Detection of Aminium Ion Intermediates: *N*-Cyclopropyl versus *N*-Carboxymethyl Groups as Reporters

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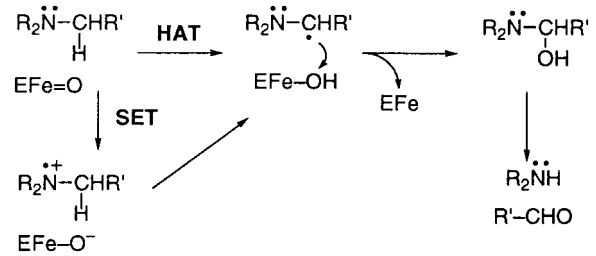
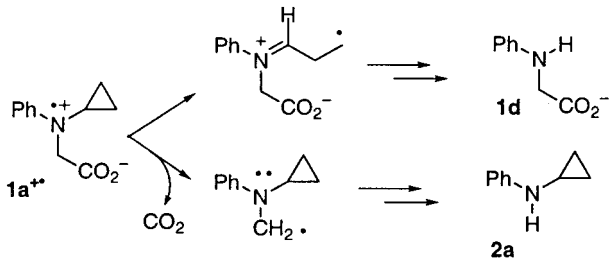
Oxidative *N*-dealkylation is the single most common reaction in the biotransformation of alkylamines. This process is catalyzed by a range of enzymes including the flavoproteins, monoamine oxidase and sarcosine oxidase, the quinoproteins diamine oxidase, lysyl oxidase, methylamine dehydrogenase, and plasma amine oxidase, the non-heme iron oxidase ACC oxidase, and the heme-thiolate cytochromes P450. *N*-Dealkylations catalyzed by cytochrome P450 enzymes involve the enzymatic generation of a carbinolamine, but the mechanism of this reaction is controversial. One alternative is the hydrogen-atom transfer mechanism (HAT, Scheme 1), but several important observations including low kinetic deuterium isotope effects on *N*-dealkylation reactions, the release of alkyl radicals during oxidation of 4-alkyl-1,4-dihydropyridines, and the suicide substrate activity of cyclopropylamines cannot be explained by this mechanism. To accommodate these observations an alternative pathway involving single-electron transfer (SET, Scheme 1) as an initiating step has been proposed.[1]

Considerable experimental effort has gone into differentiating these two mechanisms.[2−8] One approach has been to utilize "radical clock" reporter groups which reveal cation radical intermediates by undergoing unimolecular ring opening or fragmentation reactions. For this approach to be reliable, however, the probe reaction must be fast enough to intercept and divert the postulated aminium ion intermediate before it reacts to form the "normal" products, and the probe must report *unequivocally*, by giving distinct products in model HAT versus SET reactions. The cyclopropyl group has been widely applied to probe the mechanisms of amine-oxidizing enzymes.[9−13] HAT oxidation of cyclopropyl groups is known to lead to ring-intact products,[14,15] but calculations[16] and experimental results[7,8,17,18] suggest that the

Scheme 1



Scheme 2



cyclopropylaminium ion undergoes rapid ring opening[19] to a distonic cation−radical intermediate. The latter has been postulated, but never demonstrated, to be the species that inactivates P450, and until recently,[7,8] little was known about the fate of the cyclopropyl moiety lost during *N*-dealkylation reactions (Scheme 2).

Thus, we turned to the *N*-carboxymethyl group as an alternative to the cyclopropyl group. When attached to an electron-deficient aminium cation radical center, this group undergoes rapid decarboxylation[20] and fragmentation[21−23] to easily identifiable products. To evaluate its potential as a reporter group for studying enzymatic *N*-dealkylation mechanisms we first sought to compare its reactivity to that of the cyclopropyl group by means of an intramolecular competition using *N*-cyclopropyl-*N*-phenylglycine (**1a**) as the oxidizable substrate and horseradish peroxidase (HRP) as the SET oxidant (viz. Scheme 2). For context, we also studied the related substrates **1b** and **1c**.

Since comparing the relative reactivity of the alkyl, cyclopropyl, and carboxymethyl groups in **1a**−**1c** upon SET oxidation depends on quantitating the relative yields of monosubstituted anilines (**2a**−**2c**) versus *N*-phenylglycine (**1d**, viz. Scheme 2) we first investigated the stability of **2a**−**2c** and **1d** with HRP under

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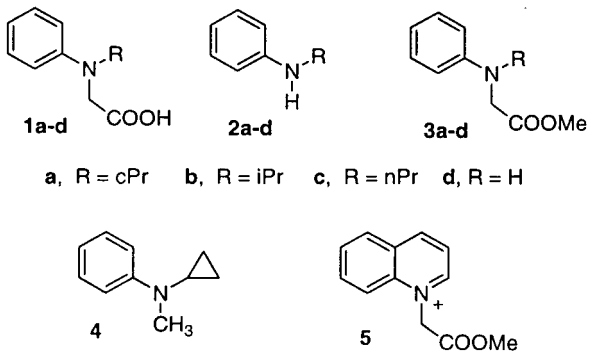

standard peroxidatic conditions.[24] Under these conditions the disappearance of **2a-2c** and **1d** follows first-order kinetics to ≥95% completion with apparent rate constants of 0.053, 0.035, 0.48, and 0.015 min^{-1}, respectively; aniline is the product in all cases. Under similar conditions the disappearance of **1b** and **1c** follows strictly zero-order kinetics through at least 95% consumption. The apparent rate constants are 41 and 102 $\mu M \cdot min^{-1}$, respectively, and in both cases the product consists almost entirely of the corresponding *N*-alkylaniline (i.e., **2b** and **2c**) along with an equivalent amount of formaldehyde and traces of aniline; *N*-phenylglycine (**1d**) is not detected.

