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Characterization of the Gly45Asp variant of human cytochrome P450 1A1 using recombinant expression

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HIGHLIGHTS

- Gly45 is within a Pro-rich region of CYP1A1, responsible for holoenzyme expression.
- A variant of CYP1A1, Gly45Asp, had lower content of heme than wild-type.
- The Gly45Asp variant showed a reduced enzyme activity than the wild-type.

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ABSTRACT

Cytochrome P450 1A1 (CYP1A1) is a heme-containing enzyme involved in metabolism of xenobiotics. CYP1A1 containing a Gly45Asp substitution has not yet been characterized. *Escherichia coli* expressing the Gly45Asp variant, as well as the purified variant protein, had lower CYP (i.e., holoenzyme) contents than their wild-type (WT) equivalents. The purified variant protein had reduced heme contents compared with their WT equivalents. Enhanced supplementation of a heme precursor during culture did not increase CYP content in *E. coli* expressing the variant, but did for the WT. Substitution of Gly45 with other residues, especially those having large side chains, decreased CYP contents of *E. coli* expressing the variants to a considerable extent. A 3D structure of CYP1A1 indicates that Gly45, along with other residues of the PR region, interacts with Arg77 of β- strand 1-1, which indirectly interacts with heme. Substitution analyses suggest the importance of residues of the PR region and Arg77 in holoenzyme expression. *E. coli* membrane and mammalian microsomes expressing the Gly45Asp variant, as well as the purified variant protein, had reduced ethoxyresorufin *O*-dealkylation activities, compared with the WT equivalents. These findings suggest the Gly45Asp substitution results in a structural disturbance of CYP1A1, reducing its holoenzyme formation and catalytic activity.

1. Introduction

The cytochromes P450 (CYPs) are a large superfamily of heme-containing mono-oxygenases that are responsible for the metabolism of a variety of endogenous and exogenous compounds. CYP1A1 is a microsomal enzyme and one of the first CYP isozymes to be characterized (Nebert et al., 1991). Endogenous substrates of CYP1A1 include fatty acids and steroids. CYP1A1 also plays an important role in bioactivation and detoxification of environmental chemicals (Nelson et al., 1996).

Genetic polymorphisms within CYPs mainly affect the metabolism of chemicals that are substrates for those particular enzymes, leading to differences in chemical response (Ingelman-Sundberg et al., 2007; Kirchheiner and Seeringer, 2007). Many allelic variants and several sub-variants have been described for the *CYP1A1* gene (http://www.cypalleles.ki.se/). To date, there are more than 120 SNPs described for CYP1A1 in NCBI dbSNP (http://www.ncbi.nlm.nih.gov/, accessed January, 2015). *CYP1A1* genotypes have been associated with various human cancers such

Abbreviations: CHO, Chinese Hamster Ovary; CYPs, cytochromes P450; CYP1A1, cytochrome P450 1A1; dALA, δ-aminolevulinic acid; EDTA, ethylenediaminetetraacetic acid; ER, 7-ethoxyresorufin; EROD, 7-ethoxyresorufin *O*-dealkylation; EV, empty control vector; ME, β-mercaptoethanol; $NADP^+$, nicotinamide adenine dinucleotide phosphate; Ni-NTA, nickel-nitrilotriacetic acid; NPR, NADPH-cytochrome P450 reductase; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PR, Pro-rich; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SNPs, single nucleotide polymorphisms; SRS-5, substrate recognition site-5; WT, wild-type.

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as those of the lung, breast, prostate, and ovary (Zhou et al., 2009). A G to A transition at nucleotide 134 of CYP1A1 (G134A, NCBI dbSNP: rs4646422), resulting in a Gly45Asp substitution, has been identified previously (Park et al., 2004; Saito et al., 2002; Jorge-Nebert et al., 2010) and an allele containing this variation has been designated as *CYP1A1*13* (http://www.cypalleles.ki.se/). The allele containing the G134A variation has previously been observed in Asian populations with allelic frequencies of 2.9–18% (Park et al., 2004; http://www.1000genomes.org/). However, the effect of the Gly45Asp substitution on the enzymatic properties of CYP1A1 has not yet been characterized.

In nearly every microsomal CYP molecule, a Pro-rich (PR) region is present following an N-terminal signal anchor sequence and a short hydrophilic linker sequence (Williams et al., 2000). One of the well-conserved motifs in the PR region is the sequence PPGPxxxPxxGx, within which the first four residues, PPGP, are the most highly conserved (Kusano et al., 2001; Kemper, 2004). The PR region is known to be involved in the folding of CYP enzymes (Yamazaki et al., 1993; Chen and Kemper, 1996; Szczesna-Skorupa et al., 1993). In human CYP1A1, the PR region has the sequence PPGPWGWPLIGH, where the sixth residue is Gly45. Substitution of the sixth residue of the PR region in rabbit CYP2C2, Pro35, with Asp results in an 85% decrease in enzymatic activity (Chen and Kemper, 1996). Although the PR region's role in holo-CYP expression has been studied in some CYP isozymes, it has not been examined in CYP1A1. In addition, the importance of the 6th residue within the region's consensus sequence remains to be elucidated in human CYPs.

