Conformational Flexibility of Mammalian Cytochrome P450 2B4 in Binding Imidazole Inhibitors with Different Ring Chemistry and Side Chains

**SOLUTION THERMODYNAMICS AND MOLECULAR MODELING**

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Recent x-ray structures of cytochrome P450 2B4 (CYP2B4) reveal an open form that undergoes a large-scale structural transition to a closed form upon binding to 4-(4-chlorophenyl)imidazole (4-CPI). Here, we report for the first time a complete solution thermodynamic study using isothermal titration calorimetry supported by spectroscopic studies to elucidate the conformational flexibility of CYP2B4 in binding imidazole inhibitors with different ring chemistry and side chains: 4-CPI, 1-benzylimidazole (1-BI), 1-CPI, 4-phenylimidazole (4-PI), 1-(2-((benzoxo)ethyl)imidazole (BEI), and 1-PI. Each of the inhibitors induced type II spectral changes, and the binding enthalpy ranged from 14 to 24 kcal mol⁻¹, following the order 1-BI < 4-CPI < 1-CPI < 4-PI < BEI < 1-PI. Calorimetric titrations using monomeric enzyme yielded a 1:1 binding stoichiometry, with the associated binding entropies ranging from 0.1 to 2.4 kcal mol⁻¹, with the exception of 4-CPI, one of the smallest ligands to the enzyme. Changes in enthalpy at 25 °C ranged from −6.5 to −8.8 kcal mol⁻¹.

The largest conformational rearrangement observed to date occurs in mammalian CYP2B4; the wide open binding pocket (Protein Data Bank code 1POS) undergoes a dramatic change to tether around 4-(4-chlorophenyl)imidazole (4-CPI; code 1SUO), one of the smallest ligands to be characterized in a P450 active site (12, 13). The spatial orientation of the dynamic structural elements between these two forms is depicted in Fig. 1. The major structural changes are limited to the lid domain of helices F and G; helices I, C, and B; and the B₁–I₄ region, with little rearrangement at the secondary structural level. These changes are consistent with similar but smaller changes observed for bacterial P450 BM3 (14) and thermophilic CYP119 (5, 6). The CYP2B4 structures may represent the widest range of conformational flexibility available in a P450 molecule. The significant movement of helix C may be the structural mechanism for coordination of redox partner and substrate binding, as charge pairing between helix C and NADPH-cytochrome P450 reductase or cytochrome b₅ is thought to be driving force for this interaction (12, 13, 15). Although such an open/closed mechanism may explain the entry of diverse substrates, it cannot explain enzyme specificity. Interestingly, significant conformational changes in the region of helices F and G and the B₁–C loop have also been shown to contribute to substrate recognition and binding in CYP2C5 (7, 8). Here, changes in the hydration network of the active site were shown to be a decisive factor in binding ligands of different size in an adaptive fit manner.

In the case of CYP2B enzymes, a wealth of site-directed mutagenesis data is available to provide insight into the structural basis of regio- and stereospecific oxidation of various substrates (16–20). Many of the active-site residues inferred from these studies are in the 4-CPI-binding site in the closed conformation of the enzyme. Sequentially, there may be additional structural rearrangement in the active site and the substrate access channel before the open structure adopts the closed conformation. Interestingly, CYP2C9 and CYP3A4 structures show no such major structural changes upon ligand binding and may need larger ligands near the heme pocket for such changes to be manifested (15, 21, 22).
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FIGURE 1. Ribbon representation of 2B4dH crystal structures in ligand-free (blue; Protein Data Bank code 1POS) and 4-CPI-bound (green; code 1SUO) conformations. The two structures are overlaid to depict the spatial orientation of the dynamic structural elements. The flexibility of the B–C and F–G loops and the repositioning of helices B and C are distinctly notable. Trp221 (yellow), located in helix C, moves toward the less solvent-accessible heme (red) by 13 Å in the 4-CPI-bound conformation. All molecular graphic images were generated using PyMOL (35).

Because the crystal structures reveal one snapshot of a minimum energy conformation, they may be influenced significantly by the crystallization conditions, and ligand-induced conformational changes may be masked. Alternatively, some P450 enzymes may not undergo marked conformational changes upon ligand binding. Hence, it is of great interest to correlate structural changes observed by crystallography to solution dynamics and also to study structurally similar inhibitors, for which CYP2B4 was the obvious choice for our studies.

In solution, structure-function relationships of P450 have been studied using a wide array of spectroscopic techniques, and many of the fundamental concepts, including redox partner recognition (23), lipid interaction (24, 25), and catalytic mechanism (26), were studied using CYP2B4 as one of the model enzymes. Solution thermodynamics of P450-ligand interactions is an important complement to crystallographic, spectroscopic, and computational tools in understanding structure-function relationships and in the rational design of better P450 enzymes and structure-based drug design. Isothermal titration calorimetry (ITC) is the method of choice to examine the complete thermodynamics of protein–ligand and protein–protein interactions in solution (27–32). This method is being extensively and successfully used in combination with structural information as a major tool in drug discovery research in a variety of systems (27–29). The recent advances in calorimetric sensitivity and data analysis have enabled innovative solution conditions to be applied to the protein–ligand system (32–34). A single ITC experiment yields a complete thermodynamic picture of the interaction, including binding stoichiometry, affinity, and changes in enthalpy and entropy. To our knowledge, ITC has not been utilized to study P450-ligand interactions. In the case of mammalian P450 enzymes, this may be because of the protein aggregation that tends to occur at high concentrations required to conduct ITC experiments and also to the use of water-insoluble ligands. Surprisingly, however, there have not been any ITC studies reported with the more soluble bacterial P450 enzymes either, except for a single report on P450cam using ITC to study protein–protein interactions (36).

