Stereochemical Evidence for Decomposition of Reactive Intermediates by Active Site Water in the Metabolism of 8-Alkyl Xanthines by P450 1A2

Jagdish K. Racha and Kent L. Kunze*

Department of Medicinal Chemistry
Box 357610
University of Washington
Seattle, Washington 98195

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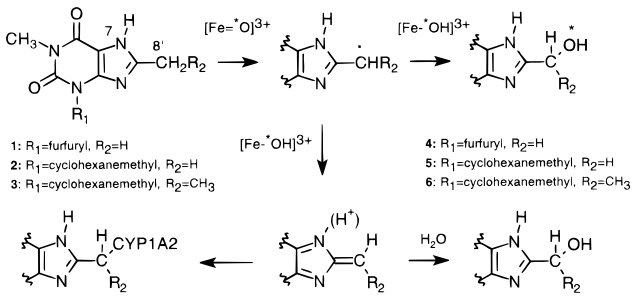
Substrate oxidation by P450 enzymes occasionally produces a flux of electrophilic reactive metabolites which, among other fates, covalently bind to cellular targets as well as the enzymes themselves. In this communication we report the first example where a P450-generated reactive intermediate is preferentially (>90%) quenched by solvent contained within the enzyme active site to produce a hydroxylated product.

Oxidation of the 8-methylxanthines furafylline **1** or cyclohexylline **2** by human CYP1A2 results in rapid loss of enzyme activity (k_{inact} > 0.9 min^{-1}) and formation of a stable one-to-one adduct of xanthine with CYP1A2 protein.[1,2] The sole metabolites of these suicide substrates are the respective 8′-carbinols (**4, 5)** which are formed with high intramolecular isotope effects ($^{H}k/^{D}k$ > 9) and low overall partition ratios (5.5 and 7.5).[2] The carbinols incorporate substantial amounts of oxygen from the medium (80 and 70%, respectively) with the balance sourced to O_2. We have proposed that oxidation of these xanthines proceeds conventionally to a substrate−radical [Fe−OH]$^{3+}$ intermediate, which decomposes by (1) oxygen rebound to give the carbinol sourced from O_2 or (2) electron transfer (SET or HAT) to produce a reactive intermediate that reacts either with active site protein residues (inactivation) or the medium to give the carbinol incorporating oxygen from H_2O (Scheme 1). On the basis of the negative results of trapping experiments we posed the hypothesis that the reactive intermediate may be substantially decomposed by addition of water within the confines of the enzyme active site. In this communication we report one test of this hypothesis via a determination of the stereochemistry of CYP1A2-catalyzed water incorporation into the prochiral methylene position of the 8-ethylxanthine **3** to produce the secondary carbinol **6**.[3]

Incubation of **3** (30 μM) with CYP1A2 microsomes and NADPH afforded the secondary carbinol **6** as the major metabolite.[4] CYP1A2-catalyzed incorporation of oxygen from the

Scheme 1



medium ($H_2{}^{18}O$) into **6** was determined by EIGC-MS analysis of extracted and derivatized **6** [5] (*N*-methyl, *O*-TMS) parent ion envelopes to be 61.9 ± 0.5%. This fraction was not significantly altered by the presence of NaCN (1 mM), glutathione (2 mM), or *N*-acetylcysteine (8 mM) in the incubation mixture.[6] Incorporation of oxygen from O_2 was determined in an atmosphere of $^{18}O_2$ where (R)-warfarin (500 μM) was coincubated with **3** (5 μM). The fraction of ^{18}O found in the CYP1A2 metabolite 6-hydroxywarfarin (97.6 ± 0.6%) was used to calculate the corrected fraction of ^{18}O in *N*-methyl *O*-TMS ether of **6** (37.8 ± 1.1%).

The enantiomeric composition of **6** was determined by HPLC-UV analysis of the (+)-di-*O*-acetyl-L-tartarate monoesters generated after HPLC purification of **6** from incubation mixture extracts. The fraction of **(*R*)-6** was determined to be 4.3% (±0.2) Figure 1b).[7] The source of oxygen in the **6** enantiomers ($H_2{}^{18}O$ in the medium) was determined from ESLC-MS-SIM analysis of the protonated parent ion envelopes of the tartarate esters. The fraction of water incorporated into **(*R*)-6** and **(*S*)-6** was 67.4% (±0.9) and 61.6% (±0.6), respectively. These results are summarized in Table 1.

