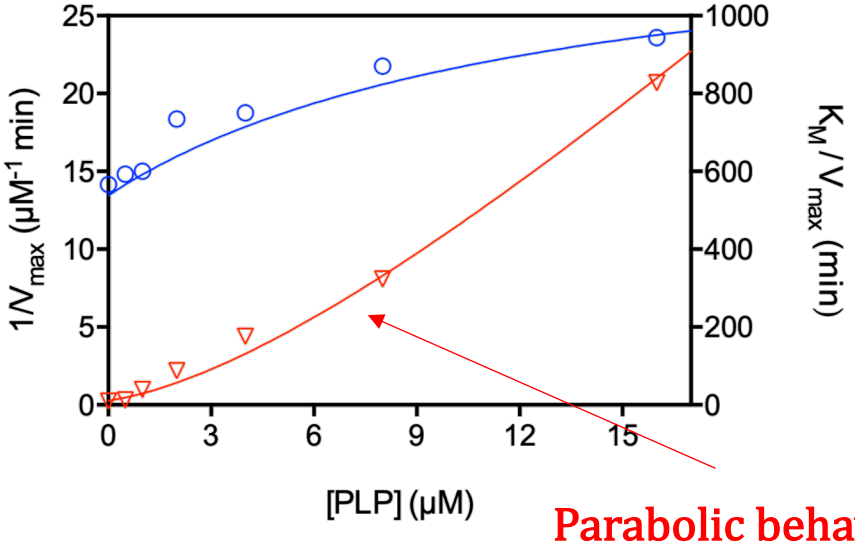
ALLOSTERIC SITE “ARGININE CAGE”



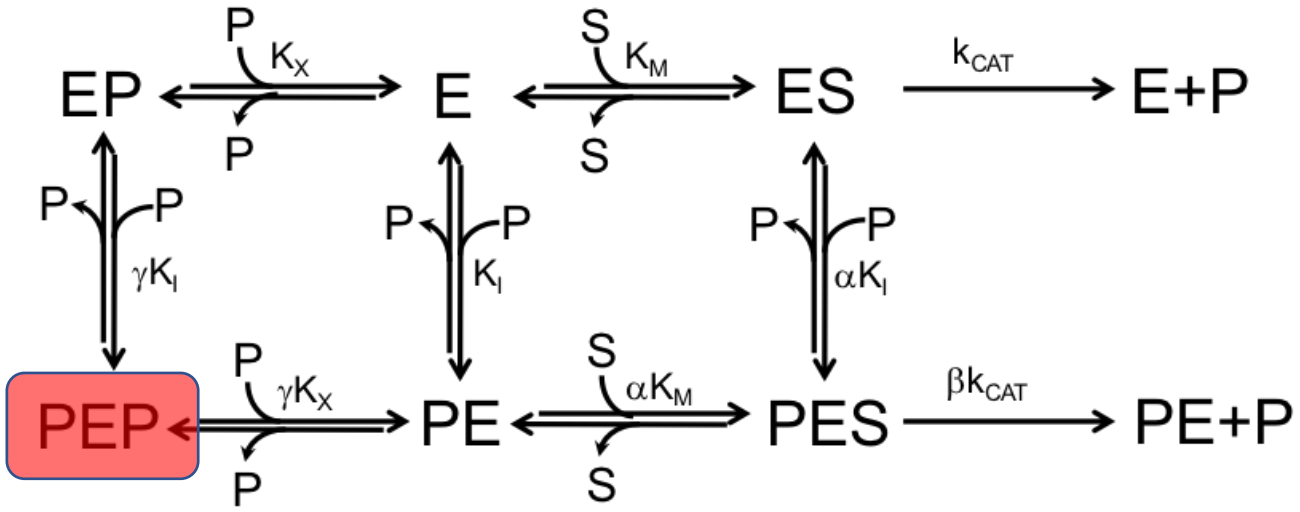
Barile et al. *J. Biol. Chem.* (2021) 296 100795

