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Substrate proton to heme distances in CYP2C9 allelic variants and alterations by the heterotropic activator, dapsone

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ABSTRACT

CYP2C9 polymorphisms result in reduced enzyme catalytic activity and greater activation by effector molecules as compared to wild-type protein, with the mechanism(s) for these changes in activity not fully elucidated. Through T_1 NMR and spectral binding analyses, mechanism(s) for these differences in behavior of the variant proteins (CYP2C9.2, CYP2C9.3, and CYP2C9.5) as compared to CYP2C9.1 were assessed. Neither altered binding affinity nor substrate (flurbiprofen) proton to heme-iron distances differed substantially among the four enzymes. Co-incubation with dapsone resulted in reduced substrate proton to heme-iron distances for all enzymes, providing at least a partial mechanism for the activation of CYP2C9 variants by dapsone. In summary, neither altered binding affinity nor substrate orientation appear to be major factors in the reduced catalytic activity noted in the CYP2C9 variants, but dapsone co-incubation caused similar changes in substrate proton to heme-iron distances suggesting at least partial common mechanisms in the activation of the CYP2C9 forms.

Allelic variant forms of polymorphic drug metabolizing enzymes, especially CYP2C9, often display altered kinetics from the wild-type enzyme. Most commonly, these variants exhibit a reduction in the rate of metabolism. Many *in vivo* and *in vitro* examples of these alterations in enzyme activity exist. For example, Dickmann et al. demonstrated that CYP2C9.3 and CYP2C9.5 both exhibit reduced metabolizing activity toward (S)-warfarin, diclofenac, and lauric acid metabolism [1]. *In vivo*, patients expressing a CYP2C9 allelic variant form (either *2 or *3) require lower than normal doses of warfarin and phenytoin to prevent toxicity due to reduced metabolism [2,3]. Changes in kinetic profile can also occur with CYP2C9 allelic variants as has been observed with naproxen demethylation exhibiting a linear kinetic profile with CYP2C9.3 and CYP2C9.5 as compared to the biphasic profile observed with CYP2C9.1 [4]. Also, piroxicam exhibited a substrate inhibition profile when metabolized by CYP2C9.1 and CYP2C9.3, but a hyperbolic kinetic profile was evident with CYP2C9.5 [4]. These allelic variants result from single nucleotide polymorphisms encoding for single amino acid changes in the protein. These amino acid changes may affect substrate turnover either directly or indirectly through effects on substrate binding, alterations in electron transport or active site waters affecting P450 cycle function or changes in active site conformation [5].

Of the CYP2C9 variant forms discovered to date, three of the most extensively characterized are CYP2C9.2, CYP2C9.3, and CYP2C9.5. The R144C substitution in CYP2C9.2 is outside of the active site and was originally proposed to affect P450 reductase binding [6], though more recent studies do not support this hypothesis and instead suggest that it affects catalytic cycle functioning through alterations in shunting products and potentially water matrix disruption [5]. The I359L and D360E changes in CYP2C9.3 and CYP2C9.5, respectively, are conservative amino acid substitutions at positions thought to not be in direct contact with substrates in the active site cavity [7,8]. However, the dramatic reduction (approximately 4% for CYP2C9.3 and 10% for CYP2C9.5 of wild-type activity) [4,9] in turnover caused by these two changes suggests that alterations in interactions with other amino acids may occur that could modify the active site topology and subsequently substrate binding.

Since these variant forms of CYP2C9 alter the rate of substrate metabolism, it is possible that these amino acid changes may also impact the effects of inhibitor/activator (effector) molecules. Drug–

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drug interactions involving CYP2C9 have been shown to be substrate, effector, and enzyme variant form dependent, *in vitro*. In an example of variant-dependent effects, dapsone activation of metabolism of the nonsteroidal anti-inflammatory drugs (NSAIDs)[2], naproxen and flurbiprofen, occurred to a much greater extent in CYP2C9 allelic variants than in the wild-type enzyme [9]. With respect to substrate-dependent effects, in a study of dapsone analogs and their effects on CYP2C9-mediated metabolism, Hutzler et al. reported that phenyl sulfone was a good activator of flurbiprofen metabolism but not of naproxen metabolism [10]. More recently, substrate-dependent inhibition of both CYP2C9.1 and CYP2C9.3 was reported [11]. Thus, one cannot simply generalize that all effectors will produce the same interaction with all substrates or that the degree of effect will be similar.

