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Binding of quinidine radically increases the stability and decreases the flexibility of the cytochrome P450 2D6 active site

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ABSTRACT

Human cytochrome P450 2D6 (CYP2D6) is an enzyme of the CYP superfamily responsible for biotransformation of about 20% of drugs of known metabolism containing a basic nitrogen and a planar aromatic ring. Here, we present a combined experimental and computational study on the compressibility and flexibility of unliganded and quinidine-bound CYP2D6. Experimentally, high-pressure induced Soret band shifts of the enzyme were measured by UV/VIS spectroscopy, while 100 ns all atomic molecular dynamics (MD) simulations in explicit water were used in the computational analysis. We identified sharp differences between ligand-free and quinidine-bound CYP2D6 forms in compressibility, flexibility parameters and active site solvation. While the unliganded CYP2D6 is compressible, quinidine binding significantly rigidifies the CYP2D6 active site. In addition, MD simulations show that quinidine binding results in pronounced reductions in active site flexibility and solvation.

1. Introduction

In humans, most drugs are metabolized by cytochrome P450 (CYP) enzymes that are mainly present in the microsomal fraction of the liver and other tissues, such as brain, intestine, lung and kidney [1]. Microsomal CYPs are quite promiscuous, but vary substantially in their substrate preferences [2,3]. Key determinants of P450 substrate specificity have been analyzed from several perspectives and various structural features responsible for their promiscuity have been identified. Among other factors, the size and flexibility of the active site [4–9], together with properties of the substrate access and egress channels [10,11], have been shown to strongly influence the enzyme's substrate preferences. Techniques that can provide valuable information about their flexibility include molecular dynamics (MD) and UV/visible (UV/VIS) high pressure spectroscopy [7,8,12].

CYP2D6 is the enzyme responsible for biotransformation of about 20% of drugs of known metabolism that contain a basic nitrogen and a planar aromatic ring, including antiarrythmics (e.g. propafenone), antidepressants (e.g. venlafaxine, fluoxetine), antipsychotics (e.g. haloperidol, clozapine), beta-blockers (e.g. metoprolol, atenolol) and analgetics (e.g. codeine, tramadol) [13]. The rate of metabolism of drugs by CYP2D6 is known to reflect genetic variations of the enzyme; the metabolism of certain drugs is much faster in some patients than others, and even absent in carriers of several alleles of the encoding gene, with adverse effects in some cases [2,3]. To eliminate this variation, and associated therapeutic complications, there is great interest in designing drug candidates that are not metabolized by CYP2D6. Understanding the interactions between CYP2D6 and its ligands could greatly facilitate the rational design of potential drug candidates with such properties, or other desirable features [14].

Quinidine (see Fig. 1) is an antiarrhythmic agent (Pubchem CID: 441074) that is a substrate of another CYP enzyme, CYP3A4 [12]. It also has several typical features of CYP2D6 substrates (a planar aromatic ring and a polar nitrogen) and hence binds strongly to CYP2D6 in a similar fashion to its chiral isomer quinine, a CYP2D6 substrate, yielding a typical type I binding spectrum characteristic for binding of substrates [15]. However, in comparison to quinine, quinidine binds in reverse orientation to the CYP2D6 active site [16] and instead of being degraded it blocks the enzyme's active site and thus is a potent competitive inhibitor of CYP2D6 [17,18].

Recent analysis of the CYP2D6 active site and mutagenesis experiments have shown that two carboxylate groups (E216, D301) play key roles in its substrate recognition [14,19–21], possibly due to their interaction with the basic nitrogen of its substrates during entry or binding to the active site. It has also been suggested that

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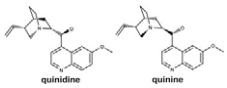

Fig. 1. Structure of quinidine and its diastereomer quinine for comparison.

aromatic residues (F120, F481, F483) may be involved in substrate binding through π–π interactions with substrates' aromatic rings [21]. Strong evidence supporting the putative roles of these residues in the enzyme's substrate recognition includes findings that (in marked contrast to the wild type CYP2D6 enzyme) E216F, E216Q/D301Q and F120A mutants are even capable of metabolizing quinidine [19].

In a previous study [7], we analyzed the flexibility of five human drug-metabolizing CYP enzymes (CYP1A2, 2A6, 2 C9, 2D6 and 3A4) and changes in properties of their active sites imposed by high pressure using both absorption spectroscopy and MD simulations. CYP2D6 showed an intermediate Soret band red shift under pressure, indicating that its active site is quite compressible, and more flexible than that of CYP1A2 and CYP2A6, but less flexible than that of CYP3A4 [7]. In addition, relatively facile denaturation of the native P450 form of CYP2D6 to the P420 form was observed, even at fairly low hydrostatic pressures (100-150 MPa).

Here, we describe changes in the structure, flexibility and compressibility of the CYP2D6 active site induced by quidinine binding observed by high-pressure UV/VIS spectroscopy and in MD simulations. Our results show that quinidine binding profoundly affects the properties of CYP2D6's active site, substantially reducing its flexibility and strongly suppressing the enzyme's denaturation to the P420 form under pressure. The large observed change in compressibility of CYP2D6 indicates that ligand binding to the active site can dramatically change enzyme properties.