In this study, we used ITC in combination with spectroscopic and molecular modeling methods to characterize the interaction thermodynamics of six imidazole inhibitors with CYP2B4 to elucidate the basis of inhibitor potency and to monitor the conformational flexibility of the protein in solution. Solution conditions were standardized to maintain the protein in a monomeric form. Each of the compounds (1-benzylimidazole (1-BI), 1-[(4-chlorophenyl)imidazole (1-CPI), 4-phenylimidazole (4-PI), 1-phenylimidazole (1-PI), and 1-(2-(benzoxyl)ethyl)imidazole (BEI)) binds with a perfect 1:1 stoichiometry through an enthalpically driven hydrophobic interaction, but with distinct differences in the thermodynamic signatures. Strikingly, the addition of a single chlorine atom (4-CPI versus 4-PI) or the repositioning of an imidazole nitrogen (4-CPI versus 1-CPI) makes a major difference in the entropy-enthalpy compensation, which is derived mainly from the plasticity of the enzymes, because the structural flexibility of these inhibitors is limited. The large changes in entropy (–ΔS) and heat capacity (–ΔCp) associated with 4-CPI binding were attributed to the burial of the large water-accessible apolar surface area ensuing from a conformational change in the protein. The binding of 4-CPI greatly decreases the accessibility of Trp221, indicating a large movement of helix C. Strikingly, helix C is unperturbed upon binding to the other five inhibitors. The thermodynamic signatures correlate nicely with the spectral dissociation constants and IC50 values. These results clearly demonstrate that the tightly closed structure of 4-CPI-bound CYP2B4 deduced from the crystal structure may represent only one of many possible ligand-bound conformations of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Tris(2-carboxyethyl)phosphine, EDTA, phenylmethylsulfonyl fluoride, 1-BI, 1-PI, BEI, and glycerol (Sigma); CYMAL-5 (cyclohexylpentyl β-D-maltoside; Anatrace, Inc., Maumee, OH); 4-CPI (Maybridge Chemical Co., Cornell, UK); and 1-CPI (Lancaster Synthesis, Pelham, NH) were used as obtained. All other chemicals used were of analytical grade.

Protein Expression and Purification—2B4dH(H226Y), generated from the wild-type protein by truncation of the N-terminal domain (residues 3–21) and attachment of a C-terminal His6 tag as described (13), was used in all studies with minor modifications in the protein purification protocol as described below. Protein ligated to nickel-nitrilotriacetic acid resin was extensively washed with buffer A (50 mM potassium phosphate, 1 mM tris(2-carboxyethyl)phosphine, 1 mM EDTA, and 500 mM NaCl, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride, 20% glycerol, and 4.8 mM CYMAL-5 at 4 °C. Protein-bound nickel-nitrilotriacetic acid resin was washed twice with the above buffer containing 30 mM histidine. P450 was eluted with 80–100 mM histidine, and the fractions were pooled according to homogeneity on SDS-polyacrylamide gel. The protein was extensively dialyzed against buffer A. Dialyzed samples were passed through a 0.22-μm sterile filter, concentrated using a centrifugal device, and stored on ice (never frozen) for further experiments. The P450 concentration was measured by reduced CO difference spectra using an extinction coefficient of 91 mM−1 cm−1. This value is ∼10% lower than the total hemoprotein concentration determined by fitting the absolute oxidized spectrum to a series of low spin P450, high spin P450, and P420 standards as described previously for CYP2B4 (37). All data presented here were reproduced at least once with a different batch of enzyme.

Analytical Ultracentrifugation Experiments—Sedimentation velocity runs were performed using a Beckman Coulter Model XLA analytical ultracentrifuge at 60,000 rpm and 20 °C. The inhibitor-free and inhibitor-bound forms of 2B4dH(H226Y) were loaded into two different 3-mm double-sector quartz cells with a respective blank sector containing buffer and an equal concentration of inhibitor. Samples were spun at 60,000 rpm and monitored at 417 nm. A 60 μM protein concentration and a 2-fold molar excess of inhibitor were used in buffer A containing...
2% ethanol. An additional experiment was carried out for 4-CPI-bound 2B4dH(H226Y) in the presence of 1 mM CYMAL-5. Experiments were also conducted for the inhibitor-titrated samples of 2B4dH(H226Y) immediately after ITC experiments. Partial specific volumes, solvent density, and viscosity were calculated using the SEDNTERP program (38). A continuous sedimentation coefficient distribution model (c(s)), a grid size of 1000, and a resolution of 150 were used for analysis with SEDFIT Version 8.7 software (38).

Inhibitor Binding by Difference Spectral Titration—Difference spectra were recorded using 1 μM 2B4dH(H226Y) on a Shimadzu 2600 spectrophotometer at 25 °C. Protein and inhibitor samples were prepared in buffer A containing 1 mM CYMAL-5 and 2% ethanol. Protein sample (3 ml) was divided into two matched quartz cuvettes, and a base line was recorded between 350 and 500 nm. Difference spectra were recorded following the addition of a series of 5-μl aliquots of inhibitor (100 μM) to the sample cuvette and the same amount of buffer A (containing 1 mM CYMAL-5 and 2% ethanol) to the reference cuvette. Differential absorbance maxima (ΔA_{max} of 431 – 400 nm) and the equilibrium dissociation constant (K_{D}) were determined by a fit to the plot of ΔA versus [I]_{o} by nonlinear regression analysis using Equation 1,

\[ 2 \Delta A[E_0] = \Delta A_{\text{max}}[E_0] + [I_0] + K_D - ([E_0] + [I_0] + K_D)^2 - 4[E_0][I_0]^{1/2} \]  

(Eq. 1)

where ΔA and ΔA_{max} are differential absorption at a particular ([I]_{o}) and saturating inhibitor concentration, respectively (KaleidaGraph, Synergy Software).

