Communication to the Editor

Piperonyl butoxide-mediated inhibition of cytochrome P450-catalysed insecticide metabolism: a rational approach†

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Abstract: The inhibitory effect of piperonyl butoxide (PBO) on cytochrome P450 was studied by theoretical methods. Binding conformations of PBO were obtained by the recently developed low-mode conformational search within the active site of cytochrome $P450_{cam}$. Increased activity of PBO relative to other methylendioxyphenyl inhibitors was rationalized by its decreased conformational mobility and the steric block created by its long side-chain in the substrate access channel of the enzyme.

Keywords: cytochrome P450; piperonyl butoxide; mechanism of action; Monte Carlo docking

Since the first observation that cytochrome P450-dependent carbaryl resistance could be abolished using the methylenedioxyphenyl inhibitor, Sesamex,[1] the in-vitro and in-vivo activity of methylenedioxyphenyl inhibitors has been demonstrated.[2,3] Although some of these compounds were introduced to the market, and piperonyl butoxide (PBO) is the leading insecticide synergist in commercial use,[3,4] the molecular mechanism of its action has not yet been interpreted. Here we report our recent investigations on this mechanism using the high resolution x-ray structure of cytochrome $P450_{cam}$. Observed structural similarity of P450 enzymes incorporating small substrates, and the diversity of the potential substrates for $P450_{cam}$,[5–7] as well as the structural similarity observed between 52 membrane-bound cytochrome P450s and $P450_{cam}$,[8] suggest that the active site of this enzyme can serve as a predictive model for the inhibition caused by methylenedioxyphenyl synergists. Recent success in the construction of cytochrome P450 isozymes (see eg CYP2B4),[9] 3D homology models and moderate sequence similarity between the specific insect P450 (CYP6A1) and $P450_{cam}$ should allow us to build a homology model for CYP6A1. This work is still in progress, but basic conclusions can already be drawn using the model based on the $P450_{cam}$ x-ray structure. Insight into the mechanism of inhibitory action is crucial in recent efforts to design and synthesize potent new inhibitors of cytochrome P450 monooxygenases.

PBO was docked to the relaxed active site of the enzyme using the efficient Monte Carlo-based methodology developed in our laboratory. Lacking the structural information of the specific insect enzyme, the crystal structure of adamantane-bound cytochrome $P450_{cam}$[10] was used to predict the most important interactions affecting the binding of inhibitors. The complexed adamantane was replaced manually by the minimum-energy conformation of the corresponding substrate. More details on the construction of the binding site and the parameters and atomic charges used in molecular mechanics calculations are given in our previous publication.[11] The initial conformational analyses were carried out utilizing the new, so-called frozen-atom feature of the 6.0 release of the Macro Model package.[12] All of the atoms of the $P450_{cam}$ enzyme shell and the heme unit were treated as fixed-point charges located at their crystallographically determined positions. The Coulomb electrostatic energy equation was utilized with the electrostatically fitted charges on the ligands (calculated at AM1 semiempirical level) and a distance-dependent dielectric constant $\varepsilon = 4r$ as a simple model for the charge–charge interaction screen-

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ing effect of the protein environment. Since the P450 active site is situated in the hydrophobic pocket and is buried entirely in the enzyme, the attenuated electrostatic treatment of the protein environment is justified as a first approximation without explicit consideration of solvent effects. During the initial conformational search, the ligands were allowed to move and deform freely in the external field of the fixed point charges of the frozen enzyme atoms. In a second analysis, however, the raw output structures of the initial conformational searches were refined in order to allow for the proper relaxation of the flexible side-chains and for the inclusion of solvation effects. The refinement involved the re-optimization of all of the output structures of the conformational searches using a tethered rather than a frozen shell. In this secondary shell, the enzyme atoms were only tethered by quadratic restoring potentials with a weak force constant of 35 kJ mol^{-1} A^{-1} allowing limited movement of the entire shell to adopt relaxed, low-energy side-chain orientations. Furthermore, the popular GB/SA continuum solvation model[13] available in Batchmin was used instead of the $\varepsilon = 4r$ electrostatic treatment to account for solvation effects 2000 low-mode conformational search steps,[14] supplemented with explicit rotations and translations of the ligand, were applied and, after energy minimization, the low-energy binding conformations within 50 kJ mol^{-1} above the global minimum were saved for further analysis. The output structures were reoptimized with the tethered shell and GBSA solvation treatment and only those within the lowest 35 kJ mol^{-1} energy window were saved and examined using molecular graphics.