2. Materials and methods

2.1. UV/VIS spectroscopy at high hydrostatic pressure

Absorption spectra were obtained with a Cary 3E spectrophotometer (Varian, Palo Alto, CA) equipped with a high-pressure cell. Details of the instrument and the applications of the method in protein generally, and CYP studies specifically, have been previously presented [12,22]. The enzyme was diluted to 2 μM concentration in a 20 mM K/PO_4 buffer (pH 7.4) containing 20% (v/v) glycerol. Quinidine was subsequently added as a 1 mM solution in the K/PO_4 buffer to the reaction mixture (final concentration, 20 μM) then the P450 was reduced by adding solid dithionite and complexed with carbon monoxide. The Soret absorption band position was determined by spectral analysis and its shift with applied pressure was plotted according to the equation $S = S_0 + \alpha P$, where S is the Soret band position at a given pressure P, S_0 is its position at normal pressure and the coefficient α is related to compressibility [23]. The results were processed using LabSpec v. 4.04 (JobinYvon Horiba, Villeneuve d'Ascq, FR) and Sigma Plot v. 8.0 (SPSS, Chicago, IL, USA).

2.2. Molecular dynamics simulations

We conducted MD simulations of CYP2D6 at normal (NP, 0.1 MPa) and high pressure (HP, 300 MPa) with/without quinidine present in the active site. The crystal structure (mean resolution 3.00 Å) of ligand-free CYP2D6 was taken from the PDB database (CYP2D6, PDB ID: 2F9Q [13]), while the structure with bound quinidine (CYP2D6qui) was kindly provided by Prof. Gordon Roberts (Department of Biochemistry, University of Leicester, UK). The positions of the backbone atoms of the missing residues (between P41 and Q52) of the CYP2D6 structure were modeled on those of the analogous residues in CYP2C8 (PDB ID: 1PQ2 [24]); the side chains of these residues were built using the Leap program of the AMBER package. The N-terminal transmembrane domains of CYP2D6 were not included in the simulations.

The force field parameters for non-standard heme residue derived according to Cornell et al. [25] were taken from our earlier articles [7,9]. Two axial ligand positions of the heme iron are occupied by two ligands: (i) cysteinate sulfur as the proximal ligand, and (ii) either a water molecule (CYP2D6) or carbon monoxide (CYP2D6qui) as the distal ligand. All structures were solvated by the TIP3P water box with three (CYP2D6) or six (CYP2D6qui) Na + counter ions to neutralize the systems. Each system was minimized, heated (0.5 ns) and after equilibration (5 ns) we ran a 100 ns long MD production simulation under periodic boundary conditions in the NpT ensemble at normal (NP, 0.1 MPa) and high (HP, 300 MPa) pressures, in both cases at 298.15 K, with 2 fs long integration steps using the AMBER 9.0 package and the parm99 force field [26]. The cut-off for noncovalent interactions was 1 nm and electrostatics were treated by the particle mesh Ewald method. Bonds to hydrogen atoms were constrained using the SHAKE algorithm.

All MD trajectories were analyzed using the ptraj module of the AMBER package. Radius of gyration and temperature B factors were calculated over backbone atoms. The solvation of the active sites of the CYP2D6 enzyme was monitored throughout long simulations at normal and high pressure. Solvation was analyzed using the radial distribution functions (RDF) for the water molecules and backbone atoms around the heme. The space-resolved distribution of water molecules was further evaluated on a grid at 1 $Å^3$ resolution in a similar fashion to the analysis of waters within CYP2D6 presented by Santos et al. [27]. Snapshots taken every 500 ps were superimposed on the backbone atoms and the positions of the water oxygen atoms on the grid were recorded. Sites within the enzyme were identified as water coordination positions if their average water occupancy was at least three times greater than the average values.

3. Results and discussion

3.1. Spectroscopy at high hydrostatic pressure shows strengthening of CYP2D6 with quinidine

Initial absorption spectroscopic analysis revealed that under high hydrostatic pressure the behavior of CYP2D6 is strikingly different in the presence and absence of bound quinidine. As shown in our preceding paper [7], unbound CYP2D6 is prone to denaturation to the inactive form, P420, even at pressures of about 100 MPa. At 250 MPa the P420 form predominates and the native P450 population represents only 15% of the starting value. However, following binding of quinidine formation of the denatured form, P420, is minimal and becomes apparent only at pressures of 250 MPa (Fig. 2).

In the next step, the red shift of the Soret band at 450 nm, characteristic of native P450 enzymes, was monitored to obtain information on the flexibility of the active site [7,19,20,28]. Our previous results indicated that in the absence of bound ligand the active site of CYP2D6 has considerable flexibility, comparable to that of CYP2 C9 [7], substantially more than that of CYP2A6, but less than that of CYP2E1 [28] and CYP3A4 [7] (for values of the compressibility-related coefficients, -α, see Table 1). However, the binding of quinidine reduces the flexibility of the CYP2D6 active site, in line with the stabilization of the CYP2D6 structure against denaturation described in the previous paragraph, and reduced the -α value to about half that of the unliganded protein (Table 1). This finding should not be generalized to other enzyme:substrate pairs as quinidine binds to CYP2D6 in an exceptionally specific and well characterized manner; the effects of other substrates binding to other enzymes may be different [13,18,19].

Overall, the experimental results indicate that quinidine binding stiffens the CYP2D6 structure and/or induces large changes in heme