[S1-3] [4/19/2001(Thur) 15:00-15:30/Hall A]

Oxidations of Phenacetin and *p*-Alkoxyanilides Catalyzed by Human Cytochrome P450 1A2: Rate-Determining Steps and Structure-activity Relationships

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Human cytochrome P450 (P450) 1A2 is involved in the oxidation of many important drugs and carcinogens. Mutants with altered activities were obtained from random libraries of human P450 1A2 with the putative substrate recognition sequences (SRS) mutated. Six mutants from SRS 2 (E225I, E225N, F226I, and F226Y) and 4 (D320A and V322A) regions were expressed as oligohistidine-tagged proteins, purified to homogeneity, and used to analyze kinetics of individual steps in the catalytic cycle, to determine which reaction steps have been altered. When the wild-type and six mutant proteins were reconstituted with NADPH-P450 reductase, rates of 7-ethoxyresorufin *O*-deethylation and phenacetin *O*-deethylation were in accord with those expected from membrane preparations. Within each assay the values of *k*cat/*K*m varied by 2-3 orders of magnitude, and in the case of E225I and E225N, these parameters were 7-8-fold higher than for the wild-type enzyme. The coupling efficiency obtained form the rates of product formation and NADPH oxidations was low (<20%) in all enzymes. No correlation was found between activities and several individual steps in the catalytic cycle examined, including substrate binding, reduction kinetics, NADPH oxidation, and H2O2 formation. Quenching reactions did not show a burst for either phenacetin O-deethylation or formation of the acetol, a minor product indicating that rate-determining steps occur prior to product formation. Inter- and intramolecular kinetic deuterium isotope effects for phenacetin *O*-deethylation were 2-3. In the case of phenacetin acetyl hydroxylation (acetol formation), large isotope effects [D*k*cat or D(*k*cat/*K*m) > 10] were observed, providing evidence for rate-limiting C-H bond cleavage.

In an effort to improve rates of catalysis of P450 1A2 enzymes, a set of *p*-alkoxyacylanilide analogs of phenacetin was considered. It was found that variations in the *O*-alkyl and *N*-acyl substituents altered the rates of the two oxidation reactions and the ratio of acetol/phenol products. Moving on methylene group of phenacetin from the *O*-alkyl group to the *N*-acyl moiety increase rates of both oxidations ~5-fold and improved the coupling

efficiency from 6 to 38%. Non-competitive deuterium kinetic isotope effects of 2-3 were measured for all *O*-dealkylation reactions examined with wild-type and the E225I mutant, which has 6-fold figher activity. A trend of decreasing kinetic deuterium isotope effect for E225I > wild-type > mutant D320A was observed for *O*-demethylation of *p*-methoxyacetanilide, which follows the trend for *k*cat. The set of *O*-dealkylation and acetol formation results for wild-type P450 1A2 and the E225I mutant with several of the protiated and deuterated substrates were fit to a model developed for the basic catalytic cycle and a set of microscopic rate constants in which the only variable was the rate of product formation (substrate oxygenation, including hydrogen abstraction). In this model, *k*cat is considerably less than any of the microscopic rate constants and is affected by several individual rate constants, including the rate of formation of the oxygenating species, the rate of substrate oxidation by the oxygenating species, and the rate of generation of reduced oxygen species (H2O2, H2O). This analysis of the effects of the individual rate constants provides a framework for the consideration of other P450 reactions and rate-limiting steps.

Figure 1. General Mechanism of P450 Catalysis

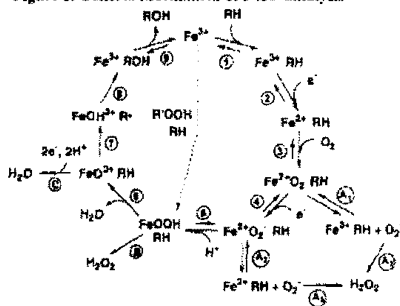

Figure 2. Oxidation of Phenacetin Catalyzed by Human P450 1A2

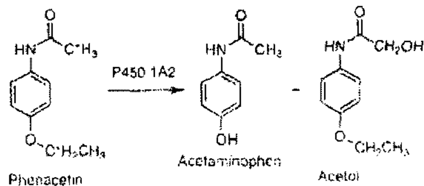

Figure 3. Oxidations of *p*-Alkoxyacylanilides by P450 1A2