Functional Allosterism of CYPs

I. Brief History/Definitions

II. Generic Examples of Homotropic Effects

III. Generic Examples of Heterotropic Effects

IV. Specific Examples

V. Other Analytical Methods

VI. Mechanistic/Structural Models

Historical references:


Although ‘allosteric’ kinetics were documented for CYP-dependent metabolism as early as 1980, the widespread appreciation that Drug-Drug interactions may result has only happened since 2000.

In early ‘80s Conney et al. demonstrated activation of CYP3A-dependent metabolism of zoxazolamine by α-naphthoflavone (αNF) and other flavonoids in rats and in microsomes [Science. 1982 216:1419-21].
I. History


Progesterone hydroxylation was cooperative in HLM α-NF stimulated steroid hydroxylation.

In 1994 Korzekwa et. al. pointed out that non-hyperbolic kinetics, and non-reciprocity between substrates must be due to simultaneous binding of two drugs at a single active site [Biochemistry. 1994 33:6450-6455].
I. History

Cupp-Vickery published the structure of CYPeryF with two amino-phenanthrene molecules or two progesterones simultaneously bound [Proc Natl Acad Sci (2000) USA 97:3050-3055 Crystal structures of ligand complexes of P450eryF exhibiting homotropic cooperativity].
I. History

Between 1999-2003 examples of possible in vivo activation were published:

Quinidine increased metabolism of diclofenac in monkeys (Tang et. al. (1999) JPET 291: 1068.)


Comparative analysis of CYP3A heteroactivation by steroid hormones and flavonoids in different in vitro systems and potential in vivo implications.

I. Recent History

Experimental Design to Explore Allostery in vivo: CYP3A Metabolism of Midazolam

In vitro:
- Product ratio changes with [MDZ], homotropic allostery.
- Product ratio changes with other drugs, heterotropic allostery

In vivo
- Absolute rate of product formation is confounded by enzyme expression/induction, competitive inhibitors etc.
- Exploit product ratio as a reporter – independent of enzyme level.
Fluconazole Effects on CYP3A4 Metabolism of MDZ in vitro

1’-OH-MDZ  4-OH-MDZ  Ratio 1’-OH/4-OH

FLZ reduces total MDZ Clearance and causes a decrease in 1’-OH/4-OH

Effects of Fluconazole on MDZ Product Ratio in Humans

• In vivo change in product ratio mimics in vitro change.

• CYP3A5 phenotype doesn’t impact change in the ratio.

• Product ratio, but not clearance per se, reports directly on allosteric effect.

Effects of FLZ on glucuronidation of hydroxy-MDZs indicate that FLZ does not effect product ratio via effects on $k_{\text{elim}}$. 
I. History

Currently, activation or at least allostery occurs in vivo.

More importantly, may be an in vitro behavior that needs to be understood to improve in vivo-in vitro extrapolations.
I. Definitions

An allosteric effector alters the kinetic/binding properties of another molecule for an enzyme/protein.

Homotropic effects occur when a molecules alters the kinetic/binding parameters of a second molecule of the same structure (i.e. two molecules are identical).

Heterotropic effects occur between chemically distinguishable molecules.

Allosteric systems are described as either ‘V systems’ or ‘K systems’ depending on whether the $V_{max}$ or the $K_M$ is altered by the effector. For a single enzyme, some effectors may be ‘V-type’ and others may be ‘K-type,’ and some may be mixed ‘V,K-type.’
I. Definitions

activation and some types of inhibition require simultaneous binding of two substrates (homotropic) or substrate and activator/inhibitor (heterotropic).

<table>
<thead>
<tr>
<th></th>
<th>heterotropic</th>
<th>homotropic</th>
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<tbody>
<tr>
<td>inhibition</td>
<td>1 or 2 drugs bound (competitive or not)</td>
<td>2 drugs bound (substrate inhibition)</td>
</tr>
<tr>
<td>activation</td>
<td>2 drugs bound</td>
<td>2 drugs bound</td>
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No requirement for binding within a single site vs. remote sites.
II. Generic Examples of Homotropic Activation

Homotropic:
The defining feature of homotropic activation is sigmoidal or biphasic velocity vs. [substrate] or ‘bowed’ Eadie-Hoffstee plots (velocity vs. v/[S])

Either ‘V’ or ‘K’.
Multiple binding required
II. Analytical Methods - the Two Site Model for Homotropic Effects

2 distinct species, ES and SE, with identical properties, but different from SES. Second ‘S’ scales $K_S$ and $k_p$ by $\alpha$ and $\beta$.