The inhibitory action of PBO is initiated by its enzymatic recognition. Enzyme–substrate interactions were therefore studied using the energy-minimized structures of the cytochrome $P450_{cam}$–PBO complexes. Low-energy binding conformations obtained by Monte Carlo conformational search were analysed by the evaluation of the distances between the ferryl oxygen and the closest carbon atoms provided by the different inhibitor orientations. Since proximity of the central carbon atom of the methylenedioxy O—CH_2—O fragment is required for the formation of the intermediate carbene, the conformational pool could be grouped into two main families. In the first family, the crucial methylenedioxy carbon atom was found to be closest to the ferryl oxygen (active conformers) while the remaining conformations can be characterized as possibly inactive low-energy conformers. Conformational search for the low-energy binding conformations of PBO resulted in 29 unique conformers and all of them belong to the active family (100%). The global minimum energy conformation of the cytochrome $P450_{cam}$–PBO complex is depicted in Fig 1. Although the long side-chain of PBO makes the inhibitor considerably flexible, because of its large molecular volume relative to the size of the binding pocket, conformational freedom is rather limited. Monte Carlo docking of two structurally related

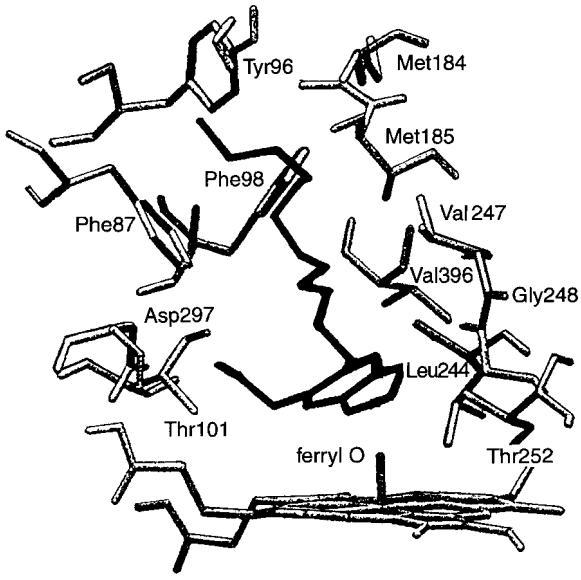

Figure 1. Minimum energy conformation of cytochrome $P450_{cam}$–PBO complex obtained by LMOD/MOLS calculations.

inhibitors, safrole and isosafrole led to 19 (54.2%) and 11 (73.3%) active binding conformations. Comparison of the relative numbers of active conformations obtained for the macromolecular complexes of safrole, isosafrole and PBO with their corresponding molar IC_{50} values for the inhibition of oxidative carbaryl metabolism in fat body homogenates from cabbage looper (8.0, 3.6 and 1.0×10^{-5}, respectively)[15] suggests that relative abundance of conformers can be used as a descriptor of inhibitory potency. Conformational analysis of the macromolecular complex of inhibitor candidates might, therefore, be particularly useful for the molecular design of more potent synergists.

Structural studies on several cytochrome P450-substrate complexes revealed that hydrophobic interactions might be crucial for substrate recognition.[10,16] The hydrophobic pocket around the complexed safrole, isosafrole and PBO was identified as a 3.0 Å sphere around the particular inhibitor. Low-energy active conformers of safrole and isosafrole occupy virtually the same volume where the natural substrate, camphor, binds.[17] Based on the global minimum structure of the most active PBO, side-chains should have at least two functions (Fig 1). The shorter side-chain and the methylenedioxyphenyl ring partly occupy the substrate binding site (Leu244, Val247, Asp297) while the longer chain extends into the substrate access channel (Phe87, Tyr96, Phe98), which should direct the substrate to the active site. Therefore, we conclude that, in addition to the limited conformational mobility of PBO, these interactions are mainly responsible for the more complete inhibition of the enzyme by blocking both the ligand binding site and the substrate access channel. These dual functions were recently recognized in the case of an inhibitor from Pfizer which has similar side-chains to those of PBO.[18]

Using the proposed model we found several factors that can be utilized in the rational design of more potent inhibitors. Hydrophobic interactions were found to be important in substrate recognition. Construction of the CYP6A1 homology models is in progress, which should enable us to gain in-depth knowledge of the inhibitory action based on our preliminary conclusions drawn here.

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