Oxidative and Nonoxidative Decarboxylation of *N*-Alkyl-*N*-phenylglycines by Horseradish Peroxidase. Mechanistic Switching by Varying Hydrogen Peroxide, Oxygen, and Solvent Deuterium†

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ABSTRACT: Horseradish peroxidase (HRP) typically oxidizes aniline derivatives using hydrogen peroxide as the oxidant. The action of HRP on *N*-alkyl-*N*-phenylglycine derivatives **1b**−**1e** (PhN(R)CH_2COOH; R = Me, Et, *n*-Pr, *i*-Pr, respectively) is highly unusual if not unique. Under standard peroxidatic conditions (HRP/H_2O_2/air), the major product (ca. 70%) is the secondary aniline **2b**−**2e** (PhNHR) resulting from the expected oxidative decarboxylation process, but a significant amount (ca. 30%) of the related tertiary aniline PhN(CH_3)R (**3b**−**3e**) arises from an unexpected nonoxidative decarboxylation process. Under anaerobic, peroxide-free conditions only the tertiary anilines **3b**−**3e** are formed in a reaction that is extremely rapid compared to those in which H_2O_2, molecular oxygen, or both are present. In D_2O buffers, the product is exclusively the monodeutero tertiary aniline PhN(CH_2D)R and the reaction is much slower ($k(H_2O)/k(D_2O) = 5.7$), suggesting that a proton transfer step is substantially rate-limiting in turnover. It is proposed that ferric HRP oxidizes **1** to a cation radical, which then decarboxylates to an α-amino radical having carbanion character on carbon; protonation of the latter, followed by electron capture from ferrous HRP, completes the cycle. Hydrogen peroxide and oxygen slow turnover by diverting ferric HRP toward the compound I/compound II forms or toward compound III, respectively. Finally, under peroxidatic conditions, **1a** (R = cyclopropyl) inactivates HRP with concurrent formation of **2a** but not *N*-phenylglycine, but under anaerobic, peroxide-free conditions, **1a** inactivates HRP almost instantly with no detectable product formation.

Heme peroxidases (oxidoreductase, EC 1.11.1.7) catalyze the oxidation of a variety of organic and inorganic compounds at the expense of hydrogen peroxide. Among plant peroxidases, horseradish peroxidase (HRP)[1] has been the major focus of numerous structural and mechanistic studies due to its unusual stability in aqueous solutions. More specifically, HRP belongs to the family of secretory plant peroxidases (class III) that also contains peanut peroxidase and soybean seed coat peroxidase (*1*). HRP catalyzes the oxidation of a wide range of compounds such as phenols and aromatic amines following the "peroxidatic" catalytic cycle represented by eqs 1−4 (*2*). Rapid transfer of oxygen

$$\text{HRP} + \text{H}_2\text{O}_2 \rightarrow \text{compound I} + \text{H}_2\text{O} \quad (1)$$

$$\text{SH} + \text{compound I} \rightarrow \text{compound II} + \text{SH}^{\bullet+} \quad (2)$$

$$\text{SH} + \text{compound II} + 2\text{H}^+ \rightarrow \text{HRP} + \text{H}_2\text{O} + \text{SH}^{\bullet+} \quad (3)$$

$$\text{SH}^{\bullet+} \rightarrow \text{H}^+ + \text{products} \quad (4)$$

from hydrogen peroxide to ferric heme (a formal two-electron oxidation) initiates the cycle and generates a molecule of water and the reactive intermediate compound I. Extensive kinetic and spectroscopic evidence (*1*) indicate that compound I contains a porphyrin cation-radical/oxoiron(IV) complex. Compound I oxidizes substrates by a single-electron transfer (SET) giving rise to compound II and a substrate-derived radical ($SH^{\bullet+}$). Although a much weaker oxidant than compound I, compound II can also oxidize substrates by SET in returning to the ferric form. The radical products generated during the catalytic cycle can disproportionate, dimerize, or react with other compounds including molecular oxygen.

HRP has long been known to catalyze the decarboxylation of indole-3-acetic acid (IAA) via a reaction that requires molecular oxygen but not hydrogen peroxide (*1*−*3*). Although the mechanism of this reaction is not completely understood, it is apparently initiated by the reduction of ferric HRP to ferrous HRP, which then reacts with molecular oxygen to form compound III (*4*). The latter oxidizes IAA to a radical that decarboxylates and then reacts further with molecular oxygen (*5*, *6*). The peroxide intermediates generated support turnover of HRP, but molecular oxygen is the ultimate oxidant (*5*, *7*−*9*). In this well-known reaction, IAA

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[1] Abbreviations: HRP, horseradish peroxidase; SET, single electron transfer; TBAP, tetrabutylammonium hexafluorophosphate; DNPH, dinitrophenyl hydrazine; GC/EIMS, gas chromatography/electron impact mass spectrometry; Me, methyl; Et, ethyl; *c*-Pr, cyclopropyl; *i*-Pr, isopropyl.