$\alpha<1$ or $\beta>1$ positive homotropic kinetics;

$\alpha>1$ and $\beta>1$ biphasic kinetics.
II. Homotropic Activation and Clearance

Reminder: when Michaelis-Menten kinetics apply, then

\[ v = \frac{V_{\text{max}} \cdot [S]}{K_M + [S]} \]

And

\[ \text{Cl}_{\text{int}} \Rightarrow \frac{V_{\text{max}}}{K_M} \]

\( \text{Cl}_{\text{int}} \) has units of vol/time-mol CYP,

Here:

\[ v = \frac{V_{\text{max}}_1 [S]}{K_{M1}} + \frac{V_{\text{max}}_2 [S]^2}{K_{M1} K_{M2}} \]

\[ 1 + \frac{[S]}{K_{M1}} + \frac{[S]^2}{K_{M1} K_{M2}} \]


So - if homotropic activation is operative, underestimate CL at high [S] and overestimate CL at low[S]:

note subtle difference!
II. Homotropic Activation and Clearance

For Biphasic Kinetics
\[ \alpha > 1 \text{ and } \beta \geq 1, \]
III. Generic Examples of Heterotropic Activation

For **heterotropic** activation with a substrate exhibiting Michaelis-Menten kinetics, the activator can decrease $K_M$ or increase $V_{max}$.

Also, the activator can ‘induce’ non-hyperbolic kinetics; then, ‘activation’ is dependent on substrate/effector concentration.

Assuming rapid equilibrium, nonhyperbolic $v$ vs. $[S]$ requires multiple $S$ binding . . . . So, . . .

If ‘effector’ induces non-hyperbolic kinetics, must be at least 3 binders, $[CYP\cdot S\cdot S\cdot E]$, And $[CYP\cdot S] = [CYP\cdot S\cdot S]$
III. Examples of Heterotropic Activation

Heterotropic activators could also make a ’non-hyperbolic’ substrate become ‘hyperbolic.’ Or, the activator may shift the response, which remains non-hyperbolic. 

\[[E\cdot S\cdot A] S_{0.5} < [E\cdot S]\]

again, ‘activation’ may only be in a limited substrate-effector concentration range.

\[K_M < S_{0.5}\]

\[+/\text{-Mg}^2+ = \text{Effector},\]
\[+\text{pyrene/3A4} = S\]

Also, Nifedipine, Felodipine, MDZ inhibit TST and ‘relieve’ positive homotropic effects.
III. Analytical Methods- Kinetic Models Based on Independent Sites

2 sites, each binds A or S

2 S sites, 1 A site

Kenworthy et. Al.
2001. DMD
29:1644.

Houston & Galetin
2005. ABB
433:351.

Galetin et. Al.
2003. DMD
31:1108.

\[ \frac{v}{V_{\text{max}}} = \frac{[S]}{K_v} + \frac{\beta[S]^2}{aK_v} \]

\[ \frac{v}{V_{\text{max}}} = \frac{[S]}{K_v} + \frac{[S]^2}{aK_v} + \frac{\beta[S][A]}{aK_v} \]

\[ \frac{v}{V_{\text{max}}} = \frac{[S]}{K_v} + \frac{[S]^2}{aK_v} + \frac{[S][A]}{aK_v} + \frac{[S][A]}{aK_v} + \frac{[A]}{aK_v} \]

\[ \frac{v}{V_{\text{max}}} = \frac{[S]}{K_v} + \frac{[S]^2}{aK_v} + \frac{[S][A]}{aK_v} + \frac{[S][A]}{aK_v} + \frac{[A]}{aK_v} \]

TST=S;
Quinidine = I
III. A General Model for Heterotropic Interactions

\[
\begin{align*}
\text{ME} + P & \xrightarrow{\gamma kp} \text{E} + P \\
\text{MES} & \xrightarrow{\delta K_M} \text{ES} \quad \text{ES} \xrightarrow{\alpha K_s} \text{SES} \xrightarrow{\beta kp} \text{ES} + P \\
\text{ME} & \xrightarrow{\delta K_s} \text{K_s} \quad \text{K_s} \xrightarrow{\alpha K_s} \text{SE} \xrightarrow{kp} \text{E} + P \\
\text{MEM} & \xrightarrow{\alpha M K_M} \text{EM} \xrightarrow{\delta K_M} \text{SEM} \xrightarrow{\gamma kp} \text{EM} + P
\end{align*}
\]

Note: \(K_M\) is not Michaelis constant here.

