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Heterotropic Cooperativity in Cytochrome P450 eryF

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Background

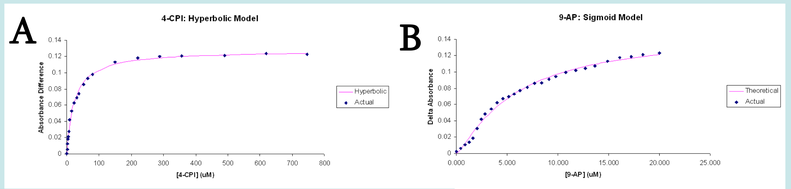
Cytochromes P450 are a family of enzymes that metabolize most chemicals that enter the body, including drugs and environmental toxins.[1] These enzymes tend to be extremely unspecific and flexible, meaning they can often bind to various types of molecules and even multiple substrates at a time.[2] Interestingly, it is possible for the binding of one molecule in the active site to influence the binding and subsequent catalysis of a second molecule – a phenomenon known as cooperativity. This is responsible for drug-drug interactions, where the effects of one drug (the substrate) are altered by another drug (an effector), often leading to harmful outcomes like toxicity.[3]

The process of cooperativity is poorly understood. Studies using the bacterial model enzyme eryF suggest that there are two distinct binding sites inside a larger binding pocket, and that while a substrate may bind to only one of these sites, effector molecules can bind to either.[4,5] The present experiment had three primary goals: (1) to reproduce literature data for the affinity of 4-CPI and 9-AP for P450 eryF, (2) to test how effectors (such as 9-AP) influence substrate binding, and (3) to ultimately develop a more precise model of the eryF active site.

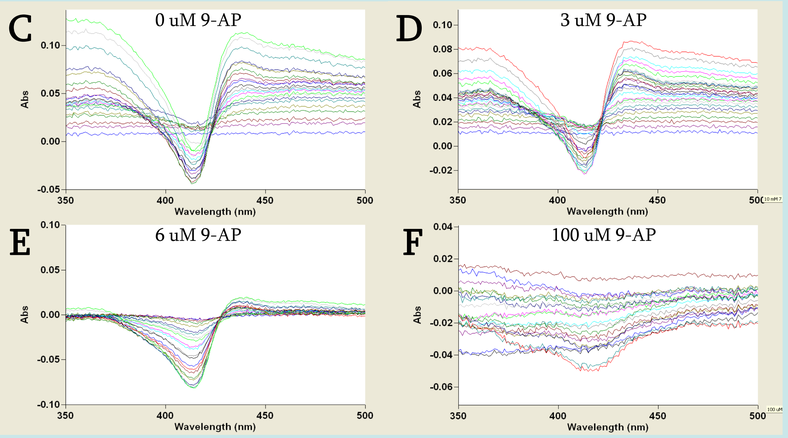
Methods

Absorbance difference titrations were used to determine enzyme affinity for substrate and effector molecules. NMR paramagnetic relaxation experiments acquired data for distance measurements, which were then entered into the molecular docking software HADDOCK to model the structure of the ligand-protein complex.

UV-Vis Titrations



Literature 9-AP K_D values[3] (5 uM and 11 uM) suggest that 0, 3, 6, and 100 uM 9-AP should fill, respectively, none, only the first, only the first, and both effector binding sites (eryF is bound to none, one, one, and two 9-AP molecules).



- Adding ligand causes absorbance to decrease at 417 nm and to increase at 437 nm in a manner proportional to the concentration of enzyme-ligand complex.
- Figure 1A and B show absorbance difference (A_{437} - A_{417}) as a function of ligand concentration. Titration data were consistent with reported affinity values.
- With 9-AP-bound eryF used as a baseline, small concentrations of 9-AP do not change the apparent affinity of 4-CPI (Figure 1D, E).
- In contrast, a saturating amount of 9-AP (that fills both binding sites) prevents 4-CPI from further altering solution absorbance (Figure 1F).

Figure 1 Absorbance difference spectra. EryF (3 uM) was titrated separately with 4-CPI (from 0 to 750 uM) and 9-AP (from 0 to 20 uM) [A, B], then presaturated with 9-AP, baselined, and titrated with 4-CPI as done previously [C through F].

NMR-PRE Experiments

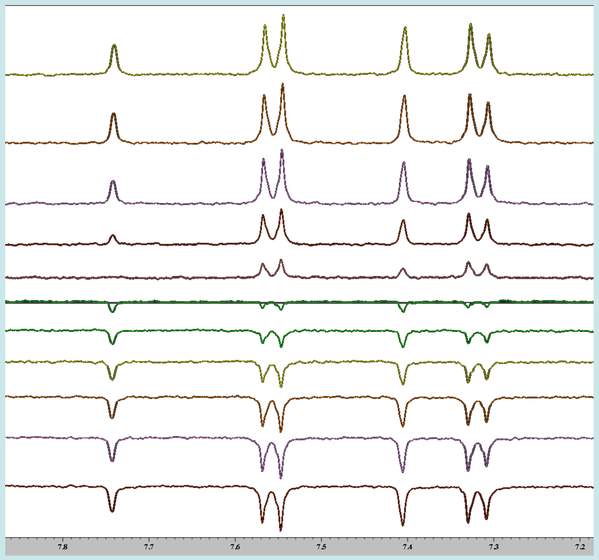


Figure 4 Stacked plot of NMR relaxation spectra for free 4-CPI. Tau interval increases from bottom to top, where the lowest spectrum shows τ = 0.1 s and the top shows τ = 12 s. Relaxation experiments used the sequence 180°-τ-90°-detect, with 20 seconds relaxation delay and τ values of 12, 10, 6, 3, 2, 1, 0.75, 0.5, 0.3, 0.2, and 0.1 seconds. These experiments took 16 scans at 28.5° C unless otherwise indicated.