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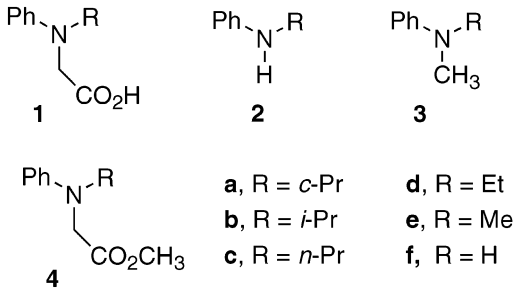
Chart 1



is nearly unique as an HRP substrate. However, it is mentioned here to contrast it to the HRP-catalyzed decarboxylation of *N*-isopropyl-*N*-phenylglycine (**1b**), which we recently reported (*10*). In this latter reaction, the decarboxylation is nonoxidative and neither hydrogen peroxide nor molecular oxygen are required; in fact, these oxidants actually inhibit the reaction. To explain these observations, we proposed a new catalytic cycle for HRP as outlined in eqs 5−8.

$$\text{ferric HRP} + \mathbf{1b} \rightarrow \mathbf{1b}^{\bullet+} + \text{ferrous HRP} \quad (5)$$

$$\mathbf{1b}^{\bullet+} \rightarrow CO_2 + \text{Ph}(i\text{-Pr})\text{NCH}_2^{\bullet} + H^+ \quad (6)$$

$$\text{Ph}(i\text{-Pr})\text{NCH}_2^{\bullet} + \text{ferrous HRP} \rightarrow \text{ferric HRP} + \text{Ph}(i\text{-Pr})\text{NCH}_2^{-} \quad (7)$$

$$\text{Ph}(i\text{-Pr})\text{NCH}_2^{-} + H^+ \rightarrow \text{Ph}(i\text{-Pr})\text{NCH}_3\ (\mathbf{2b}) \quad (8)$$

In the present manuscript, we describe the reactivity and mechanism of a series of *N*-alkyl-*N*-phenylglycines, their methyl esters, and related compounds (Chart 1) and provide a more comprehensive understanding of this novel nonoxidative decarboxylation activity of HRP.

EXPERIMENTAL PROCEDURES

Materials. Horseradish peroxidase (RZ = 2.7−3.4) and hydrogen peroxide (30%) were obtained from Sigma Chemical Co. (St, Louis, MO). Tetrabutylammonium hexafluorophosphate (TBAP) and ferrocene were obtained from Aldrich Fine Chemicals Co. (Milwaukee, WI). Chemicals and solvents were of reagent or HPLC grade and were used as received. Water for chromatography was distilled and passed through a Millipore Milli-Q water system. Analytical thin-layer chromatography (TLC) was carried out using Analtech Uniplate 250 μ silica gel plates with UV light detection. Selecto Scientific 63−200 mesh silica gel was used for flash chromatography. Unless otherwise indicated, all incubations were performed under air at room temperature. *N*-Cyclopropyl-*N*-methylaniline (**3a**), *N*-(2,4-dinitrophenyl)-*N*′-methylene hydrazine (*11*), *N*-cyclopropyl-*N*-phenylglycine (**1a**), *N*-isopropyl-*N*-phenylglycine (**1b**), and quinolinium (**7**) (*10*) were synthesized and fully characterized previously.

Analytical Methods. Absorption spectra were obtained using a Hitachi U-3010 dual beam spectrophotometer. High-pressure liquid chromatography was performed on a Waters system equipped with dual pumps, a 717 autosampler, and a photodiode array detector (PDA 996). Data were collected and analyzed electronically using Millennium software, version 4.0. GC/EIMS data were obtained using a Hewlett-Packard system (HP 5890 series II gas chromatograph, HP 5971A mass selective detector) equipped with a 30 m DB-5 capillary column (5% vinyl methyl silicone, 30 m × 0.25 μm film thickness; J & W Scientific, Folsom, CA). Data were collected in the full spectrum mode (40−350 amu, 0.77 scans/s) using splitless injections (1 μL). NMR spectra were recorded on a 400 MHz Bruker DRX 400 spectrometer, and chemical shifts are reported in ppm (δ) relative to chloroform (7.26 ppm for ^{1}H and 77.23 ppm for ^{13}C). The concentrations of ferric horseradish peroxidase (HRP) solutions were determined using $\epsilon_{402} = 102\ \text{mM}^{-1}\ \text{cm}^{-1}$. Carbonyl metabolites were analyzed by HPLC as their DNP-derivatives (*12*, *13*).

Incubation Conditions. Incubations were performed at room temperature in potassium phosphate buffer (0.4 M, pH 5.5) as described previously (*14*).