When **1a** was incubated with HRP, a single major metabolite (>66% yield) and three minor metabolites were formed. By means of GC/MS[7] and HPLC comparisons to authentic standards the major metabolite was identified as *N*-cyclopropylaniline (**2a**), and one of the minor metabolites, as aniline (formed by secondary oxidation of **2a**). *N*-Phenylglycine (**1d**) was *not* observed among the products at any time throughout the reaction. From the relative rates of oxidation of **2a** versus **1d** given above, it is clear that if **1d** had formed it would have accumulated and been detected. Since controls showed that as little as a 5 mol % yield of **1d** would have been readily detectable, $\mathbf{1a}^{+\bullet}$ must react preferentially if not exclusively by decarboxylation rather than by cyclopropyl group fragmentation.

The kinetics of HRP oxidation of **1a** proved to be very different from the oxidation of **1b** and **1c**. The disappearance of **1a** is quite rapid up to about 25−30% consumption (within ca. 10 min), whereupon reaction ceases. Neither addition of H_2O_2, 30-fold dilution, nor waiting 24 h restores enzyme activity, whereas addition of fresh HRP causes a rapid resumption of oxidation of **1a** for a brief period, followed again by inactivity that can only be reversed by addition of fresh HRP. Furthermore, HRP exposed to **1a** and H_2O_2 for even a few minutes is incapable of oxidizing other standard substrates including *N*,*N*-dimethylaniline and pyrogallol. Thus, compound **1a** is an apparent suicide substrate for HRP (partition ratio ca. 800 turnovers/inactivation). Cyclopropanone hydrate[25] and *trans*-2-phenylcyclopropylamine[12] are also suicide substrates for HRP; however, the action of these compounds and **1a** toward HRP contrasts with that of **1d**, **2a**, and **4**,[7,8] none of which detectably inactivates HRP.

To investigate the role of the ionized carboxyl group in substrates **1a**−**1c** we also submitted esters **3a** and **3b** to oxidation with HRP and monitored product formation by HPLC. The disappearance of **3b** follows zero-order kinetics (45 $\mu M \cdot min^{-1}$) through ≥95% consumption, and the only products are **2b** and methyl glyoxylate (detected as its DNP derivative), both formed in ≥95% yield. In this respect the HRP oxidation of **3b** is analogous to the HRP oxidation of other *N*,*N*-dialkylanilines, with the highly selective loss of the ester side chain attributable to the enhanced acidity of its α-hydrogens. The disappearance of **3a** also follows zero-order kinetics (16 $\mu M \cdot min^{-1}$) through ≥95% consumption, but in contrast to **1a**, **3a** does not detectably inactivate HRP. The products formed from **3a** include *N*-phenylglycine methyl ester (**3d**, trace), aniline (**2d**, 10 mol %, formed by rapid secondary oxidation of **3d**; 146 $\mu M \cdot min^{-1}$), methyl glyoxylate (18 mol %), and quinolinium compound **5** (81 mol %, detected, isolated, and identified by HPLC and FAB-MS). The formation of **5** is analogous to the HRP-induced conversion of *N*-cyclopropyl-*N*-methylaniline (**4**) to 1-methylquinolinium ion.[8] Thus, the relative rates of reaction of substituents on a tertiary aminium ion ($N^{+\bullet}$) center are: decarboxylation of *N*-carboxymethyl > cyclopropyl ring opening > α-deprotonation of $-CH_2COOMe$ group > α-deprotonation of *N*-alkyl group.

In conclusion, we have found that the carboxymethyl group fragments at least an order of magnitude more rapidly than the cyclopropyl group in response to the one-electron oxidation of a directly attached nitrogen center. Since *N*-carboxymethyl groups are synthetically much easier to introduce into amine substrates than are *N*-cyclopropyl groups, this finding should enable a new approach to investigating mechanisms of amine-oxidizing enzymes, notably the cytochrome P450s. The inactivation of HRP by compound **1a**, which contains *both* probe groups, is enigmatic in that it is the only compound among those studied that shows this activity; efforts to elucidate its mechanism of action are currently underway.

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Supporting Information Available: Syntheses and characterizations of compounds **1a**−**c**, **3a**−**c**, and **5** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(24) Incubations were conducted at room temperature under air and contained (in order of addition) 830 μL of potassium phosphate buffer (0.4 M, pH 5.5), 1.0 μmol of substrate (40 μL of a 25 mM solution in MeCN), 3.0 μmol of H_2O_2 (30 μL of 0.1 M) and 78 pmol of HRP (Sigma, RZ = 3.0, added in 100 μL of buffer). To determine rates of substrate disappearance and product formation, 100 μL aliquots of incubation mixture were removed at different times and quenched with 100 μL of MeCN. In some cases 2,4-dinitrophenylhydrazine reagent was also added (see refs 7 and 8). Aliquots of quenched incubation mixtures (20 μL) were injected onto a Vydac C-18 column (5 μm, 4.6 mm × 150 mm) eluted at 1.0 mL/min with the following rapid two-step gradient: 0−5 min, 10−60% solvent B (MeCN) in solvent A (5% MeCN in 50 mM NH_4OAc, pH 7.2); 5−10 min, 60−10% B in A. Peaks were detected at 240 nm and integrated electronically.

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