R23L/R24L "ARGININE CAGE" MUTANT
PARABOLIC INHIBITION



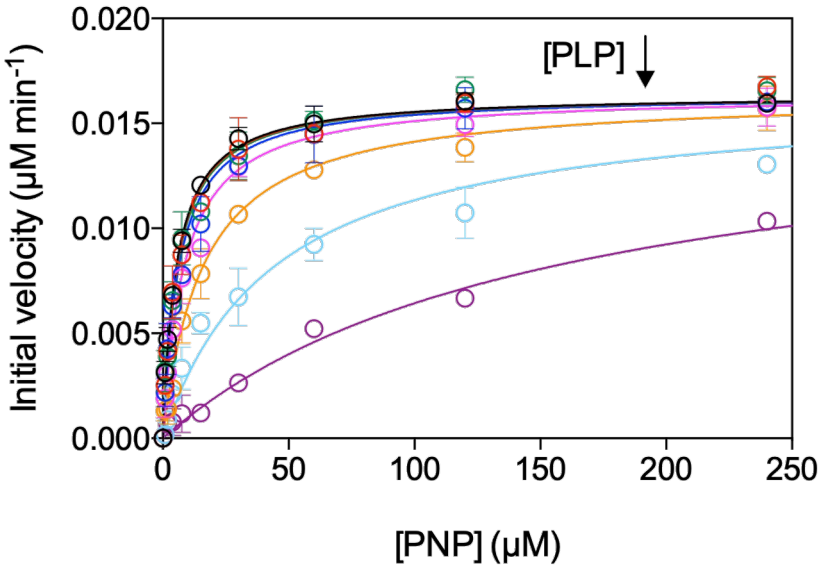


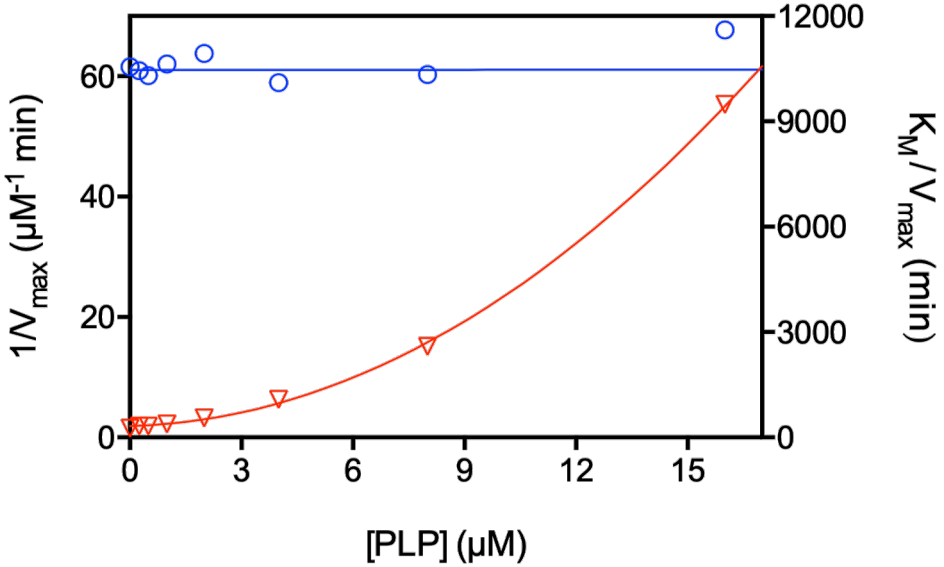
Parabolic behaviour

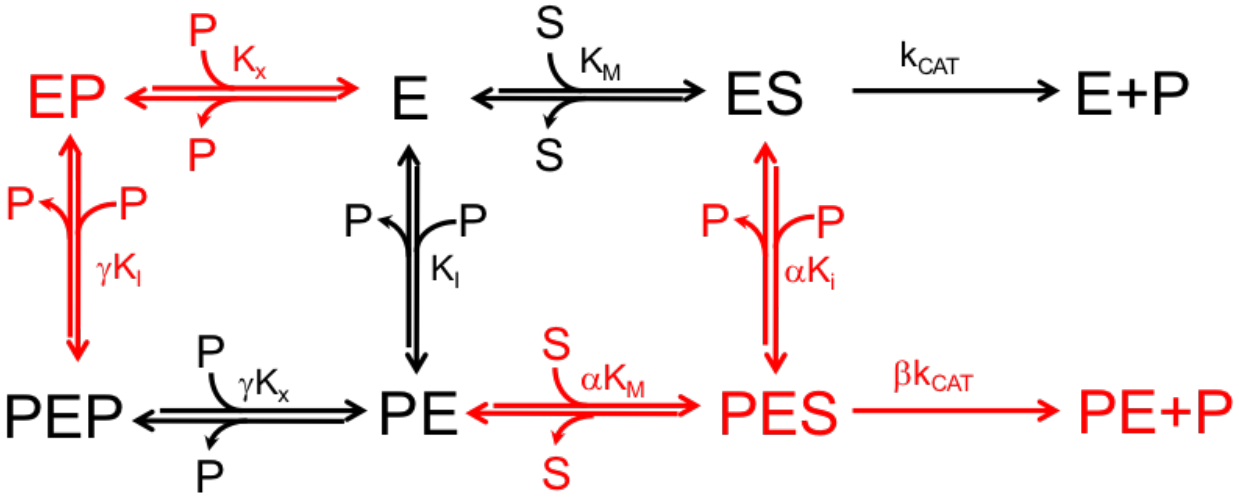


Coplex with 2 inhibitors bound

R23L/R215L "ARGININE CAGE" MUTANT