Determination of Oxidation Potentials ($E_{1/2}$). Half-wave oxidation potentials ($E_{1/2}$) were measured using linear sweep cyclic voltammetry (CV). Solutions (1 mM) were prepared in dry acetonitrile containing 100 mM TBAP as the supporting electrolyte. Solutions were placed in a one-compartment cell containing a glassy carbon working electrode, an auxiliary platinum electrode, and a saturated calomel electrode. A potential of 0.1−1.8 V was applied at a scan rate of 1.5 V/min across both the working and auxiliary electrodes using a potentiostat (model CV-50W Bio Analytical Systems, West Lafayette, IN) with a PC output. Oxidation potentials were determined from the position of the midpoint of the current wave using the software program CV-50W (ver. 2.0, 1995). Oxidation potentials were measured in quadruplicate and were reproducible within ±20 mV. The oxidation potential of ferrocene (407 ± 3 mV) was recorded daily as a standard.

Preparation of N-Alkyl-N-phenylglycines. *N*-Alkyl-*N*-phenylglycines (**1c**−**e**) were prepared by reductive alkylation of the corresponding *N*-alkylanilines using sodium cyanoborohydride and glyoxylic acid (*14*). *N*-Propyl-*N*-phenylglycine (**1c**) was obtained in 15% yield. Mp: 94−96 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.94 (t, J = 7.4 Hz, 3H), 1.65 (m, J = 7.56 Hz, 2H), 3.32 (t, J = 7.7 Hz, 2H), 4.04 (s, 2H), 6.67 (d, J = 8.1 Hz, 2H), 6.76 (t, J = 7.3 Hz, 1H), 7.23 (t, J = 7.6 Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 11.5, 20.7, 53.1, 54.2, 117.2, 117.9, 129.6, 147.9, 176.8.

N-Ethyl-*N*-phenylglycine (**1d**) was obtained in 18% yield as a colorless viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.23 (t, 3H), 3.47 (q, 2H), 4.11 (s, 2H), 6.75 (m, 2H), 6.79 (m, 1H), 7.22 (m, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 12.4, 46.5, 52.6, 112.9, 118.1, 129.6, 147.5, 176.5.

N-Methyl-*N*-phenylglycine (**1e**) was obtained in 65% yield as a colorless viscous oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.03 (s, 3H), 4.14 (s, 2H), 6.76 (d, 2H), 6.78 (t, 1H), 7.24 (t, 2H). ^{13}C NMR (100.6 MHz, DMSO) δ 54.0, 60.1, 112.5, 117.9, 129.7, 148.5, 176.5.

General Procedure for the Synthesis of Secondary and Tertiary Anilines. The general procedure used is illustrated here in detail for the synthesis of *N*-isopropyl aniline (**2b**). Sodium cyanoborohydride (942 mg, 15 mmol) was added to a stirred solution of Kugelrohr-distilled aniline (**2f**, 665 mg, 5.0 mmol) and acetone (870 mg, 15 mmol) in MeCN (10 mL). Glacial acetic acid (500 μL, 9.0 mmol) was added to the solution over a 15 min period, and the mixture was

stirred at room temperature. After 2 h, glacial acetic acid (300 μL, 5.4 mmol) was again added to maintain the pH of the solution at ~5.0, and the reaction was stirred at room temperature for an additional 2 h. Potassium hydroxide (2 N, 20 mL) was added to stop the reaction, and the mixture was extracted with ether (4 × 10 mL). The ether extracts were pooled, washed with brine (15 mL), dried with Na_2SO_4, and concentrated in vacuo. The crude residue was purified by flash chromatography (5:1 hexanes/ethyl acetate), giving **2b** as a colorless oil (0.567 g, 42%). TLC (4:1 hexanes/ethyl acetate) R_f = 0.77. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (d, J = 6.3 Hz, 6H), 3.67 (sp, J = 6.2 Hz, 1H), 6.62 (d, J = 8.6 Hz, 2H), 6.71 (t, J = 7.3 Hz, 1H), 7.20 (t, J = 7.5 Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 23.2, 44.3, 113.4, 117.1, 129.4, 147.7. GC t_R = 10.0 min. EIMS [M^+] = 135.

N-Isopropyl-*N*-methylaniline (**3b**) was synthesized following the general procedure above from *N*-methylaniline (530 mg, 5.0 mmol) in 47% yield. TLC (4:1 hexanes/ethyl acetate) R_f = 0.72. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.16 (d, J = 6.6 Hz, 6H), 2.72 (s, 3H), 4.09 (sp, J = 6.6 Hz, 1H), 6.69 (d, J = 7.3 Hz, 1H), 6.79 (d, J = 8.0 Hz, 2H), 7.23 (t, J = 8.0 Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 19.5, 29.9, 49.1, 113.5, 116.6, 129.3, 150.4. GC t_R = 11.3 min. EIMS [M^+] = 149.

