Predicting the Rates and Regioselectivity of Reactions Mediated by the P450 Superfamily

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no matter how many times the same sequence is aligned, one should never feel that ones work is done until a crystal structure has been determined for the protein!

Acknowledgment

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[36] Predicting the Rates and Regioselectivity of Reactions Mediated by the P450 Superfamily

By JEFFREY P. JONES and KENNETH R. KORZEKWA

Introduction

If the rate of a given CYP-mediated oxidation of a substrate can be predicted, a number of properties could be anticipated, including the half-life of drugs, the toxicity of a xenobiotic, and the amounts of different metabolites that would come from a given substrate. Studies in our laboratories are aimed at developing computational tools to predict the rates of CYP-mediated oxidations. To this end we have exploited two different computational methodologies, molecular dynamics (MD) and quantum mechanics (QM), to predict steric or electrostatic effects, respectively. In our studies to date, we have been able to predict the rates and regioselectivity when either steric factors and electronic factors are the predominant influence on the outcome of the reaction by using the appropriate method. It is our hope that these two methodologies can be merged to predict the rates and regioselectivity of CYP-mediated oxidations in general. While others have used similar methodology, we will focus on the protocols established in our laboratories.

While both QM and MD computational methods have been used in the study of other enzymic systems, the cytochrome P450 enzyme family is relatively unique in a number of ways. (1) At least for xenobiotic metabolism, P450 substrates have been shown to bind in a number of different orientations and with relatively low affinity. Thus, a given substrate can give a large number of different products. While most enzymes are stereospecific, xenobiotic metabolism by the cytochrome P450 superfamily is usually stereoselective, resulting in varying degrees of enantiomeric excess. (2) The substrate oxidation step is not catalyzed in the classic sense by the enzyme.

Instead, the enzyme catalyzes the activation of molecular oxygen,[1,2] and this active oxygen species acts like a chemical in a nonpolar solution. The transition state for the oxidation of substrate does not appear to be stabilized by the enzyme to significant extent.[3,4] (3) The substrate oxidations occur at very slow rates ($<100\ min^{-1}$) for all but a few enzymes in the superfamily.

These three characteristics of the P450 superfamily have an effect on the design and interpretation of computational methods for these enzymes. The first characteristic, the lack of strong binding forces, makes the use of QM methods to predict rates possible since the product formed is in some cases dependent entirely on the reactivity at a given position and is not influenced by the enzyme. However, this same characteristic can make MD methods difficult to use since no specific binding orientation can be assumed and very long simulations may be required to approximate the free motion of substrate in the active site. The second characteristic, the chemical-like nature of the oxidation step, again favors QM methods, but will also make MD methods easier to apply in the exploration of enzyme interactions with transition states and intermediates. The third characteristic, the slow rates of product formation, hinders the use of both QM and MD methods. The slow reaction rate is due to rate-limiting steps prior to substrate oxidation. These steps act to mask the electronic effects on the rates of reaction. This characteristic also means that MD will have to be extremely long to simulate the complete space available for substrate motion prior to oxidation. Thus, the choice of substrate, based on the compounds reactivity and freedom motion in the active site, can have a profound effect on the success of a QM or MD simulation. Our success to date, in part, reflects our judicious choice of substrates.

Structural Models of Mammalian P450s Based on P450cam and P450bm3 Crystal Structures

In an attempt to predict the effects of structure on the rates of CYP-mediated reactions, we have used two of the four bacterial CYP enzymes for which crystal structures have been published.[5,6] From our studies of benzo[*a*]pyrene and [*R*]- and [*S*]-nicotine, it would appear that certain

[1] K. R. Korzekwa and J. P. Jones, *Pharmacogenetics* **3,** 1 (1993).
[2] J. Aikens and S. G. Sligar, *J. Am. Chem. Soc.* **116,** 1143 (1994).
[3] J. P. Jones, A. E. Rettie, and W. F. Trager, *J. Med. Chem.* **33,** 1242 (1990).
[4] S. B. Karki, J. P. Dinnocenzo, J. P. Jones, and K. R. Korzekwa, *J. Am. Chem. Soc.* **117,** 3657 (1995).
[5] T. L. Poulos, B. C. Finzel, and A. J. Howard, *J. Mol. Biol.* **195,** 687 (1987).
[6] K. G. Ravichandran, S. S. Boddupalli, C. A. Hasemann, and J. A. Peterson, *Science* **261,** 731 (1993).