To examine the effects of CYP2C9 variant proteins (CYP2C9.1, CYP2C9.2, CYP2C9.3, and CYP2C9.5) on substrate orientation and proton–heme-iron distances, NMR derived T_1 relaxation studies were conducted with the probe substrate flurbiprofen. In addition, co-incubation of flurbiprofen with dapsone not only results in heteroactivation but also causes the flurbiprofen protons to move closer to the heme-iron in wild-type protein [12]. Thus, we also examined the effects of dapsone on flurbiprofen distance and orientation in these same variants. In addition, binding affinity was assessed through UV/Vis spectral binding studies. The purpose of these studies was to gain additional information on mechanisms involved in altered catalytic activity with the CYP2C9 variants and differences in degree of heteroactivation noted among the variant proteins.

Materials and methods

Chemicals

D_2O, polyvinylpyrrolidone, sodium dithionite, *racemic*-flurbiprofen, dapsone, and dilauroylphosphatidylcholine were purchased from Sigma–Aldrich (St. Louis, MO). Centricon MW cutoff filters were purchased from Millipore (Billerica, MA). Potassium phosphate, and EDTA were purchased from Fisher Scientific (Pittsburgh, PA). All other chemicals were purchased from commercial sources and were of the highest purity available.

Enzyme expression and purification

CYP2C9.1 and CYP2C9.3 were expressed in *E. Coli* according to established methods [13] Purified CYP2C9.2 and CYP2C9.5 proteins were provided by Dr. Allan Rettie from the University of Washington.

Spectral binding

Spectral binding studies to measure enzyme–substrate affinity were performed as previously reported [14]. Briefly, 300 pmol of enzyme along with 0.2 μg/pmol dilauroylphosphatidyl choline (200 nm vesicles) was placed into the sample and reference cuvette. Aliquots (5 μL) of flurbiprofen dissolved in 50 mM pH 7.4 potassium phosphate buffer were added to the sample cuvette while 5 μL of 50 mM pH 7.4 potassium phosphate buffer was added to the reference cuvette. After mixing, the sample and reference cuvettes were allowed to equilibrate for 3 min prior to spectral analysis. Spectra were recorded on an Aminco DW-2000 UV/Vis spectrophotometer with Olis modifications (Olis, Inc., Bogart, GA). The spectrophotometer was set to record spectra between 350 and 500 nm wavelengths with a slit width of 6.0 nm and scan rate of 100 nm/min. The temperature was held at a constant 28° C. The difference in absorbance between the peak (~390) and trough (~420) of the observed Type-I binding spectrum was calculated and plotted against flurbiprofen concentration. A binding constant (K_S) was determined by fitting the resulting data to a hyperbolic curve using Eq. (1).

$$\Delta A = \frac{(B_{\max} \bullet S)}{K_s + S} \tag{1}$$

Spectral binding experiments were also performed with flurbiprofen in the presence of 100 μM dapsone in a manner identical to that described above.

[2] *Abbreviations used:* NSAIDs, nonsteroidal anti-inflammatory drugs; SRS, substrate recognition site.

Spin state determination

Substrate-induced spin state changes with each of the CYP2C9 enzymes were measured as described above for spectral binding measurements. Spectral titrations with *(S)*-flurbiprofen and dapsone (concentrations were identical to those used for NMR experiments) were performed by adding equal volumes of either flurbiprofen, dapsone or flurbiprofen + dapsone to both sample and reference cuvettes followed by spectral scanning between 320 and 500 nm. Prior to scanning, the sample and reference cuvettes were allowed to equilibrate for 3 min with enzyme and substrate. Absolute spectra were then obtained to estimate the percent conversion of CYP2C9 from low spin to high spin using the relative low spin peak area [15]. Deconvolution of spectra was carried out with the multiple Gaussian curve fitting available in OriginPro (v7.5 OriginLab Corporation, Northampton, MA). The model was developed to include three components: a low spin component (~416–420 nm), a high spin component (~390–405 nm) and δ-bands (~360 nm). The amount of low spin heme-iron is correlated to the low spin peak areas deconvoluted from samples of different P450 concentrations ($R^2 = 0.99$).