Enzyme Inhibition Assay—Inhibition of 7-ethoxy-4-trifluoromethylcoumarin oxidation was measured fluorometrically (Cary Eclipse, Varian Inc.) as described previously (17). A typical 100-μl assay mixture consisted of 10 pmol of P450, 40 pmol of NADPH-cytochrome P450 reductase, 20 pmol of cytochrome b_{5}, and 150 μM 7-ethoxy-4-trifluoromethylcoumarin in the presence of 0–30 μM inhibitor with a constant 2% ethanol concentration. The activities were plotted against inhibitor concentration, and the data were fit to a four-parameter logistic function by linear regression analysis (KaleidaGraph) to derive the 50% inhibitory concentration (IC_{50}) values.

ITC Experiments and Data Analysis—ITC experiments were performed on a VP-ITC calorimeter interfaced with a computer for data acquisition and analysis using Origin Version 7 software (MicroCal, LLC, Northampton, MA). Freshly prepared 2B4dH(H226Y) was dia-lyzed extensively against degassed buffer A, passed through a 0.22-μm sterile filter, and stored on ice at all times. A protein concentration of 60 mg/ml was used in all experiments. Cold ethanol was added to the protein to a final concentration of 2% just prior to the temperature equilibration, and the sample was loaded into the calorimetric cell. Inhibitor stock solutions were prepared in 100% ethanol and were carefully diluted to 2 mM using 0.22-μm filtered dialysate with a 2% final ethanol concentration. Extreme care was taken to prevent evaporation of alcohol from each of the solutions. Prior to sample loading, the calorimeter cell and syringes were thoroughly rinsed with dialysate containing 2% ethanol to prevent any subtle heat changes due to a buffer mismatch between the solutions in the calorimetric cell and the titration syringe. The stability and CO binding properties of the protein were not altered in the buffer containing 2% ethanol and 1 mM CYMAL-5 for the duration of the ITC experiments (~2 h for each titration). Protein and inhibitor samples were quickly preincubated to the required temperature using a ThermoVac (MicroCal, LLC) and loaded into the calorimetric cell and titration syringe, respectively. A typical titration schedule included the addition of 4 μl of inhibitor/injection with 20–25 injections spaced at 5-min intervals. The titration cell was continuously stirred at 305 rpm. For the first injection only, 1 μl of inhibitor was added, and the corresponding data point was deleted from the analysis. Reference titrations were carried out by injecting each inhibitor into buffer alone in the calorimetric cell, and heat of dilution was subtracted from the inhibitor-protein titration data. There was no change in the pH of the protein or buffer samples after titration with inhibitor. The binding isotherms were best fit to a one-set binding site model by Marquardt nonlinear least-squares analysis to obtain the binding stoichiometry (N), association constant (K_{a}), and thermodynamic parameters of the interaction using Origin Version 7.0 software. Calorimetric titrations were carried out at 20, 25, 30, and 37 °C, and the change in heat capacity (ΔC_p) associated with the binding reaction was determined by the relationship ΔC_p = ΔH/ΔT. The thermodynamic parameters are derived from the total ligand concentration and are thus independent of whether P450 or total hemoprotein is used in the data analysis (39).

Acrylamide Quenching—The accessibility of the sole tryptophan (Trp^{121}) in the inhibitor-free and inhibitor-bound forms of 2B4dH(H226Y) was probed by acrylamide quenching experiments in buffer A containing 2% ethanol and 1 mM CYMAL-5. Protein (5 μM) was incubated for 1 h at 25 °C with different inhibitors at a molar ratio of 1:2 prior to measuring the tryptophan fluorescence using a Fluorolog-3 Model FL3-22 spectrofluorometer (Jobin Yvon, Edison, NJ) with dual grating monochromators on both the excitation and emission pathways. The tryptophan was excited at 295 nm, and emission was measured between 310 and 400 nm. Collisional quenching is described by the Stern-Volmer equation (Equation 2),

\[ F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_{SV}[Q] \]  

(Eq. 2)

where F_0 and F are the fluorescence intensities in the absence and presence of acrylamide, respectively; k_q is the bimolecular quenching constant; \tau_0 is the lifetime of the fluorophore in the absence of quencher; [Q] is the concentration of acrylamide; and K_{SV} is the Stern-Volmer constant.

Molecular Modeling Studies—Molecular docking of the inhibitors was performed using AutoDock Version 3.05 to search for the minimum energy conformations and the inhibitor orientations according to the Lennard-Jones genetic algorithm (40). Kollman united atom charges were assigned to the protein using AutoDockTools, and the charges for the heme were assigned as described previously (41). For the closed structure, a 60 × 58 × 60-point grid with a spacing of 0.375 Å centered at −18.8, 90.0, and 4.6 Å around the 4-CPI-binding site was used. For the open structure, a larger 120 × 120 × 120-point grid with a spacing of 0.375 Å centered at 20.9, 48.8, and 51.9 Å around the putative binding pocket at the distal end of the heme was used. A total of 10 unique minimum energy ensembles that exhibited <1 Å root mean square deviation were generated in each case, and the first two lowest energy ensembles were used for the analysis. Residue side chains within 5 Å of the docked position were mapped. Two tautomeric forms of 4-CPI and 4-PI were considered for docking, yielding similar results. Inhibitors were designed, and the charges were assigned using the PRODRG server (available at davapc1.bioch.dundee.ac.uk/programs/prodrg/) (42).

RESULTS

Spectral Studies of Inhibitor Binding and Enzyme Inhibition Assay—The experiments in this study were performed with 2B4dH(H226Y) because the 4-CPI-bound Tyr mutant was more amenable to x-ray crystallography and showed unaltered catalytic properties relative to 2B4dH (13). Spectral titrations of 2B4dH(H226Y) were carried out with 4-CPI (Fig. 2A), 1-BI (Fig. 2B), 1-CPI (Fig. 2C), 4-PI (Fig. 2D), 1-PI (Fig. 2E), and
BEI (Fig. 2F), whose chemical structures are shown as insets. Both 4-CPI and 4-PI have a dissociable proton and can exist in two tautomeric forms. The imidazole nitrogen linked to the side chain in 1-BI, 1-CPI, 1-PI, and BEI allows a continuous electron flow from the imidazole ring to the side chain. Chemically, 4-CPI and 1-CPI differ from 4-PI and 1-PI, respectively, by the addition of a chlorine, whereas 4-CPI and 4-PI differ...
TABLE 1
Thermodynamic parameters of the inhibitor interaction with 2B4dH(H226Y) derived from ITC and spectral titrations