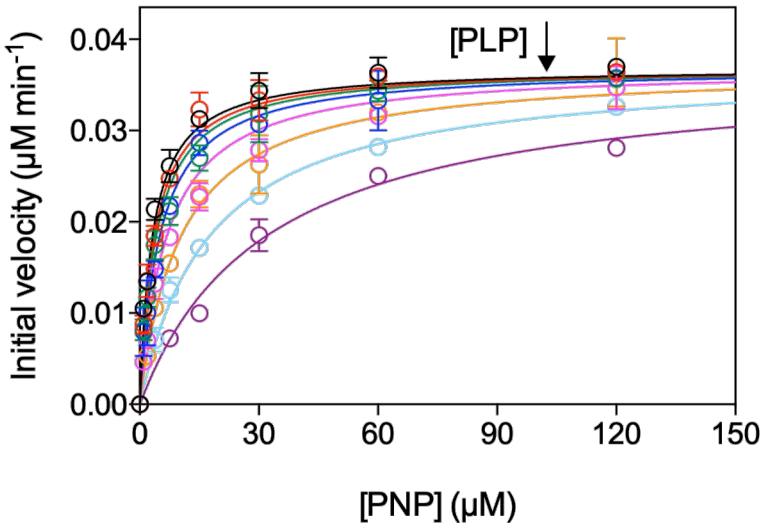


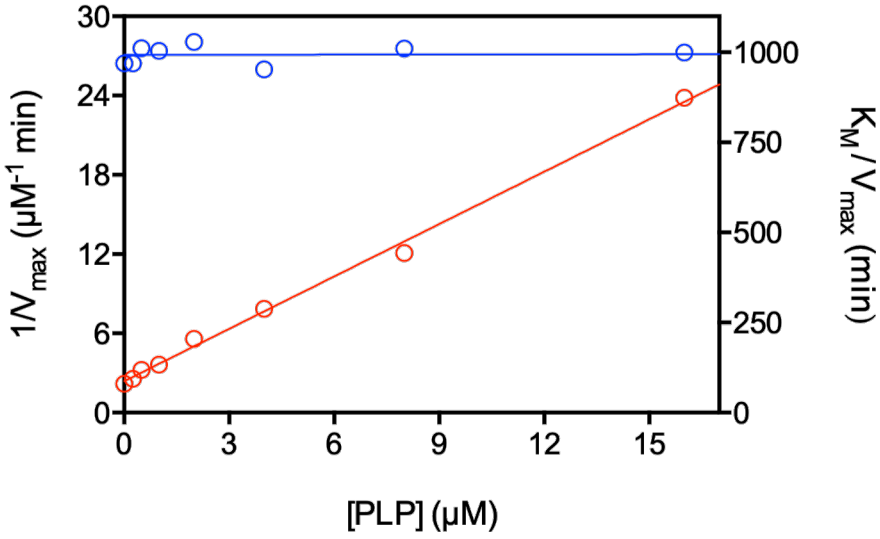


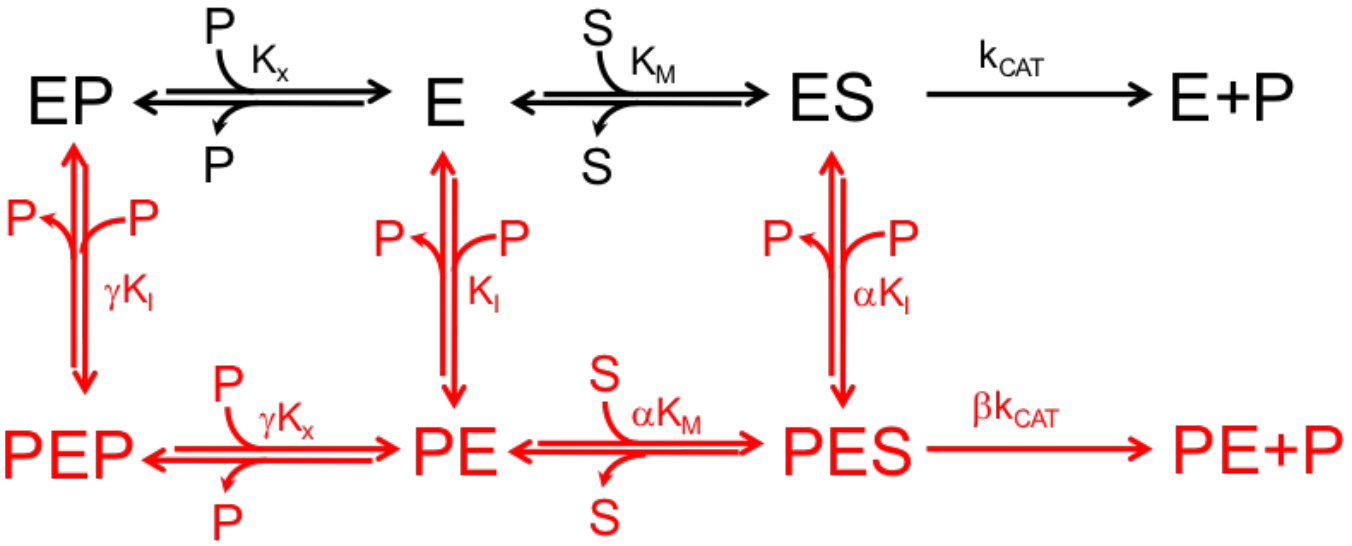


“ARGININE CAGE” R23L/R24L/215L MUTANT

COMPETITIVE INHIBITION







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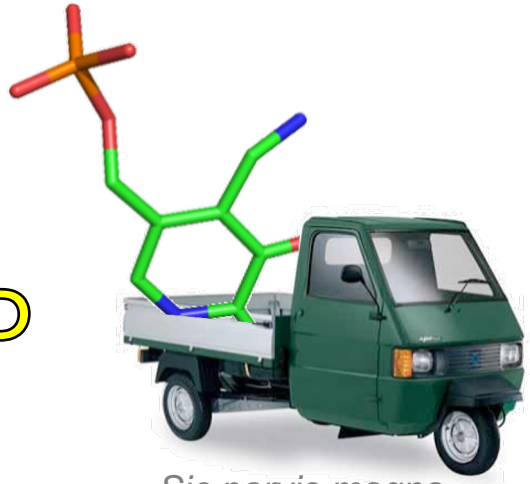