N-*n*-Propyl-*N*-methylaniline (**3c**) was synthesized from Kugelrohr-distilled *N*-methylaniline (**2e**, 530 mg, 5.0 mmol), propionaldehyde (870 mg, 15 mmol), and sodium cyanoborohydride (942 mg, 15 mmol) as above. The product was obtained as a colorless liquid (0.246 g, 17% yield). TLC (4:1 hexanes/ethyl acetate) R_f = 0.71. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.99 (t, J = 7.4 Hz, 3H), 1.66 (m, J = 7.5 Hz, 2H), 2.99 (s, 3H), 3.33 (t, J = 7.5 Hz, 2H), 6.75 (m, J = 7.4 Hz, 3H), 7.28 (t, J = 8.0 Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 11.9, 20.4, 38.7, 54.9, 112.5, 116.2, 129.6, 149.8. GC t_R = 10.3 min. EIMS [M^+] = 149.

N-Ethyl-*N*-methylaniline (**3d**) was synthesized from Kugelrohr-distilled *N*-ethylaniline (605 mg, 5.0 mmol), formaldehyde (450 mg, 15 mmol), and sodium cyanoborohydride (942 mg, 15 mmol). The product was obtained as a colorless liquid (0.617 g, 51% yield). TLC (4:1 hexanes/ethyl acetate) R_f = 0.66. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.11 (t, J = 7.1 Hz, 3H), 2.89 (s, 3H), 3.39 (q, J = 7.1 Hz, 2H), 6.72 (d, J = 8.6 Hz, 2H), 6.78 (t, J = 7.3 Hz, 1H), 7.22 (t, J = 8.0 Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 11.4, 37.6, 47.0, 112.6, 116.2, 129.4, 149.3.

N-Cyclopropylaniline (**2a**) was synthesized as follows. Cyclopropylamine (12.0 mL, 130 mmol) was added to a solution of *p*-nitrofluorobenzene (9.0 mL, 116 mmol) in a mixture of 1:1 acetonitrile/2-propanol (50 mL) containing potassium carbonate (40 g, 300 mmol). The reaction mixture was refluxed for 48 h. After the mixture cooled, water (50 mL) was added, and the mixture was extracted with ether (3 × 50 mL). The combined ether layers were dried over $MgSO_4$, concentrated in vacuo, and recrystallized from 1:1 hexanes/ethyl acetate to afford *p*-nitro-(**2a**) as a bright yellow crystalline solid (15.3 g, 81% yield). Mp: 126−127 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.61 (m, 2H), 0.87 (m, 2H), 2.55 (m, 1H), 4.99 (br, s, 1H), 6.76 (d, J = 9 Hz, 2H), 8.16 (d, J = 9 Hz, 2H). Anal. Calcd. for $C_9H_{10}N_2O_2$: C, 60.67; H, 5.66; N,15.72. Found: C, 60.71; H, 5.80; N, 15.82.

A mixture of *p*-nitro-**2a** (14.6 g, 82.4 mmol), THF (50 mL), pyridine (8.7 mL, 107 mmol), and 4-(dimethylamino)-pyridine (1.36 g, 10.7 mmol) was stirred under nitrogen in a flask immersed in an ice bath, and acetyl chloride (7.6 mL, 107 mmol) was added slowly. After the mixture was stirred on ice for 30 min and at room temperature for 24 h, 1 N HCl (20 mL) was added, the volatile organic solvents were evaporated under vacuum, and the remaining aqueous phase was extracted with ethyl acetate (3 × 25 mL). The combined organic layers were washed with saturated $NaHCO_3$ solution, dried over $MgSO_4$ and concentrated in vacuo. Recrystallization from ethyl acetate/hexanes afforded *N*-acetyl-*p*-nitro-**2a** as a pale yellow crystalline solid (13.7 g, 76% yield). Mp: 105−106 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.63 (m, 2H), 1.06 (m, 2H), 2.36 (s, 3H), 3.14 (m, 1H), 7.44 (d, J = 9 Hz, 2H), 8.26 (d, J = 9 Hz, 2H). Anal. Calcd. for $C_{11}H_{12}N_2O_3$: C, 59.99; H, 5.49; N,12.72. Found: C, 60.28; H, 5.59; N, 12.56.

N-Acetyl-*p*-nitro-**2a** (13.0 g, 60.0 mmol) in ethyl acetate (100 mL) was hydrogenated at ambient temperature and 45 psi in the presence of 10% Pd/C (1.3 g, 10% w/w) for 1.5 h. The solution was filtered, and the filtrate was dried in vacuo affording *N*-acetyl-*p*-amino-**2a** as a white crystalline solid (11.1 g, 97% yield). Mp: 86−88 °C. ^{1}H NMR (400 MHz, DMSO, 90 °C) δ 0.37 (m, 2H), 0.64 (m, 2H), 1.81 (s, 3H), 3.11 (m, 1H), 4.85 (br, s, 2H), 6.57 (m, 2H), 6.74 (m, 2H). Anal. Calcd. for $C_{11}H_{14}N_2O$: C, 69.45; H, 7.42; N,14.72. Found: C, 69.51; H, 7.35; N, 14.66.