| Inhibitor/temperature | N | $K_a \times 10^{-5}$ | $-\Delta H$ | $-\Delta G^\circ$ | $\Delta S^\circ$ | $K_p \times 10^{-6}$ | $1/C_{50} \times 10^5$ |
|-----------------------|---|---------------------|--------------|-----------------|--------------|-----------------|------------------|
| °C                    |   |                     |              |                 |              |                 |                   |
| 4-CPI                 |   | 1.05 ± 0.03         | 6.08 ± 1.56  | 8.83 ± 0.20     | 7.75 ± 0.25  | 13.38 ± 1.22   |                   |
| 20                    |   |                     |              |                 |              |                 |                   |
| 25                    |   | 1.00 ± 0.01         | 11.25 ± 2.05 | 8.48 ± 0.19     | 8.25 ± 0.16  | 5.87 ± 0.21     | 4.3 ± 0.7        |
| 30                    |   | 1.08 ± 0.02         | 8.31 ± 0.71  | 10.31 ± 0.25    | 8.21 ± 0.30  | -6.93 ± 1.40    | 11 ± 1           |
| 37                    |   | 0.98 ± 0.02         | 7.42 ± 0.51  | 13.90 ± 0.31    | 8.33 ± 0.35  | -17.97 ± 2.50   |                   |
| 1-BI                  |   | 1.03 ± 0.02         | 29.8 ± 3.14  | 8.20 ± 0.10     | 8.83 ± 0.15  | 2.13 ± 0.06     | 6.5 ± 0.5        |
| 25                    |   |                     |              |                 |              |                 |                   |
| 1-CPI                 |   | 1.02 ± 0.01         | 7.98 ± 1.65  | 8.15 ± 0.08     | 8.05 ± 0.05  | -0.35 ± 0.01    | 12.5 ± 0.6       |
| 25                    |   |                     |              |                 |              |                 | 21 ± 2           |
| 4-PI                  |   | 1.01 ± 0.01         | 7.56 ± 1.05  | 8.60 ± 0.05     | 8.02 ± 0.03  | -1.97 ± 0.02    | 13.2 ± 1.8       |
| 25                    |   |                     |              |                 |              |                 | 37 ± 2           |
| 1-PI                  |   | 1.04 ± 0.01         | 2.06 ± 0.80  | 8.08 ± 0.07     | 7.25 ± 0.02  | -2.79 ± 0.02    | 48.6 ± 8.1       |
| 25                    |   |                     |              |                 |              |                 | 237 ± 15         |
| BEI                   |   | 0.94 ± 0.06         | 3.37 ± 0.12  | 7.77 ± 0.43     | 7.40 ± 0.40  | -1.26 ± 0.15    |                   |
| 20                    |   |                     |              |                 |              |                 |                   |
| 25                    |   | 0.98 ± 0.04         | 3.50 ± 0.25  | 8.77 ± 0.32     | 7.56 ± 0.24  | -4.06 ± 0.20    | -331             |
| 30                    |   | 1.01 ± 0.08         | 3.73 ± 0.63  | 11.40 ± 0.55    | 7.72 ± 0.50  | -12.14 ± 2.60   | 103 ± 10         |
| 37                    |   | 0.75 ± 0.20         | 4.41 ± 1.20  | 13.00 ± 1.50    | 8.00 ± 1.20  | -16.10 ± 5.20   |                   |

$\Delta G$ and $\Delta S$ were calculated by the following equations, respectively: $\Delta G = -RT \ln K_a$ and $\Delta S = (\Delta H - \Delta G)/T$. $^b \Delta C_p (dT/\Delta H)$ was determined from the slope of the linear fit in Fig. 4. $^c K_p$ was determined by a fit of Equation 1 to the spectral titration in Fig. 2.

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from 1-CPI and 1-PI, respectively, by the repositioning of an imidazole nitrogen. The flexibility of all the inhibitors is highly restricted, except in the case of BEI and 1-BI. An oxyethyl linker in BEI and an ethyl moiety in 1-BI provide flexibility between the aromatic and imidazole rings.

The binding of these inhibitors to 2B4dH(H226Y) induced type II spectral changes, with the absorbance minimum at 400 nm and maximum at 431 nm, characteristic of nitrogen ligation to the heme iron (left panels). A plot of differential absorbance at 431 and 400 nm, versus inhibitor concentration is shown for each inhibitor (right panels), and the data were fit to Equation 1. The potency of these ligands to inhibit the monooxygenase activity of 2B4dH(H226Y) was investigated using 7-ethoxy-4-trifluoromethylcoumarin as a substrate. The $IC_{50}$ values and the dissociation constants are listed in Table 1. The $K_a$ values follow the order 4-CPI < 1-BI < 1-CPI < 4-PI < BEI < 1-PI. The rank order of the $IC_{50}$ values was similar, except that 1-BI showed a lower $IC_{50}$ value (0.05 μM) compared with 4-CPI (0.11 μM). A 3-fold increase in the potency was observed upon the addition of chloride (4-CPI versus 4-PI) or by the rearrangement of the imidazole nitrogen (4-PI versus 1-PI).