The remarkably high stereoselective incorporation of oxygen from water into the secondary carbinol [(*S*)/(*R*) = 20, enantiomeric excess 91%] suggests that the portion of substrate that is promoted by the enzyme to the oxidation state required for reaction with water is largely decomposed within the chiral confines of the active site. The fraction of intermediate stereoisomers which might escape the active site must be less than 9%. If a finite dissociation constant for the enzyme-reactive intermediate complex exists, these results indicate that (1) intermediate release is severely limited by the enzyme and/or (2) decomposition in the active site is extremely rapid.

P450 enzymes are known to exert variable degrees of stereochemical control over abstraction and recombination events. Without knowledge of (1) the stereochemical course of hydrogen removal for either pathway and (2) a definitive mechanism for generation and decomposition of the two-electron-oxidized intermediate, it is not possible with the data at hand to establish the manner in which product stereochemistry is established. Moreover, while the location of the introduced oxygen in a rebound event is known and can be used to evaluate crossover probabilities when the site of abstraction is known,[8] the same

* To whom correspondence should be addressed.

(1) Kunze, K. L.; Trager, W. F. *Chem. Res. Toxicol.* **1993**, *6*, 649−656.
(2) Racha, J. K.; Rettie, A. R.; Kunze, K. L. *Biochemistry.* In press.

(3) Substrate **3** was synthesized by condensation of 1-methyl-3-cyclohexanemethyl-4,5-diaminouracil[2] with propionic anhydride. The desired secondary carbinols **6**, **(*R*)-6**, and **(*S*)-6** were prepared by ring closure of the diaminouracil with *rac*, (*R*), or (*S*) lactic acids, respectively. Treatment of **6** with (+)-di-*O*-acetyl-L-tartaric anhydride in a solution of 20% glacial acetic acid in CH_2Cl_2 gave quantitative conversion to the corresponding diastereomeric monoesters which were separated by HPLC (Figure 1a). The enantiomeric excess of the synthetic (*R*) and (*S*) carbinols after chiral derivatization and prior to purification of the diastereomers was found to be greater than 99%.

(4) Incubations of **3** with microsomes prepared from lymphoblastoid cells expressing human CYP1A2 (1 mg of protein, Gentest) were conducted for 30 min in the presence of NADPH (1 mM) in potassium phosphate buffer (100 mM.; pH 7.4). Amounts of **6** were determined by HPLC-UV analysis of supernatants following precipitation of protein with TFA.[2] Metabolite identification was made by comparison with authentic **6** by ESLC-MS/MS daughter ion spectra. Formation of **6** was NADPH dependent and linear with time through 6 min and modestly declined thereafter, suggesting that **3** may be a weak mechanism based inactivator of the enzyme. Preliminary kinetic experiments demonstrated saturable metabolism and provided estimates of K_m and k_{cat} of 2.5 μM and 1.2 min^{-1}, respectively. No **6** (detection limit 0.5% of CYP1A2 rate) was observed by HPLC analysis of control microsomes derived from native cells not expressing CYP1A2.

(5) Protocols for $H_2{}^{18}O$ water and $^{18}O_2$ experiments and derivatization procedures for EIGC-MS analysis of xanthine carbinols and 6-hydroxywarfarin have been described.[1, 2]

(6) See the following and references therein for an example where formation of a similar type of reactive intermediate (3-methyleneindolenamine from 3-methylindole) has been proposed that is efficiently trapped by the exogenous nucleophiles employed here as well as solvent: Skiles, G. L.; Yost, G. S. *Chem. Res. Toxicol.* **1996**, *9*, 291−297.

(7) Control experiments where conducted in which each of the chiral carbinols (4 μM) were exposed to the enzyme for a period of 30 min in the presence of NADPH. Greater than 98% of the carbinols survived the incubation with no significant racemization. Significant incorporation of oxygen from the medium when **6** was the substrate was not observed.