A cold solution of sodium nitrite (0.7 M in water (2 mL)) was added dropwise to a solution of *N*-acetyl-*p*-amino-**2a** (10.1 g, 53 mmol) in 50% hypophosphorous acid (7 mL) at 0 °C. The mixture was stirred for 10 min, and a catalytic amount of cuprous oxide (0.2 g, 1.4 mmol) was added. The mixture was stirred at room temperature for 3 h, after which the solution was made basic by careful addition of a saturated solution of potassium carbonate and extracted with ethyl acetate (3 × 25 mL). The organic layers were combined, dried over $MgSO_4$, filtered, and concentrated. The desired product was isolated by column chromatography on silica gel using 50% ethyl acetate in hexanes to yield a white solid. Recrystallization from ethyl acetate/hexanes afforded *N*-acetyl-**2a** as a white crystalline solid (6.63 g, 72% yield). Mp: 36−38 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.52 (m, 2H), 0.83 (m, 2H), 2.01 (s, 3H), 3.19 (m, 1H), 6.92−7.40 (m, 5H). Anal. Calcd. for $C_{11}H_{13}NO$: C, 75.40; H, 7.48; N, 7.99. Found: C, 75.34; H, 7.79; N, 8.03.

During the acid hydrolysis of *N*-acetyl-**2a**, it is important to exclude air completely to prevent formation of oxidation products (*15*). Thus, *N*-acetyl-**2a** (2.1 g, 12 mmol) was dissolved in 6 M HCl (20 mL) in a 25 mL screw cap culture tube, thoroughly degassed with and sealed under N_2, and stirred at 90 °C for 3 h. Upon cooling, the mixture was neutralized with concentrated Na_2CO_3 solution and extracted with chloroform (3 × 30 mL). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. Kugelrohr distillation (80 °C, 0.6 mmHg) afforded **2a** as a colorless oil (1.3 g, 85% yield). TLC (4:1 hexanes/ethyl acetate) R_f = 0.77. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.48 (m, 2H), 0.69 (m, 2H), 2.39 (m, J = 3.3 Hz, 1H), 4.09 (br, s, 1H), 6.72 (t, J = 7.3 Hz, 2H), 6.77 (d, J = 7.6 Hz, 2H), 7.18 (t, J = 7.9 Hz, 2H). ^{13}C NMR (100.6 MHz, $CDCl_3$) δ 7.57, 25.39, 113.31,

Table 1: Apparent Rate Constants for Oxidation of **1a**−**4f** by HRP under Standard Peroxidatic Conditions[a]

substrate	**1** (μM min^{-1})	**4** (μM min^{-1})	**2** (min^{-1})	**3** (min^{-1})
a, R = c-Pr	22[b]	16	0.053	0.14
b, R= i-Pr	41	45	0.035	0.10
c, R = n-Pr	102		0.48	0.18
d, R = Et	189		0.69	0.27
e, R = Me	489		0.93	0.46
f, R = H	0.02	146		

[a] Initial conditions: 1 mM substrate, 3 mM H_2O_2, 39−78 nM HRP in 0.4 M potassium phosphate buffer, pH 5.5. For individual kinetic runs, $r^2 > 0.98$; rate constants vary <10% day to day. [b] Estimate of initial rate (see text).

117.88, 129.29, 148.85. GC t_R = 9.8 min. EIMS [M^+] = 133.

Synthesis of DNP−Glyoxylic Acid Adduct. Glyoxylic acid (390 mg, 5.2 mmol) was dissolved in 2 mL of H_2O and added dropwise to a solution of DNPH reagent (0.15 M, 10 mL). Almost immediately, orange crystals precipitated from solution. The mixture was stirred for 30 min, after which the orange crystals were filtered, washed with water (500 mL), and dried. The orange solid was recrystallized from ethyl acetate/hexanes to yield an orange crystalline derivative (710 mg, 60% yield). Mp: 190−191 °C. ^{1}H NMR(400 MHz, DMSO) δ 8.01 (s, 1H), 8.02 (d, J = 9.6 Hz, 1H), 8.47 (dd, J = 9.6 Hz, 12.2, 1H), 8.85 (d, J = 2.6 Hz, 1H). ^{13}C NMR (100.6 MHz, DMSO) δ 117.5, 122.6, 129.9, 131.4, 138.8, 138.9, 144.0,164.3. FAB MS [MH^+] = 255.0.