NMR sample preparation

Samples were prepared by a 50-fold dilution of the purified enzyme of interest into 50 mM potassium phosphate (pH 7.4), in D_2O, to reduce the concentration of glycerol and H_2O in the sample. Enzyme and substrate concentrations were based on the calculated K_S for each variant so that all enzymes present in the sample were saturated with substrate. For CYP2C9.1, 0.014 μM enzyme was used along with 145 μM flurbiprofen, 145 μM flurbiprofen and 100 μM dapsone, or 100 μM dapsone. For CYP2C9.2, 0.005 μM enzyme was used along with 50 μM flurbiprofen, 50 μM flurbiprofen, and 100 μM dapsone, or 100 μM dapsone. For CYP2C9.3, 0.014 μM enzyme was used along with 140 μM flurbiprofen, 140 μM flurbiprofen, and 100 μM dapsone, or 100 μM dapsone. For CYP2C9.5, 0.016 μM enzyme was used along with 160 μM flurbiprofen, 160 μM flurbiprofen, and 100 μM dapsone, or 100 μM dapsone. All samples were made up in a final volume of 750 μL.

T_1 relaxation measurements

T_1 times of substrate protons were determined with the NMR instrument operating at 600.5 MHz, internally locked on the deuterium signal of the solvent. NMR spectra were acquired on a Varian Inova® 600 MHz NMR (Varian Instruments, Palo Alto, CA). The probe was maintained at 298° K for all experiments except for those involving temperature dependence. The Varian T_1 inversion-recovery sequence (d_1-180-d_2-90) was used along with presaturation of the residual HOD signal. The PW 90 was calibrated on each sample. Spectra were acquired for 12 τ (d_2) values ranging from 0.0125 to 25.6 s and a period of 10 T_1 was used between pulses (d_1). The Varian software routines were used to determine the T_1 times. Once the paramagnetic effect of the heme-iron on substrate protons was measured, CO was bubbled through the sample for 15 min, sodium dithionite was added and the sample allowed to equilibrate for 30 min then measured again, in order to determine the diamagnetic contribution to the T_1 relaxation times. Stability of the enzyme as well as the CO reduced complex was tested and both were found to be stable for the duration of the NMR acquisition time.

T_1 temperature dependence

The validity of the T_1 measurements and the distances calculated between protons in flurbiprofen and dapsone is dependent on the substrates being in fast exchange. This can be demonstrated by conducting T_1 measurements over a range of temperatures [16]. Therefore, T_1 measurements were performed as described above at three different temperatures (283, 298, and 310 K). Data were collected both in the absence ($1/T_{1,2C9}$) and presence ($1/T_{1,2C9+CO}$) of CO/sodium dithionite. To assure adequate diffusion of CO and mixing of dithionite, samples were removed from the NMR tube and placed in a test tube, CO bubbled into the sample and sodium dithionite added, then the sample was placed back into the NMR tube.

Distance calculations

In our previous work [12] on distances between heme and flurbiprofen/dapsone, estimates were calculated using the equation: $r = C[T^*_{1p}\alpha^*_m f(\tau_c)]^{1/6}$, where r is the distance and C is a constant, that is, a function of the metal, oxidation state, and whether it is low or high spin. In this case, Fe^{+3} is in the low spin state and thus the appropriate value for C is 539 [17]. However, we have since obtained spin-state data and this permits a more precise calculation. In particular, here, distances were calculated from the Solomon–Bloembergen equation (Eq. (2)) which can be written [18]:

$$\frac{1}{T_{1P}} = \alpha \frac{9.87 \times 10^{16} S(S+1)}{r^6}(\tau_c) \tag{2}$$

T_{1p} is the portion of T_{1obs} due to paramagnetic effects alone and is given by $T_{1P}^{-1} = T_{1obs}(Fe^{+3})^{-1} - T_{1obs}(Fe^{+2})^{-1}$ assuming that all of the diamagnetic contribution is represented by $T_{1obs}(Fe^{+2})$ [16]. This assumption has been used in many similar studies and appears to be generally valid [19,20]. The correlation time (τ_c) for CYP2C9 has been previously reported (2×10^{-10} s^{-1}) [19] and was used here.