structural features are conserved between the mammalian and the bacterial systems. If this is true, it is likely to be the regions of the protein responsible for heme binding and orientation, as suggested by Poulos.[7] However, the low primary sequence homology and the fact that a single amino acid change can alter substrate selectivity[8] means that predicting structural effects for mammalian CYP enzymes will require crystal structures for the mammalian enzymes. In the absence of crystal structures for mammalian CYP enzymes, we are forced to use the bacterial enzymes as surrogates for the mammalian enzymes and, through a process we call functional homology, make deductions about what factors may influence binding. These studies serve at least two purposes: (1) they produce hypotheses about the active site that can be tested through experiment and (2) they help us sharpen the tools that we will use when crystal structures become available for mammalian enzymes.

The following strategy is used to prepare for a MD run. (1) The compound of interest is tested to determine if it is a substrate for CYP101 or CYP102. (2) Point charges are determined for, and a force field assigned to, the substrate molecule. (3) The enzyme is prepared for dynamics. (4) The molecule is docked in the active site. (5) Dynamics runs are made to equilibrate the molecule in the active site. (6) Production dynamics runs are made. (7) Statistics are collected and analyzed. (8) The results are compared with experiment.

To date, we have studied three substrates, benzo[*a*]pyrene,[9] [*S*]-nicotine, and [*R*]-nicotine,[10] in detail. We have also performed preliminary studies with norcamphor, a series of dimethylanilines,[4] and a series of toluenes. In all cases a large amount of CYP101 or CYP102 was required to see substantial turnover. Normally we must use between 1 and 10 nmol of enzyme to see enough product for accurate quantitation. To ensure that the turnover occurs in the active site, we perform inhibition experiments with camphor or palmitic acid for CYP101 or CYP102, respectively. Since molecular dynamic calculations are usually run with a single substrate molecule, the experiments should be run to determine V/K values or to determine some intramolecular ratio since these values reflect turnover at low substrate concentrations. For example, for the nicotine enantiomers we made pseudoracemic mixtures [the (*R*) enantiomer was labeled with 1 dueterium in the 2′ position] so that the levels of metabolism could be compared using an intermolecular competitive type of experiment. For benzo[*a*]pyrene we looked at the intramolecular competition between oxidation on two sepa-

[7] T. L. Poulos, *Methods Enzymol.* **206,** 11 (1991).
[8] R. L. P. Lindberg and M. Negishi, *Nature* (*London*) **339,** 632 (1989).
[9] J. P. Jones, M. Shou, and K. R. Korzekwa, *Biochemistry* **34,** 6956 (1995).
[10] J. P. Jones, W. F. Trager, and T. J. Carlson, *J. Am. Chem. Soc.* **115,** 381 (1993).

rate faces of the molecule. These intermolecular and intramolecular competition types of experiments are usually required since the low level of turnover with the bacterial enzymes makes the use of high substrate concentrations desirable, but the theoretical construct predicts behavior at low substrate concentrations.

The second step, assignment of point charges to the substrate, remains problematic in that no consensus has been established on how to make these assignments. The standard method for determining point charges is to do a quantum chemical calculation and then perform a Mulliken population analysis on the resulting wave function. However, Mulliken population analysis has a number of problems that have been described in the literature.[11] In our laboratory we normally use the semiempirical AM1 Hamiltonian[12] to optimize our structure since this method gives very good geometries for a number of different functional groups. We then do a single point calculation (a calculation with no geometry optimization) using the semiempirical MNDO Hamiltonian.[13] The MNDO Hamiltonian has been shown to give a good wave function for charge determination.[14] The point charges are assigned to this wave function using the ESP program of Besler and Merz.[14] This program probes the wave function with a charge and assigns point charges based on a least-squares fit to the resulting potential. All of these methods are available in either the Gaussian 94 or Mopac 93 quantum chemical packages.