Monomeric State of 2B4dH(H226Y) at High Concentrations—For the accurate interpretation and comparison of the calorimetric data, the protein should remain monomeric upon ligand binding. As such, prior to ITC experiments, it was important to establish conditions in which the enzyme remained monomeric in solution at the high protein concentrations required. Thus, sedimentation velocity experiments were carried out in the presence of inhibitors (supplemental Fig. 1S). Protein purified by the modified procedure used in this study was purely monomeric in buffer A even at 60 μM as shown by the sedimentation distribution pattern monitored at 417 nm. The enzyme sediments with a sedimentation coefficient of 3.4 S. The protein behaved as a monomer upon binding to 1-BI, 1-CPI, 4-PI, 1-PI, and BEI, each with an S value of 3.5. However, >90% was recovered as a dimer upon 4-CPI binding, with an S value of 5.6. When CYMAL-5 (1 mM) was added to the 4-CPI-bound dimers of 2B4dH(H226Y), only a monomer with an S value of 3.6 was observed. The ligand-free protein in buffer A containing 1 mM CYMAL-5 also showed a S value of 3.4. Sedimentation velocity experiments carried out on the inhibitor-bound protein samples after calorimetric titrations in buffer A containing 1 mM CYMAL-5 also yielded similar patterns, with an S value of 3.6 for all inhibitors. All sedimentation experiments described above were simultaneously monitored at 280 nm, yielding similar results compared with the measurements at 417 nm. The standard error in the S value determination was ±0.1.

Calorimetric Titration and Thermodynamics of Inhibitor-2B4dH(H226Y) Interaction—With recent advances in the sensitivity and versatility of calorimeters, ITC has become an indispensable tool for the direct measurement of thermodynamic parameters such as Gibbs free energy ($\Delta G$), enthalpy ($\Delta H$), and entropy ($\Delta S$) changes along with the association constant ($K_a$) and number of binding sites (N) from a single experiment. Because ITC works solely on the principle of universal heat exchange during a reaction, it provides a complete picture of the bimolecular interaction independent of the accompanying spectroscopic changes. Here, by clarifying the issues of enzyme solubility and oligomerization, we were able to successfully employ ITC to study P450-ligand interactions. The results of a typical calorimetric titration, which consisted of adding 4-μL aliquots of 2 mM inhibitor to 60 μM 2B4dH(H226Y) at 25 °C, together with the nonlinear least-squares fit of the data are shown for inhibitors 4-CPI (Fig. 3A), 1-BI (Fig. 3B), 1-CPI (Fig. 3C), 4-PI (Fig. 3D), 1-PI (Fig. 3E), and BEI (Fig. 3F). The upper panels exhibit a monotonic decrease in the exothermic heat of binding with successive injections until saturation was reached. The lower panels show the integrated enthalpic changes for each injection, which fit to the single-class binding site model (solid lines). Fitting of integrated enthalpic data to two-class binding site models or to a sequential binding site model each yielded a poor fit with unreasonable error. The results of ITC experiments are summarized in Table 1. A clear 1:1 stoichiometric binding was observed in all cases, with the $K_a$ values ranging from 0.3 to 4.8 μM following the order 1-BI < 4-CPI < 1-CPI < 4-PI < BEI < 1-PI (the same order as the $IC_{50}$ values). The stoichiometric ratio was decreased by ~10%, whereas all thermodynamic parameters remained unchanged, when the total hemoprotein concentration was used, suggesting that the 1:1 stoichiometry represents the active pool of P450 determined by reduced CO difference spectra.

A clear 3-fold higher affinity of 1-BI compared with 4-CPI was observed. The $\Delta G$ values were a direct reflection of $K_a$ values, but significant differences were seen in the $\Delta H$ and $\Delta S$ values in a compensa-
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At 25 °C, positive ΔS values of 5.9 and 2.1 cal mol⁻¹ K⁻¹ were observed for 4-CPI and 1-BI binding, respectively. For the other inhibitors, the ΔS values were negative, with a maximum value of −4.1 cal mol⁻¹ K⁻¹ observed in the case of BEI binding. A much larger unfavorable ΔS value of −33.2 cal mol⁻¹ K⁻¹ was obtained for the binding of a bulkier imidazole inhibitor, bifonazole, to 2B4dH(H226Y) at acidic pH (43). However, detailed heat capacity analysis under identical conditions was not feasible with bifonazole because of its limited solubility in 2% ethanol at pH 7.4. BEI was the compound that showed the next largest negative ΔS value and was chosen for heat capacity analysis in comparison with 4-CPI. To delineate the mechanistic differences in large ΔTΔS values between the interaction of 4-CPI and BEI with 2B4dH(H226Y), the calorimetric titrations were carried out at 20, 25, 30, and 37 °C, and the change in the heat capacity (ΔCₚ) associated with the binding reaction was determined from the slope of the plot of ΔH versus temperature (Fig. 4). The binding reaction was exothermic, with a clear 1:1 stoichiometry over the entire temperature range for both 4-CPI and BEI. The ΔCₚ values were −604 and −331 cal mol⁻¹ K⁻¹, respectively, for 4-CPI and BEI. For both inhibitors, the ΔH and ΔS values showed a clear temperature dependence. The ΔG values did not vary significantly with temperature in either case. The entropy-enthalpy compensation plots depicting the variation in ΔH as a function of ΔTΔS.
for 4-CPI and BEI interaction are shown in Fig. 5 (A and B, respectively). The linear increases in $\Delta H$ as a function of $T\Delta S$ (slope of $\sim 1$) in both cases indicate strong entropy-enthalpy compensation. Usually, entropy-enthalpy compensation occurs in any system when $\Delta C_p >\Delta S$. In the entropy-enthalpy compensation plots, the energy values at the intersection between $\Delta G$ and $\Delta H$ (where $\Delta G = \Delta H$ and $\Delta S = 0$) were $-8.4$ kcal mol$^{-1}$ for 4-CPI and $-7.5$ kcal mol$^{-1}$ for BEI. The corresponding temperatures of these values were 28.2 and 20.1 °C, respectively, for 4-CPI and BEI interaction as derived from the plot of $\Delta H$ versus temperature (Fig. 4). At these temperatures, the favorable entropic changes (i.e. hydrophobic and vibrational entropic effects) are negated by the unfavorable entropic changes (i.e. rotation translational and conformational entropic changes). Above this transition temperature, the interaction is mainly driven by favorable enthalpic changes (Table 1) dominated by hydrophobic interaction (for both 4-CPI and BEI). Below this temperature, the favorable large entropy, observed with only 4-CPI binding, is compensated by the decrease in binding enthalpy.

