Evidence That Aspartic Acid 301 Is a Critical Substrate-Contact Residue in the Active Site of Cytochrome P450 2D6

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Model building studies have intimated a role for aspartic acid 301 in the substrate binding of cytochrome P450 2D6 (CYP2D6). We have tested this hypothesis by generating a range of CYP2D6 mutants substituting a variety of amino acids at this site. The mutant proteins, which included substitution with a negatively charged glutamic acid residue or neutral asparagine, alanine, or glycine residues, were expressed in Saccharomyces cerevisiae. In addition, a mutant where aspartic acid 301 was deleted was also tested. All the mutants expressed approximately equivalent amounts of recombinant apoprotein and, apart from the alanine 301 and the asparagine 301 deletion mutants, gave carbon monoxide reduced spin changes in the mutant proteins were markedly reduced. These data collectively demonstrate that aspartic acid 301 plays an important role in determining the substrate specificity and activity of CYP2D6 and provide experimental evidence supporting the role of this amino acid in forming an electrostatic interaction between the basic nitrogen atom in CYP2D6 substrates and the carboxylate group of aspartic acid 301.

Cytochrome P450 2D6 (CYP2D6) mediates the metabolism of over 30 drugs of wide therapeutic use including many anti-arrhythmics, antidepressants, β-adrenergic antagonists, neuroleptics, and analgesics (1). Although structurally diverse, all known ligands (substrates and inhibitors) of CYP2D6 possess a basic nitrogen, usually either an amine or a guanidino group, which is presumed to be protonated when the ligand is bound in the active site of the enzyme (2). Furthermore, substrate-template models (3–5) have revealed that this basic nitrogen is normally located 5–7 Å from the site of oxidation in the substrate molecule. A pharmacophore based on competitive inhibitors of CYP2D6 (6) also complies with this model; the positively charged nitrogen of such inhibitors is distanced up to 7.5 Å from a flat hydrophobic region of the inhibitor. Based on this conformity, it has been proposed that the basic nitrogen of CYP2D6 substrates and inhibitors interacts with a negatively charged residue, such as aspartate or glutamate, in the active site of enzyme and that this electrostatic interaction facilitates binding and orientation of the ligand in the active site (3–7).

Recent computer-derived homology models of the active site of CYP2D6 (4, 8, 9), based on alignment with the crystal structures of the bacterial P450s (P450BM-3) have identified aspartic acid 301 (Asp301) as a candidate residue for the proposed electrostatic interaction with the ligand. Such homology models and a recent structure-based alignment (10) locate Asp301 in the central region of the I-helix of CYP2D6. This region maps to one of the substrate-recognition sites (SRS4) identified by Gotoh (11) as being important in substrate binding in the CYP2 family of P450s; these substrate-recognition site regions may also be predictive across other P450 families (10).

In three bacterial P450s for which crystal structures are known (12–14), the central region of the I-helix is one of the most spatially conserved areas of the P450 core (10). In these P450s, it is located close to the heme moiety and runs across the distal face of the heme, completely or partially covering pyrrole ring B (10). Several residues in the central region of the I-helix have also been shown by mutation to play a role in substrate specificity and/or reaction kinetics (15–17). Recent work also indicates that residues in this region may play a role in the supply of catalytic protons via helix-associated solvent molecules (10).

In this report we provide experimental evidence, through the use of site-directed mutagenesis, that Asp301 is of critical importance in the efficient oxidation of substrates by CYP2D6. The data are consistent with the proposal that Asp301 forms an
ion pair with the basic nitrogen of CYP2D6 ligands, facilitating binding and orientation in the active site.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strains DH5α and Cj 236 and Saccharomyces cerevisiae strain AH22 have been described elsewhere (18, 19). Human CYP2D6 cDNA (CYP2D6-Val), considered to represent a wild type sequence (20), and the yeast expression vector pMA91 have been reported previously (18). Metoprolol tartrate (racemate) and O-demethyl metabolite and α-hydroxymetabolite hydroxybenzoate metabolites were gifts from AB Hässle (Mändal, Sweden). The hydrochloride salts of R-(-)- and S-(-)-metoprolol were gifts from Ciba-Geigy (Basel, Switzerland). Debrisoisequino and its 4-hydroxymetabolite were donated by Hoffmann-La Roche (Basel, Switzerland). All other chemicals were obtained commercially and were of the highest grade of purity.

Recombinant DNA Manipulation and Generation of Mutant CYP2D6 cDNAs—Manipulations leading to the generation and isolation of recombinant constructs were performed according to standard protocols as described by Sambrook et al. (21). Mutant CYP2D6 cDNAs were generated by site-directed mutagenesis using the dut- ung E. coli strain Cj 236 according to the method of Kunkel et al. (19). Point mutations at bp 903 C→A, 901 G→A, 902 A→C, and 902 A→G and a deletion of bp 901–903 were performed; when expressed these cDNAs gave rise to the CYP2D6 mutants D301E, D301N, D301A, D301G, and D301I, respectively. The mutated cDNAs were ligated into the BglII site of the yeast expression vector pMA91 and transformed into S. cerevisiae AH22 cells as described previously (20). All constructs were sequenced (Sequenase 2.0 kit; Amersham International, UK) before and after subcloning in pMA91 to confirm the presence of the desired mutational lack of any additional changes in the CYP2D6 sequence. Sequencing also confirmed that the ATG start codon of the CYP2D6 gene of each construct was distanced minimally (12 bp) from the end of the phosphoglycerate kinase promoter of pMA91.