Preparation of the protein involves downloading the protein structure from the Protein Data Bank at Brookhaven (a PDB file) and loading charges and force constants for the various amino acids. For the CYP superfamily of enzymes, an added complication is that the parameters for the iron–protophyrin IX–sulfur complex are not available in the standard databases and needs to be constructed. Our method of construction is outlined in Jones *et al.*[10] We tested our parameters using a thermodynamic cycle for [*R*]- and [*S*]-nicotine binding and found that we could quantitatively reproduce the experiment.[10] While our results are encouraging, parameters for the heme-thiol group can likely be improved. After the input parameters are constructed, the protein must be minimized. The starting crystal structure will be at a very high energy and should be relaxed before docking a molecule in the active site. For CYP101 we used the 3CPP structure from the PDB at Brookhaven. To relax the protein the following

[11] A. E. Reed, J. L. Curtiss, and F. Weinhold, *Chem. Rev.* **88,** 899 (1988).
[12] M. J. S. Dewar, E. G. Zoebisch, E. F. Healy, and J. T. Stewart, *J. Am. Chem. Soc.* **107,** 3902 (1985).
[13] M. J. S. Dewar and W. Thiel, *J. Am. Chem. Soc.* **99,** 4899 (1977).
[14] B. H. Besler, K. M. Merz, and P. A. Kollman, *J. Comput. Chem.* **11,** 431 (1990).

protocol was used. (1) The waters of crystalization were minimized using the steepest descent method in the MINMD module of AMBER[15] without any electrostatic energy terms and holding the entire protein fixed. (2) The electrostatic terms were included for the water and minimized. (3) All hydrogen atoms were minimized by steepest descent. (4) The side chains were minimized holding the C_α backbone fixed. (5) All atoms were minimized by steepest descent. (6) All atoms were minimized by the conjugate gradient method. After complete minimization the RMS deviation from the crystal structure was 0.57 Å.

Docking the substrate in the enzyme active site of the bacterial enzymes is an art. At least two problems are encountered when docking a substrate: (1) the small active site of P450cam makes docking difficult and (2) the starting orientation can have a profound effect on the results. In docking our substrates, every effort is made to keep the protein as close to the crystal structure as possible. Thus, we fix the protein when we dock the substrate, minimize the substrate, and perform dynamics with the protein fixed. Only after the substrate has low energy are the side chains of the protein allowed to move. Finally, the whole protein is minimized. After docking, the entire enzyme–substrate complex should maintain a significant negative energy. The effect of starting orientation on the subsequent dynamics runs was apparent in both our nicotine and B[*a*]P studies. The nicotine enantiomers were docked in a number of orientations in CYP101. (Note: Nicotine was not metabolized by CYP102 so no studies were done with this enzyme.) In each case, the pyridine ring nitrogen established a hydrogen bond with TYR96 during equilibration. However, the pyrolidine ring would adapt one of two different conformations depending on the starting structure. These two conformations exchanged very slowly at 310 K. Thus, the starting conformation influenced the sampling statistics. While the slow interchange is consistent with our experimental results,[16] it confounds analysis of the data. An even larger dependence on the starting orientation was observed for B[*a*]P. In this case, no interchange was observed among four possible starting orientations. Again, these results are consistent with experimental results, but it means that predictions about the amount of each oxidation product are difficult to make.

After docking, the enzyme–substrate complex must be equilibrated for the dynamics runs. We use the SANDER module in AMBER for our dynamics runs. Initially, velocities are randomly assigned and the system

[15] D. A. Pearlman, D. A. Case, J. C. Caldwell, G. L. Seibel, U. C. Singh, P. Weiner, and P. A. Kollman, "AMBER 4.0." University of California, San Francisco, 1991.