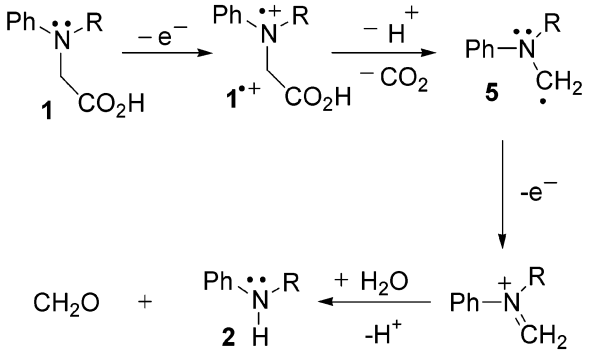
DNP−Methyl Glyoxylate Adduct (DNP-8). This adduct was synthesized from methyl glyoxylate (123 mg, 1.39 mmol; prepared from treating glyoxylic acid with a fresh ethereal solution of diazomethane) and DNPH reagent (0.15 M, 10 mL) following the above procedure. Recrystallization from ethyl acetate/hexanes afforded a crystalline orange derivative (253 mg, 67%). Mp: 183−185 °C. ^{1}H NMR (400 MHz, DMSO) δ 3.70 (s, 3H), 7.95 (d, J = 9.5 Hz, 1H), 8.10 (s, 1H), 8.48 (dd, J = 9.5 Hz, 12.1 Hz, 1H), 8.84 (d, J = 2.65 Hz, 1H). ^{13}C NMR (100.6 MHz, DMSO) δ 52.17, 117.36, 122.57, 130.05, 131.61, 137.45, 139.05, 143.74, 163.11. GC t_R = 24.52 min. EIMS [M^+] = 268.

RESULTS AND DISCUSSION

*Reaction of Compounds (**1a**−**e**) under Peroxidatic Conditions.* Under standard peroxidatic conditions (HRP/H_2O_2/air), HRP catalyzes the decarboxylation of compounds **1a**−**e** (see Chart 1) following zero-order kinetics up to >95% substrate consumption within 30−40 min. The apparent rates of disappearance (k_{app}) in Table 1 reveal that the HRP-catalyzed reaction is sensitive to the steric bulk and degree of branching of the alkyl group directly attached to the amine nitrogen—the smaller the alkyl group, the faster the HRP oxidation of that substrate. It was also observed that the initially colorless incubations become pale yellow within 1−2 min.

Across the entire series of *N*-alkyl-*N*-phenylglycines **1a**−**e**, exposure to HRP/H_2O_2/O_2 leads exclusively to the decarboxylation of these substrates to give two products in essentially quantitative yield. The major product (ca. 70 mol %) is the corresponding secondary aniline (**2**). As shown in Scheme 1, HRP compound I is proposed to abstract an electron to generate **1**$^{\bullet+}$, followed by rapid decarboxylation,

Scheme 1



a second one-electron oxidation, and hydrolysis to release formaldehyde and the secondary aniline **2**. In each case, the minor product, tertiary aniline **3** (ca. 30 mol %), arises from the unusual nonoxidative decarboxylation as described earlier for compound **1b** (*10*). Interestingly, the potential *N*-dealkylation product *N*-phenylglycine (**1f**) was not observed at any time during any of the reactions, although separate controls confirmed that if as little as 5 mol % of **1f** had formed it would easily have been detected. As previously reported (*14*), **1a** uniquely displays suicide substrate activity toward HRP, leading to very rapid and irreversible inactivation. Neither glycine analogues lacking the cyclopropyl group (**1b**−**e**) nor the secondary and tertiary anilines containing the cyclopropyl group (**2a**, **3a**) have such an effect on HRP activity.

Given that secondary and tertiary aniline metabolites generated during HRP oxidations of the glycine derivatives are also potential substrates for HRP, their stability under peroxidatic conditions was examined separately. Both the tertiary and secondary anilines were easily oxidized to completion by HRP/H_2O_2/air within 30−40 min; their apparent rates of disappearance (k_{app}) are given in Table 1. Unlike compounds **1b**−**e**, which are oxidized following zero-order kinetics, the disappearance of the corresponding secondary and tertiary anilines obey first-order kinetics up to >95% substrate consumption. These oxidations are also accompanied by color formation similar to that observed in HRP reactions with the glycine analogues. With the exception of **3a**, the tertiary anilines undergo exclusively *N*-demethylation to the corresponding secondary anilines, instead of *N*-dealkylation at the larger alkyl group. *N*-Cyclopropyl-*N*-methylaniline (**3a**), on the other hand, reacts exclusively by fragmentation of the cyclopropyl ring (*11*).

To identify carbonyl metabolites formed during the decarboxylation of compounds **1a**−**e**, DNPH trapping reagent was added to quenched incubations, which were then analyzed by HPLC. In each case, formaldehyde was the only carbonyl metabolite identified; no trace of glyoxylic acid was detected although control reactions confirmed that if it had formed it could easily have been detected by DNPH trapping. Formaldehyde was generated in a 1:1 ratio to the secondary anilines during the reaction. However, at long reaction times, after complete consumption of both the original glycine derivatives and the tertiary anilines initially formed as metabolites, the mass balance between *N*-alkyl-*N*-phenylglycine consumption and formaldehyde generation was ca. 92−98% for compounds **1b**−**e**. This result further confirms that the *N*-alkyl-*N*-phenylglycines do not react by *N*-

dealkylation but rather by decarboxylation releasing carbon dioxide and formaldehyde.