The heme prosthetic group is the catalytic center of CYPs; heme plays an important role in electron transfer during catalysis. The spatial organization of the heme binding sites of most CYPs has been suggested to be well conserved (Nebert et al., 1988; Hasemann et al., 1995). The consensus sequence FxxGxxxCxG is an important sequence motif for heme binding. In CYP1A1 protein, the heme group is bound to residues such as Ile449, Phe450, Arg455, Cys457, Ile458, Gly459, and Ala463 that lie within and around the motif. Other residues such as Arg106, Ser122, Trp131, Ala317, Thr321, Phe381, Val382, Thr385, Ile386, His388, and Gln411 are also involved in heme binding (Walsh et al., 2013). In contrast to the synthesis of CYP apoprotein and its incorporation into endoplasmic reticulum membranes, the insertion of heme appears to be less tightly coupled to the synthesis of the CYP apoprotein (Sakaguchi et al., 1987). When heme is supplied insufficiently, heme saturation of the CYP apoprotein is incomplete (Correia and Meyer, 1975; Sadano and Omura, 1983).

In the present study, we characterized a CYP1A1 variant harboring a Gly45Asp substitution by using heterologous expression in *Escherichia coli* and mammalian cells. Our findings suggest that the variant apoprotein has a low affinity for its prosthetic heme group and that the variant has decreased enzymatic activities compared with wild-type (WT) CYP1A1. Based upon site-directed mutagenesis studies, the variant's reduced capacity for holoenzyme formation and enzymatic activities may be due to Gly45Asp-induced structural disturbances.

2. Materials and methods

2.1. Chemicals

All chemicals used were of analytical grade or higher and were purchased from Sigma-Aldrich (St. Louis, MO) unless specified.

2.2. Bacterial constructs

Open reading frame cDNA clones for WT CYP1A1 and a variant containing the G134A substitution were prepared using total RNA from lymphocytes of an individual having the substitution (Park et al., 2004). The cDNA sequences were modified for expression in *E. coli* according to Guo et al. (1994). The second *N*-terminal residue of CYP1A1, Leu, was replaced with Ala, and the nucleotide sequences encoding residues 3–9 were changed to AT-rich sequence (5′-ATGGCTTTTCCAATTTCAATGTCAGCA-3′) without substitution of residues. Each cDNA fragment was inserted into the *Nde*I and *Xba*I restriction sites of pCW-NPR, a human NADPH-cytochrome P450 reductase (NPR)-containing bicistronic expression vector (Parikh et al., 1997). For purification of WT CYP1A1 and Gly45Asp variant proteins, six-His codons were added just before the stop codon of the modified cDNAs. The resulting fragments were inserted into the monocistronic vector pCW using the *Nde*I and *Hin*dIII restriction sites (Gillam et al., 1995).

2.3. Bacterial expression

Bacterial harvest and membrane preparation were performed as described previously (Gillam et al., 1993; Guengerich and Martin, 2006). *E. coli* DH5α cells were transformed with the expression constructs and grown overnight at 37 °C in Luria–Bertani broth containing 50 μg/mL ampicillin. The overnight culture was inoculated 1:1000 into Terrific Broth medium containing 50 μg/mL ampicillin and 1 mM thiamine. Cultures were incubated at 37 °C with shaking at 200 rpm until they attained an OD_{600} of 0.5–0.7, then were supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside and 0.5 mM δ-aminolevulinic acid (dALA), a heme precursor, and cultured for 24 h at 30 °C with shaking at 200 rpm.

Culture was then chilled on ice and centrifuged at 3800 × *g* for 20 min. The cell pellets were washed with phosphate-buffered saline (PBS), and the cells were weighed and resuspended in 100 mM Tris-acetate buffer, pH 7.6, containing 500 mM sucrose, and 0.5 mM ethylenediaminetetraacetic acid (EDTA). Lysozyme was added to 0.2 mg/mL, and the suspensions were diluted two-fold with distilled H_2O before incubation on ice for 30 min. The resulting spheroplasts were sedimented at 3800 × *g* at 4 °C for 20 min, and resuspended in 100 mM potassium phosphate buffer, pH 7.6, containing 6 mM magnesium acetate, 20% glycerol (v/v), and 10 mM β-mercaptoethanol (ME).

Suspensions of spheroplasts were sonicated four times for 20 s each, on ice, and centrifuged at 10,000 × *g* at 4 °C for 20 min. Supernatants were centrifuged at 100,000 × *g* at 4 °C for 75 min. Sedimented membrane fractions were resuspended in 100 mM potassium phosphate buffer, pH 7.6, containing 6 mM magnesium acetate, 20% glycerol (v/v), and 10 mM ME. The membrane preparation was stored at −70 °C until use.

2.4. CYP and heme contents

CYP content was determined by reduced CO difference spectra (Omura and Sato, 1964). Sodium dithionite was added to reduce ferric CYPs. Ferrous-CO CYP complexes were generated by passing CO gas through solutions of the ferrous CYPs. The spectra were collected on a spectrophotometer at room temperature. Heme content was quantified using a pyridine hemochromogen assay and calculated from the difference in absorption between 557 and 575 nm (Schenkman and Jansson, 2006; Sinclair et al., 2001).

2.5. Immunoblots

Immunoblots were performed using a primary anti-CYP1A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (GenDepot, Barker, TX, USA). Blots were developed using a chemiluminescence reagent kit. Protein concentrations were