[16] T. J. Carlson, J. P. Jones, L. Peterson, N. Castagnoli, K. R. Iyer, and W. F. Trager, *Drug. Metab. Dispos.* **23,** 749 (1995).

is slowly heated to 310 K over 10 psec. The temperature is maintained by loosely coupling the system to a 310 K constant temperature bath. Dynamics runs are made at 310 K until the system maintains a relatively constant temperature, potential energy, and kinetic energy. For the majority of our calculations we have used the belly routine in AMBER to limit the number of residues that are allowed to move. This has two effects: (1) the overall structure of the molecule will remain close to the crystal structure and (2) the computational effort is greatly reduced. The first effect may be deleterious if the crystal structure is not a substrate-bound structure. Preliminary reports from Ornstein indicate that this may be the case for P450BM3.[17] However, if the structure is close to the substrate-bound one, this method provides the opportunity to explore more of the substrate–enzyme interaction surface since fewer amino acids are in motion and the protein does not need to be solvated. We establish which amino acids should be included in the calculation by trial and error. Initially we use a sphere around the substrate of ca. 12 Å. After the system is reasonably equilibrated, the dynamics runs are observed and different amino acids are either added or deleted based on proximity to the substrate. If allowing an amino acid to move alters the simulation by causing a significant move from equilibrium, it is added in the simulation. We usually do not allow the amino acids on the proximal side of heme to move and the heme itself is held rigid.

Production dynamics are run after equilibration. Thus far, the length of our dynamics runs has been dictated by CPU and disk space constraints. Simulation of around 200 psec can be performed in about 1 week of CPU time on today's workstations.

The analysis of production runs involves compiling statistics about the motion of the substrate in the active site. The assumption is that the oxidizable positions will be in close proximity to the active oxygen species for a reaction to occur. We have used two methods to predict reactivity: (1) the average distance between the position of oxidation and the active oxygen species is determined and (2) the number of times a position is within a given distance, during the simulation, is counted. The first is qualitative and presents a simple picture of the predicted regioselectivity. The second can be used to quantify the simulation's predicted regioselectivity. Neither takes into account the differences in the reactivity of different positions in the molecule. Post facto inclusion of reactivity is extremely complicated since the rise in energy as a hydrogen atom approaches the active oxygen species is 40–50 kcal/Å in quantum chemical calculations. Thus, to a first approximation it would appear that distance would overwhelm reactivity

[17] M. D. Paulsen and R. L. Ornstein, *Proteins* **21,** 237 (1995).

differences. However, White and co-workers[18] have shown that enzymatic reactivity is similar to that expected for Boltzman distributions of orientations, at least for some substrates.

Electronic Models of Mammalian P450s Based on Semiempirical Methods

QM models for cytochrome P450 oxidations are models that use the electronic properties of the P450 enzymes and their substrates to predict the rates of oxidations. The rationale for this approach has intuitive and physical chemical origins, and has been used qualitatively for many years. For example, the tendency for oxidation of a certain functional group generally follows the relative stability of the radicals that are formed, e.g., N-dealkylation > O-dealkylation > 2° carbon oxidation > 1° carbon oxidation. This is a consequence of the generally broad regioselectivity of the P450 enzymes. If an enzyme has access to several positions of a substrate, the most easily oxidized position will be metabolized. The more formal relationship between stability and reaction rates can be described as variations of the Bronsted relationship.[19] Within certain classes of reactions, the activation energy of a reaction is proportional to the heat of reaction. In other words, the more stable the products, the faster the rate of reaction.

Our first quantitative QM model (the PNR model)[20] was based on hydrogen abstraction reactions using the *p*-nitrosophenoxy radical (PNR) and the semiempirical AM1 Hamiltonian. The model uses the calculated (AM1) enthalpies of reaction and the ionization potentials of the resultant radicals to predict the AM1 activation enthalpies (H_{act}). This model oxygen radical was chosen because it gave a thermodynamically symmetrical reaction ($H_{reac} = 0$) for the abstraction of a hydrogen atom from primary methyl groups in alkanes, within the AM1 formalism. Isotope effect experiments for cytochrome P450-mediated oxidations suggested that the P450-catalyzed reaction is also symmetrical.[21] The *p*-nitrosophenoxy radical was used to abstract hydrogen atoms from a series of 20 substrates, providing transition state geometries and thermodynamic properties. Although a moderate correlation was obtained for the pure Bronsted relation ($r^2 = 0.86$), the correlations could be greatly improved by including resonance parameters. When compared to inductive effects, a smaller fraction of resonance stabilization

[18] R. E. White, M. McCarthy, K. D. Egeberg, and S. G. Sligar, *Arch. Biochem. Biophys.* **228,** 493 (1984).