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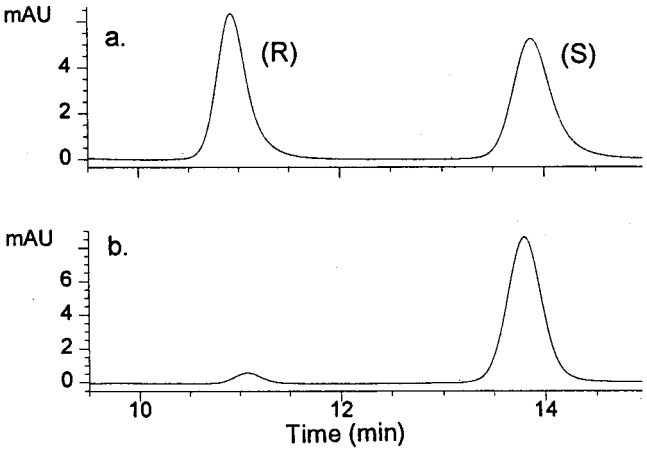

Figure 1. HPLC profiles of the di-*O*-acetyl-L-tartarate monoester derivatives of (a) synthetic **6** and (b) CYP1A2 generated **6** purified from incubation mixtures. Solvent (0.1% aqueous acetic acid−acetonitrile (75:25)) was delivered at 0.7 mL/min through a Hewlett-Packard ODS Hypersil (125 × 4 mm) 5 μm column with UV detection at 280 nm.

Table 1. Enantiomeric Composition of CYP1A2-Generated **6** and the Source of Carbinol Oxygen[a]

enantiomer	H_2O	O_2
(*R*)-**6**	2.9% (±0.2)	1.4% (±0.1)
(*S*)-**6**	58.9% (±0.7)	36.7% (±0.7)

[a] Calculated from enantiomeric fractions and $H_2{}^{18}O$ incorporation data given in text.

cannot be said for the water pathway where multiple reacting solvent molecules may exist in the active sites of substrate bound P450's, particularly those forms with broad substrate selectivities.

Nevertheless, the most likely scenario is the one where water incorporation into the major carbinol enantiomer occurs as a consequence of an encounter of the *si* face of one of the two isomeric forms of the reactive intermediate with a particular solvent molecule located on the interior surface of the enzyme. This stereoselective rendezvous of reactants may occur prior to or following release of the intermediate from the position assumed by the substrate during the oxidation sequence. The possibility that the reacting water may be partially populated with oxygen atoms derived from O_2 generated during catalysis cannot be excluded. The presence of a localized solvent molecule in close proximity to the heme iron in the crystal structure of substrate bound $P450_{eryF}$ has been shown.[9a] $P450_{cam}$ structures with substrates and inhibitors have also revealed localized water and other more mobile solvent molecules may also be present.[9b,c,10]

It is certainly striking that the ratio of water to oxygen addition is similar for both enantiomers. One possible location for the reacting water suggested by these results is the sixth ligand position of the heme prosthetic group itself. However, if we are correct in the assumption that the reactive intermediate is formed by uptake of two electrons from the substrate by the iron bound oxygen, this site would be initially occupied by water or hydroxide originating from O_2. In this unlikely, but certainly intriguing, scenario the balance of carbinol oxygen derived from the two oxygen sources would depend on the rate of exchange of this aqua ligand with solvent as well as the extent of true oxygen rebound. Therefore, the extent of true oxygen rebound, here and above, may be partially masked by exchange processes where the reacting water is enriched in water derived from molecular oxygen. In either case, Scheme 1 would have to be amended to reflect exchange processes and the contribution of the normal oxygen rebound pathway would be overestimated by our results.

With respect to our results with the 8-methylxanthines this study provides a clear rationale for the observation that reactive intermediates could not be trapped with alternative nucleophiles in the medium.[2] Since intermediate release to the medium can be assumed to be low, the partition between water incorporation into the carbinol and inactivated enzyme (4−5) (Scheme 1) may simply report on the internal competition between active site solvent and protein nucleophiles for the reactive intermediate with the caveat that the partition may also depend on reorganization of the putative enzyme intermediate complex. More generally, these findings suggest the novel possibility that a portion of the flux of electrophilic intermediates produced by the family of P450 enzymes during oxidation of other substrates may also be deactivated by reaction with active site water molecules.

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Supporting Information Available: Synthetic procedures, spectral data for new compounds, and experimental protocols (8 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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