Substrate inhibition: \(\beta < 1\)
Substrate activation, \(\beta > 1\) or \(\alpha < 1\)
Heteroactivation: \(\gamma > 1\), heteroinhibition: \(\gamma < 1\)

\[
\begin{align*}
V_{\text{max}} &= \frac{[S]}{K_s} + \frac{\beta [S]^2}{\alpha K_s^2} + \frac{\gamma [S][M]}{\delta K_s K_M} \\
\frac{V}{V_{\text{max}}} &= 1 + \frac{2[S]}{K_s} + \frac{[S]^2}{K_s^2} + \frac{2[S][M]}{\delta K_s K_M} + \frac{2[M]}{K_M} + \frac{[M]^2}{\alpha_s K_M^2}
\end{align*}
\]

IV. Specific Examples

The ‘classic’ activator of CYP3A4 is α-NF; other flavones also activate. For some substrates, α-NF is an inhibitor!

α-NF is also a substrate, being metabolized to the 5,6- epoxide.
IV. Examples of Activation: Losartan Metabolism by CYP3A4

α-NF activates P2, inhibits P1


Effect is ‘pathway-dependent’
IV. Examples of CYP3A4 Activation by $\alpha$-NF

$\alpha$-NF effects are complex, substrate-dependent and product-dependent. [Ueng et. al. 1997, Biochemistry 36: 306.]
IV. Examples - other CYPs

It is becoming clear that other CYPS may exhibit homotropic and heterotropic activation. Examples include:

CYP2C9 heterotropic activation by dapsone.

positive homotropic O-dealkylation of phenacetin by CYP2D6.

positive homotropic activation of testosterone 16\(\beta\)-hydroxylation by CYP2B6.

Examples of negative homotropic effects include 7-ethoxycoumarin O-deethylation by CYP1A1, O-demethylation of 1-methoxy-4-nitrobenzene by CYP1A2, naproxen demethylation by CYP2C9.
V. Other Analytical Methods

The Hill Plot
If homotropic allosteryism is thought to be operative, a common method for ‘quantifying’ the degree of cooperativity is based on the ‘Hill analysis.’

\[ \text{P} + n\text{L} \rightleftharpoons [\text{P} \cdot \text{L}_n] \]

Probably a useful intuitive tool, but there is no physically meaningful interpretation of ‘n.’ As long as we limit the stoichiometry to 2:1 ligand or a defined ‘n’:1 CYP it’s a helpful way to compare systems.

\[ v = \frac{V_{\text{max}} [L]^n}{K_M + [L]^n} \]

Log \[ \frac{v}{(V_{\text{max}}-v)} \]

Log [L]

Slope = n
V. Analytical Methods, Kinetic Models

**Strengths**

Kinetic Models provide predictive power over rate of product formation for any combination of substrate/effector concentrations.

**Weaknesses**

Kinetic Models Based on Multiple Binding are complex, with too many parameters to distinguish between models. There may be several solutions, i.e. combinations of parameters that yield equally good ‘fits.’

The recovered parameters $\alpha$, $\beta$, $\gamma$, $\delta$ contain no mechanistic information, they are just scaling factors.
VI. Mechanistic Models- Overview

‘Traditional allosteric site’

‘Traditional allosteric’ enzymes employ specific regulatory sites distinct from the catalytic site to yield heterotropic effects. Effector binding changes $V_{\text{max}}$ or $K_M$.

Multiple binding site

This model assumes that two substrates or substrate and effector bind simultaneously to a single active site. If both are substrates, then both must have access to the [Fe=O]$^{3+}$. Many Variations of this have been described, including three-site models (SEE BELOW).

Protein-oligomer model

Some data based on co-expression of multiple CYPs in a single cell or spectroscopic methods suggest CYPs form high order complexes with different kinetic parameters than monomers. This provides a source of ‘heterogeneity’ in the population of enzymes that can give ‘allosteric kinetics’ without any allostery actually at work.
VI. Mechanistic Models-Multiple Binding in a Single Site

A commonly held belief is that multiple ligands bind within a single, large, active site, so both have access to the single [Fe=O]$^{3+}$ intermediate. Consider $\alpha$NF/phenanthrene with CYP3A4.