[19] R. P. Bell, *in* "Correlation Analysis in Chemistry" (N. D. Chapman and J. Shorter, eds.), p. 55. Plenum, New York, 1972.

[20] K. R. Korzekwa, J. P. Jones, and J. R. Gillette, *J. Am. Chem. Soc.* **112,** 7042 (1990).

[21] J. P. Jones and W. F. Trager, *J. Am. Chem. Soc.* **109,** 2171 (1987).

of the product radical is present at the transition state.[22] Since resonance parameters are not available for most cytochrome P450 substrates, the ionization potential of the radical (IP) was used as a measure of the resonance contribution for stabilizing the radical. Inclusion of the ionization potential gave excellent correlations between calculated heats of reaction and calculated activation energies.

Equation (1) can be used to predict the AM1 activation energy for hydrogen atom abstraction by the *p*-nitrosophenoxy radical:

$$H_{act.} = 2.60 + 0.22\ H_{reac.} + 2.38\ IP. \quad (1)$$

To predict the AM1 activation energy for hydrogen abstraction, it is only necessary to model the substrate and resultant radicals. Any standard implementation of the AM1 semiempirical program can be used, e.g., AMPAC, MOPAC, etc. Standard RHF electronic calculations should be used for closed shell calculations, and the UHF formalism should be used for open shell calculations. No post-Hartree–Fock methods such as configuration interaction should be used when generating electronic structures. Substrates and radical geometries should be minimized to default tolerances. Care should be taken that the conformation of the radical corresponds to a geometry accessible by the abstraction of a hydrogen atom from the substrate. This can usually be accomplished using the substrate geometry, less a hydrogen atom, as the starting geometry for the radical. Relaxation of this geometry will usually result in the conformation of the radical that is accessible from the transition state for hydrogen atom abstraction. Standard output for most programs includes the heat of formation and ionization potentials, which are used to estimate the AM1 activation energy for the hydrogen abstraction reaction by Eq. (1). This process is far less time-consuming than generating the reaction coordinates necessary to model the actual transition states.

The values of these calculated energies do not correspond to absolute rates since semiempirical calculations overestimate transition state energies. Instead, these values should be used on a relative basis. When calculating $H_{act.}$ values, the heats of formation for the nitrosophenoxy radical and nitrosophenol are 19.04 and −14.65 kcal/mol, respectively. The value for the radical is for one of two stable geometries for two different electronic states of the radical. The other geometry is lower in energy, but was not used to generate the reaction coordinates for the model. Since only relative activation energies are obtained, the actual values used in the calculation

[22] C. F. Bernasconi, *in* "Nucleophilicity" (J. M. Harris and S. P. McManus, eds.), p. 115. American Chemical Society, Washington, DC, 1987.

are not important, as long as they are consistent within the reactions being compared.

The PNR model has been used to describe the reaction rates of several cytochrome P450-mediated reactions. In our first attempt to use an electronic model for cytochrome P450 oxidations, the PNR model and calculated hydrophobicities were used to predict the experimental toxicities of a series of nitriles.[23] The acute toxicities of most nitriles are due to cyanide release, mediated by the hydroxylation of nitriles adjacent to the cyano functionality. The resultant cyanohydrin chemically decomposes, releasing cyanide. We used the calculated thermodynamic properties of 26 nitriles and their radicals to predict the relative rates for oxidation at all possible positions. Since oxidations by the cytochrome P450 enzymes can result in either detoxification or activation, it was necessary to include all potential oxidations in the correlations. Significant correlations were obtained between the experimental LD_{50} values and $k\alpha$ corr, the rate constants corrected for the fraction of the metabolism to the toxic metabolite:

$$k\alpha_{corr} = k\alpha[k\alpha/(k\alpha + k\beta + k\gamma \ldots)]. \quad (2)$$

Better correlations were obtained with k corr than either k or the fraction $(k\alpha/k\alpha + k\beta + k\gamma \ldots)$ alone, suggesting that both rates and regioselectivity of metabolism are important.