*Reaction of **1a**−**e** with HRP under Aerobic Peroxide-Free Conditions.* In preliminary studies, aerobic incubations containing potassium phosphate buffer, *N*-alkyl-*N*-phenylglycine analogues, and HRP were initiated by addition of hydrogen peroxide. However, HPLC chromatograms of incubation samples taken prior to the addition of hydrogen peroxide revealed that some formation of both the secondary and tertiary aniline products had already occurred. As a result of this finding, the order of reagents added to incubations was reversed; HRP was added last to initiate the reaction and prevent any interaction of substrates with enzyme without hydrogen peroxide. In addition, separate control reactions exposing compounds **1a**−**e** to HRP in the absence of added hydrogen peroxide were also performed. Surprisingly, results from these latter experiments showed that substantial amounts (60−90%) of substrates **1b**−**e** were still converted to the corresponding secondary and tertiary anilines (ca. 70:30 ratio, respectively) in ca. 20 min.

Reactions omitting hydrogen peroxide differed from those containing added hydrogen peroxide in three respects. First, the glycine derivatives do not undergo complete oxidation; the reactions are very rapid in the first several minutes but then slow and stop completely. Second, the secondary and tertiary aniline products formed during the reaction do not undergo further oxidation; once formed, they are stable. Third, the incubations do not undergo the characteristic color changes noted above; no yellow color is observed during incubations lacking added hydrogen peroxide.

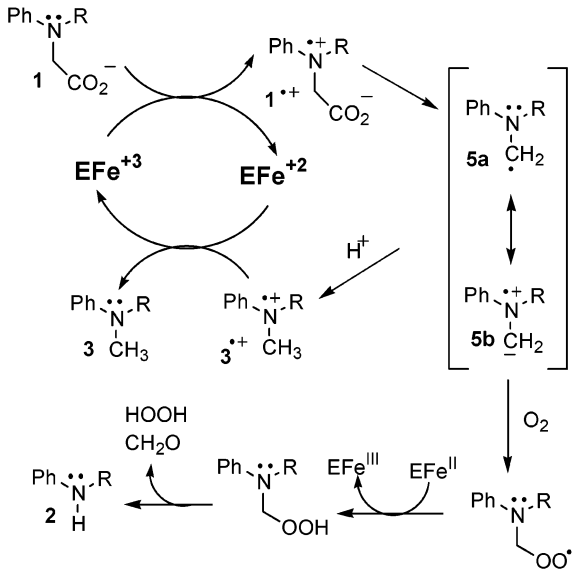
To determine whether enzyme inactivation was responsible for incomplete substrate consumption, aliquots were removed from incubations after consumption of the *N*-alkyl-*N*-phenylglycine derivatives ceased and diluted 10-fold into secondary incubations containing hydrogen peroxide and *N*-*N*-dimethylaniline, a prototypical substrate for HRP. These reactions showed that the HRP retained at least 90% of its original enzymatic activity. Moreover, direct addition of hydrogen peroxide after the originally peroxide-free reactions had stopped reinitiates oxidation leading to the complete consumption of substrates as well as secondary and tertiary aniline metabolites. Supplementing the reaction with hydrogen peroxide also initiates color formation in these initially colorless incubations.

Although aerobic reactions of **1b**−**e** with HRP in the absence of hydrogen peroxide stop prematurely, enzyme activity is not permanently affected. We propose that the decarboxylation of **1b**−**e** in the absence of hydrogen peroxide is initiated by ferric HRP, generating ferrous HRP and $\mathbf{1}^{\bullet+}$ (viz., eq 5 and Scheme 2). The radical cation then rapidly decarboxylates to generate a carbon-centered radical that can be trapped by molecular oxygen to generate a peroxyl intermediate, which may substitute for hydrogen peroxide in the formation of compound I of HRP and thus sustain turnover. Molecular oxygen may also play a role in the premature stopping of these reactions by combining with ferrous HRP ($5.8 \times 10^4\ M^{-1}\ s^{-1}$) (*16*) to generate compound III (eq 9), an inactive form of HRP that decays slowly and

$$\text{ferrous HRP} + O_2 \rightarrow \text{compound III} \qquad (9)$$

spontaneously to native ferric HRP and superoxide with a

Scheme 2



first-order rate constant of $9.0 \times 10^{-3}\ s^{-1}$ (*17*). Addition of hydrogen peroxide may divert the catalytic cycle of eqs 5−8 toward compound I and compound II (eqs 1−4) leading to resumption of the oxidation of both the glycine substrates and the aniline metabolites. Therefore, unlike the secondary and tertiary anilines, the oxidation of which unquestionably requires hydrogen peroxide and thus the compound I form of HRP, ferric HRP alone is a sufficiently strong oxidant to initiate the decarboxylation of compounds **1b**−**e**.