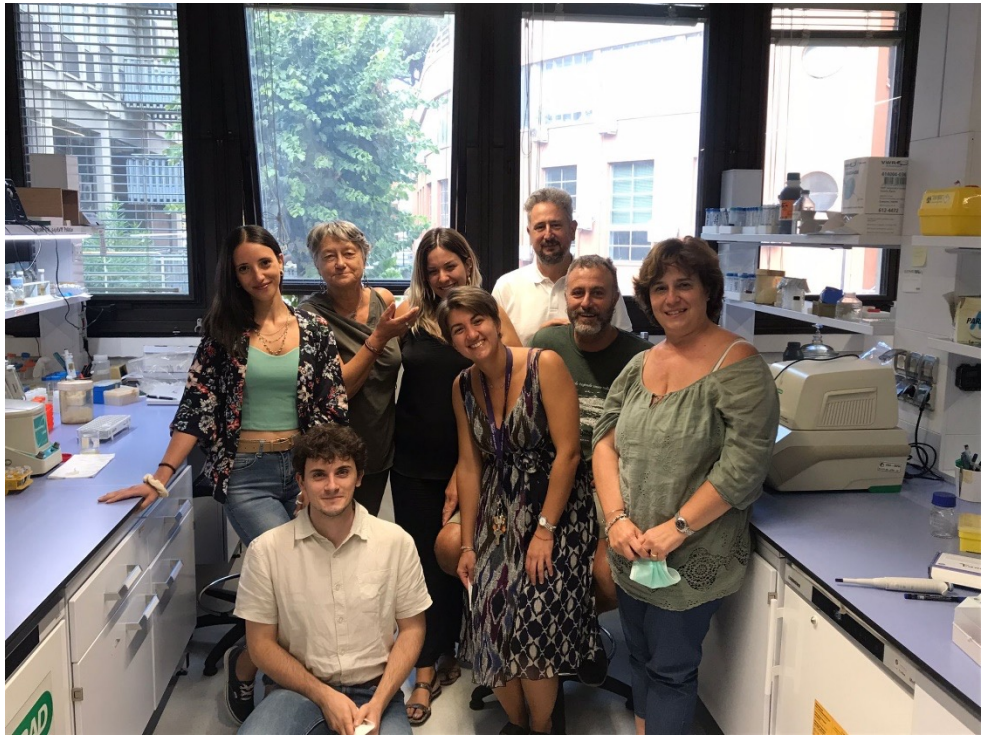


Sic parvis magna

THE PLP GROUP



Angela TRAMONTI (IBPM, CNR)

Martino Luigi DI SALVO

Anna BARILE

Claudio GRAZIANI

ISTITUTO PASTEUR ITALIA
FONDAZIONE CENCI BOLOGNETTI

Consiglio Nazionale delle Ricerche