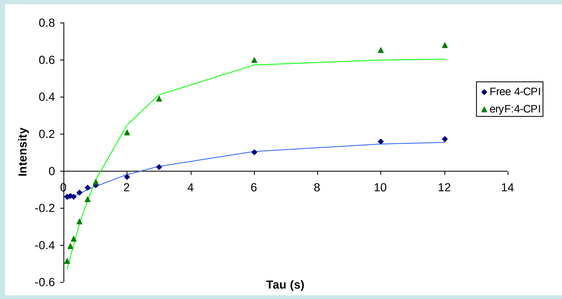


Figure 2 Example of proton relaxation curve. Data points (triangles and diamonds) show peak intensity after a given tau interval and are fit to a exponential model (curve) using the equation:

$$I = I_{max} * (1 - 2 * e^{(-\tau / T1)})$$

where I_{max} describes the horizontal asymptote, τ is the relaxation delay, and T1 is a fitting parameter unique to the proton.

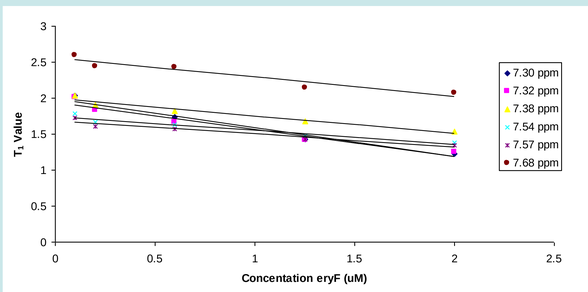
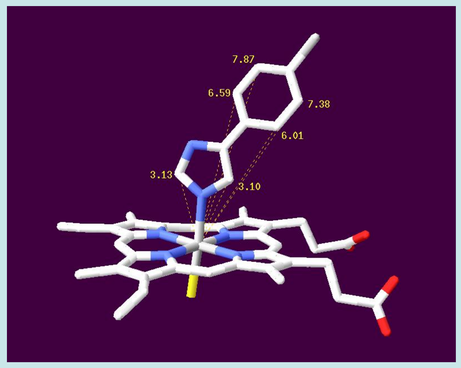


Figure 3 T1 relaxation time as a function of enzyme concentration. As the concentration of enzyme increases, the T1 value decreases, representing the larger fraction of 4-CPI being affected by the paramagnetic iron in the eryF active site.

- The rate at which a nucleus relaxes depends on its environment, where closer proximity to a paramagnetic center speeds relaxation. With a paramagnetic iron in the eryF active site, protons on bound 4-CPI re-equilibrate with the surrounding magnetic field more quickly than do the protons on free ligand.
- Because relaxation rates represent an average of all protons in solution, increasing the fraction of ligand bound decreases T1 values, as shown in Figure 3. This may also be explained by the equation shown by Figure 5, where adding enzyme increases alpha.
- T1 values of the protons on free 4-CPI were all between 2 and 2.5 seconds, except for that of the peak furthest downfield, which relaxed more slowly and gave a T1 near 4.3 seconds.



Molecular Modeling

Figure 5 The crystal structure of CYP2B4 in complex with 4-CPI with Fe-C distances shown.[6] NMR T1 results were converted to distances using a simplified form of the Solomon-Bloembergen equation[7]:

$$\frac{1}{T1_P} = \alpha \frac{9.87 \times 10^{16} S(S+1)}{r^6} (\tau_c)$$

(α = fraction of ligand bound out of the total ligand concentration, r = distance from proton to iron, $\tau_c = 3 \times 10^{-10}$ seconds , and S = spin state of the iron, which in this case is ½)

Ligand-protein complexes were modeled using distance measurements and the molecular docking program HADDOCK, producing images such as that shown in Figure 5. Preliminary trials have successfully generated ligand-protein models, showing that our protocols work and docking is possible.

Conclusions

- Literature ligand binding affinities were reproduced, with K_D of 4-CPI = 26.8 uM and S_{50} of 9-AP = 5.8 uM.
- Pre-saturating eryF with low concentrations of 9-AP, so that only the first site is occupied, does not affect 4-CPI binding affinity. Therefore, the first equivalent of 9-AP must bind somewhere other than near the heme. However, filling the second 9-AP binding site induces the same absorbance change as does 4-CPI binding, showing that the second equivalent of 9-AP must bind in the same location and coordinate to the heme iron.
- The 4-CPI protons relax differently depending on whether the ligand is enzyme-bound or free in solution, and this change in T1 is proportional to the fraction of ligand bound.
  - This and temperature data (not shown) show that the change in relaxation is due to the influence of the heme, making distance measurements possible.
- Reducing the protein (Fe^{3+} → Fe^{2+}) eliminates the paramagnetic influence on T1 values and allows calculation of ligand-iron distances.

Future Directions

The T1 values of 4-CPI bound to reduced eryF did not match those obtained of free ligand, contrary to our expectation. Future experiments will seek to improve reproducibility of the T1 data, especially in reduced samples, by adopting one or more of the following changes:

- Use a different reducing agent
- Flushing samples with CO
- Use a different ligand that would convert the iron to a high-spin complex (S = 5/2), which would increase the paramagnetic effect and possibly make T_1 changes easier to detect

Acknowledgements

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