Although phenanthrene inhibits $\alpha$NF turnover, and $\alpha$NF activates phenanthrene turnover, neither $K_M$ is affected. This indicates the two ligands are not competing for the same site, yet both are substrates. This behavior is a major piece of evidence in support of simultaneous binding of substrates and effectors in a single active site.
VI. Mechanistic Models- Multiple Binding

Evidence supporting a single large active site: crystal structures of CYPeryF with two ligands that demonstrate ‘cooperative’ spin state changes.
VI. Mechanistic Models - Multiple Binding

Evidence supporting a single large active site: fluorescence spectroscopy with pyrene in CYP3A4. Two pyrenes in direct contact give spectroscopic features observed in the presence of CYP3A4. Pyrene yields sigmoidal kinetics.
VI. Mechanistic Models - Multiple Binding

Evidence supporting a single large active site: crystal structures of human CYPs - although multiple ligands had not been directly observed as of 2006, the active sites are big enough. CYP3A4 ~1000Å³ substrate free, vs. ~350Å³ in P450cam.
VI. Mechanistic Models- Multiple Binding

CYP3A4 with two Ketoconazole ligands in the active site.

Two ligands in direct contact.

Two ligands in ‘opposite’ orientations.

KTZ at 5 mM!

Proof of principle - two large ligands can fit in the active site without major disruption of the structure.

VI. Mechanistic Models - Multiple Binding

Within the framework of this model, two extremes border a continuum of possibilities:

**Discrete static** sites within the larger ‘active site.’

**Single fluid** site with ligands dynamically accessing different regions.
VI. Mechanistic Models- Multiple Binding

Support for a fluid active site with rapid reorientation of substrates within a large active site comes from:

1) Large intramolecular kinetic isotope effects. Concentration-dependent? Dynamic heterogeneity.

2) Paramagnetic proton relaxation NMR studies. All the protons on flurbiprofen or dapsone are nearly equidistant from heme iron in CYP2C9; protons on TST or MDZ are equidistant from heme iron in CYP3A4. Dynamic or static heterogeneity.

VI. Mechanistic Models - ‘Allosteric Site’

Evidence for ‘Allosteric site’:

Crystal structure of CYP3A4 with progesterone-ligand is far from heme/active site in a pocket near the presumed membrane-binding patch (F-G region). Note: same structure supports ‘multiple binding in a single active site.’
Ligand induced conformational change, by itself can not cause ‘allosteric’ kinetics. Many enzymes exhibit ligand-dependent conformation (induced fit), but they are classic Michaelis-Menten enzymes.

Conformational change provides one mechanism by which multiple binding can cause allosteric kinetics - different conformations of [E•S] vs [E • S • S]. However, . . .
VI. Mechanistic Models- Conformational Changes

If two conformations with different kinetic characteristics are not in rapid equilibrium, then this can cause allostERIC kinetics even in the absence of multiple binding. Behaves like a mixture of two enzymes.

Evidence for this possibility:

• high pressure effects on spin state/P420 conversion (Davydov et al. 2003, BBRC 312, 121-130.)


\[ \text{E} \quad \leftrightarrow \quad \text{E'} \quad \text{slow} \]
VI. Mechanistic Models - Conformational Changes

How big is a ‘conformational change’?

‘minor’ changes in the I-helix (Oxygen binding site) and in side-chain conformations of the active site pocket. How big does a conformational change need to be?

Remember that $\Delta\Delta G^\# = 0.5\text{kcal/mol}$ for translates into a 2.5-fold difference in rate, so very small structural changes may afford significant rate changes.
VI. Mechanistic Models - Uncoupling

‘Activation’ of product formation may result from decreased uncoupling or increased rate of flux through the cycle.

e.g. flurbiprofen activation by dapsone

Hutzler et. al. 2003 ABB, 410:16.

VI. Summary for CYPs

• ‘Activation’ is CYP isoform-, substrate-, activator-dependent.

• Multiple binding models can very accurately fit experimental data, but uniqueness of fits is questionable, and limited mechanistic information.

• Multiple binding within a single large active site - a ‘near certainty’ - but allosteric sites also possible.

• Mechanisms are not mutually exclusive.

• Other drug metabolizing enzymes may follow?
Other Drug Metabolizing Enzymes Exhibit Non-Michaelis-Menten Kinetics: Homotropic Effects

Modest activation of E2 glucuronidation by some flavones.

Pfeiffer et al., 2005
Carcinogenesis 26:2172.