Accessibility of Trp$^{121}$ Located in Helix C—As observed in the crystal structures, dramatic large-scale structural rearrangement takes place upon 4-CPI binding in the motifs that flank the heme group (12, 13). This rearrangement relocates helix C bearing the sole tryptophan (Trp$^{121}$) to a more proximal position, bringing Trp$^{121}$ closer to the heme by 13 Å (Fig. 1). Accessibility of Trp$^{121}$ to the bimolecular quenching agent acrylamide was monitored in the inhibitor-free and inhibitor-bound forms of 2B4d(H226Y) by fluorescence measurements. A Stern-Volmer plot indicating the ratio of fluorescence intensities as a function of acrylamide concentration is shown in Fig. 6. The Stern-Volmer constants obtained as the slope of the linear fit were $3.3 \pm 0.2$ M$^{-1}$ for the inhibitor-free form (closed circles) and $1.8 \pm 0.2$ M$^{-1}$ for the 4-CPI-bound form (open circles) of the protein. Protein bound to the other five inhibitors behaved similarly to the inhibitor-free form. Thus, a dramatic 2-fold decrease in the accessibility of Trp$^{121}$ to the quencher is seen only when 4-CPI binds to the protein.

Molecular Docking of Inhibitors to the Open (Protein Data Bank Code 1POS) and Closed (Code 1SL0) Structures—In the closed structure, 4-CPI gets locked between key residues in substrate recognition sites 1 and 4–6 to form a strong hydrophobic pocket, whereas some of these residues are far from the heme group in the open structure (12, 13). Because the 4-CPI-bound structure has been used as a template for modeling other P450 enzymes (44), it was of interest to see whether the 4-CPI-binding pocket in the closed structure could accommodate inhibitors with different chemistry and size chains and to see how well these inhibitors could be docked in the open structure. A representative inhibitor position from the ensemble of minimum energy positions from docking of 4-CPI or BEI to both the open and 4-CPI-deleted closed structures is shown in Fig. 7. Notably, in the closed structure, 4-CPI found the exact same binding pocket originally seen in the crystal structure, validating the docking process after the heme potentials were normalized using the standard AutoDock program (Fig. 7A). The docked 4-CPI (cyan) is in the same orientation as the one seen in the crystal structure (pink); the 4-CPI long axis is at an $\sim 75^\circ$ angle with the heme plane, and the imidazole nitrogen is coordinated to the heme iron at a distance of $2.05$ Å compared with $2.14$ Å and at an angle of $30^\circ$ compared with $25^\circ$ between the two rings of 4-CPI. Strikingly, 1-CPI, 4-PI, and 1-PI were also docked to the same position with the exact orientation and angle of two rings as seen in 4-CPI (data not shown). Docking of 1-BI resulted in a similar orientation of the imidazole ring, but because of the flexibility of the ethyl link, the aromatic ring flips to a $90^\circ$ angle and points toward helix I (data not shown). BEI docking to the closed structure resulted in two ensembles of minimum energy conformations, both located close to each other in the 4-CPI-binding pocket (Fig. 7C). However, the imidazole ring does not point toward the heme iron in either ensemble. The benzyl ring bends forward at the flexible oxyethyl link, and the imidazole moiety squeezes into the gap between helix I and the $\beta_{1-4}$ sheet to a position unreasonable for heme coordination.

4-CPI docking in the open structure resulted in 10 ensembles. The first two minimum energy ensembles differ in docking energy by only 0.2 kcal mol$^{-1}$, but docked onto two entirely different locations (Fig.
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FIGURE 6. Accessibility of Trp121 to acrylamide. Shown is a Stern-Volmer plot of tryptophan fluorescence quenching by acrylamide in the inhibitor-free (○), 4-CPI-bound (□), 1-CPI-bound (▲), 4-PI-bound (◆), 1-PI-bound (●), and BEI-bound (□) forms of 284dH(H226Y). Tryptophan quenching was calculated as a function of acrylamide concentration in buffer A at 25 °C using 5 μM protein. Solid lines represent a fit to the Stern-Volmer equation (Equation 2).

7B). The first minimum energy ensemble became caught in the distal end of the molecule at the tip of the putative binding pocket between helix I and the β14-1 sheet, at least 15 Å away from the center of heme. However, in the second ensemble, 4-CPI positioned perfectly in the open binding pocket, with the imidazole nitrogen pointing toward the heme iron within 2 Å. The aromatic chlorophenyl group stands out at almost a 90° angle to the heme plane without being surrounded by five Phe residues, as seen in the closed structure. Docking of 1-CPI, 1-BI, 4-PI, and 1-PI also resulted in two ensembles, and the second low energy ensemble docked adequately to the open binding pocket, similar to 4-CPI (data not shown). BEI docking to the open structure also resulted in the first two minimum energy ensembles (Fig. 7D). Here, the first ensemble was also trapped at the tip of the putative binding pocket ~15 Å from the center of the heme. In the second ensemble, the imidazole ring positioned perfectly, facing the heme iron at a distance of 2.5 Å, and the benzyl(oxyethyl) moiety was almost left freely floating in the open binding pocket. The modeling data suggest that a much larger binding site on 284dH is required to accept the bulkier BEI. Conversely, the protein in the open conformation may require minimal structural rearrangement to bind BEI, consistent with the results obtained from the calorimetric and spectroscopic studies.