Yeast Culture and Microsome Preparation—Transformation of S. cerevisiae was by electroporation (22). Yeast transformants were grown in batch culture in 1 liter of selective synthetic medium (0.67% w/v yeast nitrogen base without amino acids, 1.04% v/v glucose, 3% v/v glycerol) for 42 h (stationary phase) at 200 rpm and 30°C. After harvesting by centrifugation, the yeast cells were disrupted mechanically with glass beads (0.45–0.50 mm) at 4000 rpm for 40 s with liquid nitrogen and stored at 80°C prior to use.

Immunoblotting and Spectrophotometric Measurements—The relative apoprotein content of microsomes was estimated by separation on a 10% SDS-polyacrylamide gel (23) and electrophoretic transfer to Hybond™ ECL nitrocellulose membrane (Amersham International, UK). Blots were probed with rabbit anti-human CYP2D6 polyclonal antibodies and visualized by ECL using a streptavidin-horseradish peroxidase conjugate and luminid (Amersham International, UK). The holoprotein content of microsomes was quantified by carbon monoxide-difference spectroscopy according to the method of Omura and Sato (24). Values of apparent Km, kcat, and SAmax of debrisoquine and quinidine were determined from ligand-induced difference spectra as described by J ecoate (25).

Assay of Metabolite and Debrisoquine Metabolites—Incubation conditions for the oxidation of metoprolol (40 μM or 2 mM racemate, 40 μM R-(-) or S-(-)-enantiomers and debrisoquine (250 μM), two probe substrates of CYP2D6, were as described previously (26). Incubates contained 10 or 30 pmol of CYP2D6 holoprotein, except those containing D301I and yeast control microsomes (derived from yeast cells transformed with pMA91 lacking a CYP2D6 cDNA insert), which contained 200 pmol microsomes of microsomal protein (0.1–4.0 mg) equivalent to the CYP2D6 holoprotein-containing incubates. Metabolites were monitored by fluorescence detection at excitation/emission filter wave-
-demethylation was significantly increased 50-fold from 40°C (Table II). In contrast, enantioselective oxidation was not altered by the substitution of Asp301 with neutral amino acids (Asn, Ala, Gly), differing in size and polarity, resulted in marked reductions in catalytic activity. While it appears that a negative charge at amino acid residue 301 is important for efficient CYP2D6 catalysis, the precise role of the negative charge cannot be ascertained from these results alone. Nevertheless, the data do support the proposal that the carboxylate anion of Asp301 forms a charge pair with the positively charged substrate nitrogen and, by doing so, facilitates the binding and orientation of the ligand in the active site.

The ligand binding role of Asp301 is substantiated by the observation that replacement of the negatively charged residue with a neutral side chain resulted in substantial decreases in the binding capacity of debrisoquine (loss of type I spectrum) and quinidine (1000-fold greater Kd value). The requirement of a negative charge at position 301 for substrate binding can also be inferred from the catalytic data, which show that substantially higher concentrations of metoprolol were required to achieve significant catalytic activity in the mutants in which Asp301 was replaced with a neutral residue. Although full kinetic analyses have not been conducted, these results are indicative of a decrease in the affinity of the enzyme for the substrate. In addition, a reduction in the Vmax value of the mutated enzyme cannot be precluded.

The altered regioselective oxidation of metoprolol in the D301E mutant compared with wild type suggests a slightly different orientation of the substrate in the active site of the enzyme. As no gross change in the integrity of the active site was apparent in this mutant (as evidenced by a normal Soret absorption maximum of 448 nm, good heme incorporation, and retention of catalytic activity), the altered regioselectivity could be due to a subtle difference in the location of the substrate oxidation sites relative to the Fe–O2•− entity, as a consequence of the extension of the carboxylate residue by a methylene group. Implicit in such a rationale is an interaction between a carboxylate residue in the active site of the enzyme and a positive charge of the substrate molecule. Thus the observed alteration in the regioselective oxidation of metoprolol by the D301E mutant adds weight to the proposal that Asp301 serves as a negatively charged substrate-contact residue in the active site.

The catalytic activity decreased significantly when Asp301 was replaced with a neutral residue (Asn, Ala, or Gly), the structural integrity of the active site was also perturbed to varying degrees (as seen by the slight shift in the Soret absorption maximum of the carbon monoxide complex and in the different extent of heme incorporation). Thus an alternative explanation for the requirement of an anionic residue at position 301 for substrate binding is that this amino acid residue helps to maintain the integrity of the active site and that in its absence the topography of the site is altered. The different effects of the Ala and Gly substitutions on heme incorporation are difficult to explain. However, while both residues can be classed as neutral, Gly with only a hydrogen atom as a side
Role of Asp$^{301}$ in CYP2D6 Catalysis

In the absence of a substrate-bound crystal structure of CYP2D6, the precise role(s) of Asp$^{301}$ in CYP2D6 catalysis will remain unproven. Nevertheless, the present data strongly support the proposal that Asp$^{301}$ is a ligand-binding residue in the active site of CYP2D6, interacting via a charge pair with the positively charged nitrogen of CYP2D6 ligands.

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