Hydrophobicity parameters are often included in QSAR studies, presumably to account for substrate transport and binding[24] In the nitrile study, the CLOGP program[24] was used to predict the hydrophobicities of the various substrates. However, recent studies with an extended set of nitrile LD_{50} values suggest that using the Syracuse Program[25] to calculate the hydrophobicities provide improved correlations (unpublished results).

Using the PNR model, we have obtained excellent correlations with several other sets of experimental data. Using data provided by White and McCarthy[26] for the metabolism of substituted toluenes by CYP2B4, a fit of the experimental k_{cat} values to the predicted enthalpy of activation and the log P of each compound gave a regression coefficient of 0.94. The AM1 model was used to calculate the predicted activation energies for P450-mediated hydrogen abstraction from a series of pentahaloethanes.[27] *In vivo*

[23] J. Grogan, S. C. DeVito, R. S. Pearlman, and K. R. Korzekwa, *Chem. Res. Toxicol.* **5,** 548 (1992).

[24] "CLOGP Version 3.42." Pomona College Medicinal Chemistry Project, Clarmont, CA.

[25] W. Meylan and P. Howard, "KOWWIN Program." Syracuse Research Corporation Environmental Sciences Center, Merrill Lane, Syracuse, NY.

[26] R. E. White and M. B. McCarthy, *Arch. Biochem. Biophys.* **246,** 19 (1986).

[27] J. W. Harris, J. P. Jones, J. L. Martin, A. C. LaRosa, M. J. Olson, L. R. Pohl, and M. W. Anders, *Chem. Res. Toxicol.* **5,** 720 (1992).

administration to rats of the pentahaloethanes in this study resulted in the trifluoroacylation of liver proteins, presumably by trifluoroacylhalides formed by P450 oxidations. Both the degree of trifluoroacylation, as determined by immunoblotting, and the amount of trifluoroacetic excreted in the urine paralleled the activation energy for hydrogen abstraction as predicted by the AM1 model. The agreement between theory and experiment indicates that the electronic characteristics of the halogenated hydrocarbon are the important determinants in the rate of trifluoroacetic acid produced. Finally, the predicted activation energies for a series of inhalation anesthetics was calculated with our model. An excellent correlation is obtained between the predicted rates of metabolism and the percentage *in vivo* metabolism in humans.[28]

Unfortunately, the PNR model cannot be extended to other (nonhydrogen atom abstraction) P450-mediated reactions. The nitrosophenoxy radical, within the AM1 formalism, does not remain on the same potential energy surface for some calculations, including addition to aromatic and olefinic compounds. Although other small oxygen radicals remain on the same potential energy surface, excessive amounts of spin contamination suggest that the calculations will not be size consistent. Using RHF AM1 calculations with partial configuration interaction prevents the spin contamination problem, but these calculations are also not size consistent. Therefore, we are in the process of using *ab initio* calculations to develop expanded electronic models for P450 oxidations.

Conclusions

Thus, while we have had a number of successes with both QM and MD calculations, our goal of obtaining a general predictive methodology has not been obtained. Our PNR model appears to give a good quantitative prediction of rates for hydrogen atom abstractions, but is inappropriate when applied to reactions that do not occur by hydrogen atom abstraction or to substrates, such as nicotine and benzo[*a*]pyrene, which have limited motion in the active site. In turn, MD models can provide information on binding factors and motion of the substrate in the active site, but mammalian structural models do not exist and regioselectivity can only be predicted in a semiquantitative way, especially when the positions of interest have different chemical reactivity.

[28] H. Yin, M. W. Anders, K. R. Korzekwa, L. Higgins, K. E. Thummel, E. D. Kharasch, and J. P. Jones, *Proc. Natl. Acad. Sci. U.S.A.* (in press).