DISCUSSION

Considerable insight into the structural basis of diversity in substrate recognition and catalytic specificity has been provided by the recent mammalian P450 x-ray structures. As ligand-bound structures almost invariably adopt a closed conformation, the dynamic structural elements (helices F, G, B′, and C) have been proposed to play a vital role in ligand access to the buried catalytic (heme) site, consistent with initial studies of ligand-free and ligand-bound P450cam (2). However, several ligand-free structures are closed (3, 45), and ligand binding appears to cause relatively little change in CYP3A4 (22). In addition, there is seldom a correlation between the active-site volume deduced from crystal structures and either the size or the shape of the ligands each P450 binds (2, 12–15, 21, 22), suggesting tertiary structural rearrangement of P450 to define ligand binding specificity. Upon binding to 4-CPI, CYP2B4 exhibited the largest degree of conformational change seen in any P450-ligand system to date (12, 13). Solution thermodynamic studies monitoring such ligand-induced structural transitions in P450 are very limited, and ITC is the method of choice to dissect the thermodynamic events of binding. However, the major limitation in applying ITC to the P450 system is that high protein concentrations are required to detect a measurable heat of interaction. Thus, conditions must be optimized to maintain the enzyme in the monomeric form to accurately interpret the thermodynamic data. Aqueous solubility of ligands is another limiting factor. With a minor modification of the previous purification procedure (13), buffer composition, and storage conditions, 284dH(H226Y) remained monomeric in solution at the 60 μM concentration used for ITC experiments. Although only 4-CPI binding induced dimers, they were readily dissociable with 1 mM CYMAL-5. Concurrently, 4-CPI binding in the presence of 1 mM CYMAL-5 did not induce dimers, facilitating clear interpretation of the thermodynamic data.

Based on the different degree of structural rearrangement observed with imidazoles of different size in CYP119 (5) and P450eryF (46), we chose a series of phenyl/benzylimidazoles (4-CPI, 1-CPI, 4-PI, 1-PI, 1-BI, and BEI) that exhibited at least a 10-fold difference in IC50 and spectral KD values. There are one rotatable bond and one dissociable proton in 4-CPI and 4-PI, which are absent in 1-CPI and 1-PI. The flexibility of these four inhibitors is highly limited. There were no changes in the pH of inhibitor-titrated protein samples, especially with 4-CPI and 4-PI, which have a dissociable proton. Both 1-BI and BEI are flexible imidazoles of intermediate size. The ethyl linker in 1-BI and the oxyethyl linker in BEI between the imidazole and benzene rings give flexibility to these molecules, which in turn may adopt a different orientation in the binding site than structurally rigid phenylimidazoles. Neither of these inhibitors has a dissociable proton.

The thermodynamic signature obtained by ITC gave a comprehensive picture of the nature of the interaction of these inhibitors and the plasticity of CYP2B4. Although the difference in free energy changes was minimal for these inhibitors (mean ΔG = −8 kcal mol−1 at 25 °C), a marked difference was observed in the ΔH and ΔS values as depicted in Fig. 8. With a clear 1:1 stoichiometric binding of each inhibitor at 25 °C, the ΔH values varied from −6.5 to −8.8 kcal mol−1 to compensate for the marked changes in the ΔS values. The ΔH value of +2.1 kcal mol−1 for 4-CPI was transformed to −0.6 kcal mol−1 for 4-PI by a simple deletion of the chlorine. The favorable entropy with 4-CPI derives solely from the conformational change in the protein because both of these inhibitors have the same limited flexibility and similar ΔH values for binding. The binding of 1-BI produces an additional −2.3 kcal mol−1 of favorable ΔH compared with 4-CPI, which compensates more for the unfavorable ΔS changes. Considering the fact that the accessibility of Trp121 to acrylamide was not decreased by 1-BI, it may induce a different degree of compactness in the protein compared with 4-CPI. Interestingly, the favorable ΔH but unfavorable ΔS observed with 1-CPI, 4-PI, and 1-PI binding would suggest no major conformational changes, as opposed to the compactness observed in the 4-CPI-bound protein, despite the striking structural similarity among these inhibitors. Conversely, for BEI (the more flexible inhibitor) binding, the enthalpy is contributed mainly by the favorable hydrophobic interaction at all temperatures (Fig. 5B), is less compensated by entropy, and does not indicate any compactness in the protein structure. Thus, only 4-CPI binding shows the complete entropy-enthalpy compensation. In the 4-CPI-bound crystal structure, the binding site residues strictly constrain the inhibitor on all sides, such that nearly any rotation of the inhibitor would require a conformational change on the part of protein (13). The striking differences observed among the phenylimidazoles used in this study appear to depend on the tautomeric imidazole structure and the position of chlorine, whereas the flexibility of BEI appears to be decisive in inducing conformational strain in the binding pocket. The largest unfa-
Vorable entropy with a $T\Delta S$ value of $-10.1$ kcal mol$^{-1}$ was obtained upon the binding of a bulkier antifungal imidazole (bifonazole) to 2B4dH(H226Y) (43). This would suggest a more open-like conformation of the protein, rupturing the binding pocket formed in the 4-CPI-bound structure. We have recently published a crystal structure of bifonazole-bound 2B4dH(H226Y) showing a wide open conformation of helices F, G, and C (Protein Data Bank code 2BDM) (43).

There are observed discrepancies between the $K_I$ and IC$_{50}$ values for several nitrogenous heterocycles, suggesting that the type II spectral changes may not constitute good indicators of inhibitory potency (47–49). In this study, 1-BI showed a higher spectral $K_I$ values compared with 4-CPI, although its IC$_{50}$ values were 2-fold lower than those of 4-CPI. Steric factors near the heme, from either the protein side chains or the inhibitors, largely contribute to the type II spectral change, which does not reflect the comprehensive picture of inhibitor side chain interactions (48, 49). Several fold differences in the $K_I$ values were observed between spectroscopic and ITC titrations for all of the inhibitors. However, they all induce similar type II spectral changes resulting from the imidazole nitrogen coordination as a sixth ligand to the heme iron, which displaces a bound water molecule. Because ITC works on the principle of heat exchange, it measures the cumulative effect of a variety of reactions independent of spectroscopic changes that may occur during the reaction. Such discrepancies between ITC and spectroscopic titrations have also been observed in other protein-ligand interaction systems (30, 50). Use of ethanol does not account for such differences because the spectroscopic titrations yielded similar $K_I$ values even in the absence (<0.003%) of ethanol (data not shown). In addition, use of organic solvents does not contribute to the thermodynamic parameters.
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derived from ITC for different hydrophobic ligand-binding proteins (51, 52). In our case, the higher \(K_D\) values in ITC could possibly be due to the competitive displacement of bound detergent molecules from the CYP2B4 binding pocket. However, this does not hinder the comparative analysis of binding parameters for different ligands using identical solution conditions in equilibrium (32, 52). We chose 4-CPI and BEI, which showed maximum maximum entropy-compensation, for further detailed heat capacity (\(\Delta C_p\)) analysis under identical solution conditions.

The \(\Delta C_p\) derived from the temperature dependence of enthalpic changes for protein-ligand interaction is one of the most valuable thermodynamic parameters for inferring the structural changes in the protein. The \(\Delta C_p\) for P450-ligand interaction can be directly derived only from ITC experiments because thermal unfolding of P450 is irreversible, limiting the use of differential scanning calorimetry. Following the discovery by Edsall (53) that the transfer of apolar groups to aqueous media results in a large increase in the \(\Delta C_p\), the study of a series of model compounds and proteins has demonstrated that burial of the apolar surface area results in a decrease in the \(\Delta C_p\) of the system (27–32, 53, 54). The dissociable proton in 4-CPI (also in 4-PI) did not change the solution pH, facilitating the clear comparison of \(\Delta C_p\) values between 4-CPI and BEI. The large negative \(\Delta C_p\) for 4-CPI binding (\(-604\) cal mol\(^{-1}\) K\(^{-1}\)), almost twice that for BEI binding (\(-331\) cal mol\(^{-1}\) K\(^{-1}\)), is a clear indication of burial of a large part of the apolar surface area of 284H2266Y upon 4-CPI binding, which is consistent with the large structural rearrangement of helices B', F, and G seen in the crystal structure (12, 13). The negative \(\Delta C_p\) can also be taken as an indicator of strong hydrophobic interaction to bring the net thermodynamic driving force for 4-CPI binding to shift from entropic to enthalpic with an increase in temperature (Fig. 5A). Considering that there is only minor rearrangement of the protein at the secondary structural level (13), the contribution from burial of the polar surface is minimal.

The apolar and polar surface areas of the open and closed structures of CYP2B4 were calculated from the crystal structures by rolling a water molecule of 1.4-Å radius over the surface (55). Residue-specific calculations indicated a 13% decrease in the total surface accessibility in the closed structure, due mainly to the burial of 80% of the apolar residues located on helices B', C, and G. A larger differential apolar surface area of 2504 Å\(^2\) and a smaller differential polar surface area of 527 Å\(^2\) were obtained between the open and closed structures. This finding supports the inference that the 2-fold larger \(\Delta C_p\) obtained upon binding to 4-CPI compared with BEI originates primarily from the burial of apolar surface area resulting from strong hydrophobic interactions and a conformational transition. In solution, Trp\(^{211}\) (located in helix C) is freely accessible to the bimolecular quencher acrylamide, but upon 4-CPI binding, the accessibility significantly decreases as revealed by a 2-fold decrease in the Stern-Volmer constant (Fig. 6). This suggests the movement of helix C bearing Trp\(^{211}\) to a more hydrophobic environment, which is not observed in the protein bound to any of the other five inhibitors. Remarkably, these observations in solution are in tune with the crystal structures of 4-CPI-bound CYP2B4, where the switch from the open to closed conformation relocates helix C to a more proximal position near the heme, bringing Trp\(^{211}\) 13 Å closer to the more hydrophobic environment (Fig. 1) (12, 13). In the closed form, Trp\(^{211}\) is also involved in the hydrogen bonding with the D-ring propionate of heme (13). Surface accessibility calculations also showed a more buried helix C. These spectroscopic observations complement the calorimetric data showing the compactness of the CYP2B4 structure upon binding to 4-CPI (but not BEI). Interestingly, bifonazole binding to 284(H2266Y) also did not hinder the Trp\(^{211}\) accessibility (data not shown), and the crystal structure shows an unperturbed helix C (43).

4-CPI is one of the smallest ligands to be crystalized with a P450, forming a compact active site. Logically, docking of a larger ligand, irrespective of chemistry, would induce a conformational strain on the heme to adopt a non-ideal position in the active site because the protein backbone flexibility is limited in the modeling program. 4-CPI was docked perfectly to the CPEI-deleted closed structure as one single conformational ensemble. Although the bulkier BEI was docked perfectly to the binding cleft in the open form, the compound was docked in an unreasonable orientation to the closed structure unsuitable for nitrogen-iron coordination, reflecting the conformational strain. Docking results were unable to resolve the striking differences observed in the inhibition and thermodynamic properties among 4-CPI, 1-CPI, 4-PI, and 1-PI, reflecting the limitations of the docking program.

It is becoming increasingly clear from the P450 crystal structures that ligand binding can induce a more open or closed (putative) conformation depending on the ligand chemistry (2). The solution thermodynamic approach described in this study should provide an efficient way to analyze new P450-ligand pairs. Lead compounds can be designed or screened to optimize the thermodynamic signatures between potential targets and specific P450 enzymes to minimize the drug-drug interactions. This approach would also facilitate the structure-based design of specific inhibitors of different P450 enzymes and the identification of compounds that are likely to yield interesting substrate-specific conformations. For example, based on the data presented here, we predict that the BEI-bound CYP2B4 will have more of an open conformation, with helix C oriented away from the heme pocket similar to the substrate-free open form, whereas the 1-BI-bound structure will have a compact binding pocket. Based on the same rationale, the bifonazole-bound 284H2266Y structure we solved recently shows an open conformation (43). Upon successful resolution of the experimental challenge of keeping CYP2C9 or CYP3A4 in a monomeric state, solution biophysical studies of ligand-free and ligand-bound forms of these enzymes should help illuminate why no major conformational changes are observed in the corresponding x-ray crystal structures.

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