In contrast to results with **1b**−**e**, HRP subjected to **1a** in the absence of hydrogen peroxide loses all enzymatic activity within 10 min if air is present. Dilution into a secondary assay or direct addition of hydrogen peroxide does not reverse the loss of HRP activity.

*Reactions of **1a**−**e** with HRP under Anaerobic, Peroxide-Free Conditions.* To assess the role of molecular oxygen in the reactions of compounds **1a**−**e** with HRP, incubations were performed under anaerobic peroxide-free conditions. As previously observed with compound **1b**, HRP also efficiently catalyzes the quantitative conversions of compounds **1c**−**e** to their respective tertiary anilines extremely rapidly (<1 min). Due to the rapid nature of these reactions, it was necessary to reduce the concentration of HRP 20-fold from 78 nM (in peroxidatic incubations) to 3.9 nM to measure rates of disappearance. Under these modified conditions, the disappearance of **1b** and **1c** follows strict zero-order kinetics through complete (>98%) substrate consumption in less than 5 min (k_{app} = 227 and 489 μM min^{-1}, respectively). These nonoxidative decarboxylation reactions did not occur when heat-denatured HRP or free hemin were added as catalysts, eliminating the possibility that these reactions are catalyzed by anything other than intact, viable HRP.

Under standard peroxidatic conditions, compound **1a** slowly inactivates HRP as it turns over. In contrast, when **1a** is exposed to HRP under anaerobic, peroxide-free conditions, no turnover is observed. However, removal of aliquots to assay remaining HRP activity (under anaerobic, peroxide-free conditions) shows that the enzyme was immediately and irreversibly inactivated despite the absence of detectable turnover. Thus under anaerobic, peroxide-free

Table 2: Solvent Deuterium, Oxygen, and pH Effects on Decarboxylation of **1b** by HRP/H_2O_2[a]

atmosphere	[HRP], nM	k_H/k_D[b]	(k/k')[c]
O_2	78	2.12 ± 0.26	0.99
air	39	3.09 ± 0.44	1.01 ± 0.09
N_2	3.9	2.6 ± 0.35	1.5 ± 0.25

[a] Initial conditions: 1 mM substrate, 3 mM H_2O_2, HRP as noted in 0.4 M potassium phosphate buffer, pH 5.5; results are mean ± SD (n = 3) except where a single value is given. [b] For these experiments, H_2O buffer was lyophilized and reconstituted in an equal volume of D_2O. [c] Ratio of rate constants at pH 5.5/pH 5.9 in H_2O buffer.

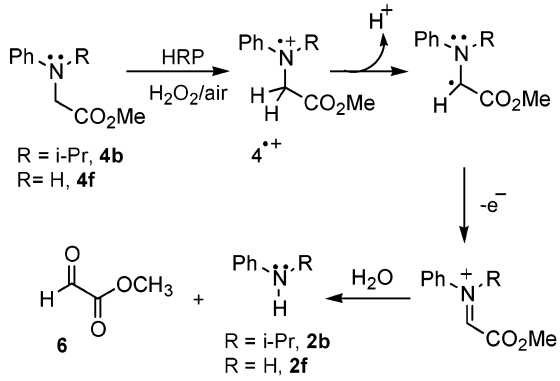
conditions, where ferric HRP is the only oxidant present, **1a** inactivates HRP with an extremely low partition ratio (moles of product formed per mole of HRP inactivated).

In a recent communication (*10*), we presented a novel catalytic cycle that accounts for the quantitative formation of tertiary anilines from *N*-alkyl-*N*-phenylglycine derivatives under anaerobic, peroxide-free conditions. The new catalytic cycle involves the ferric HRP (EFe^{3+}) as the initiating oxidant (Scheme 2). In the absence of hydrogen peroxide and molecular oxygen, HRP is the only oxidant present. Thus it must oxidize **1** to $\mathbf{1}^{\bullet+}$ while forming ferrous HRP (EFe^{2+}). Rapid decarboxylation of $\mathbf{1}^{\bullet+}$ generates a carbon radical intermediate the resonance form of which has carbanion character on its methyl carbon. Protonation of **4** generates $\mathbf{3}^{\bullet+}$, which must then reoxidize ferrous HRP generating **3** and ferric HRP. In support of this mechanism, molecular ions of anilines **3b**−**e** formed during reactions conducted in D_2O buffers exhibit a 1 amu increase compared to molecular ions of **3b**−**e** generated in H_2O buffers, while recovered starting material remains deuterium-free. This result confirms that one and only one proton in the tertiary aniline metabolites formed in the nonoxidative decarboxylation pathway is derived from solvent, as suggested in Scheme 2.

*Solvent Kinetic Isotope Effects on the Reaction of **1b** with HRP*. The rate of decarboxylation of **1b** was measured under peroxidatic conditions (HRP/H_2O_2/air) in D_2O vs H_2O buffer. Phosphate buffer (pH 5.5) made up in H_2O was lyophilized and reconstituted with D_2O. Since D_2O increases the pK_a of monoprotic acids by ca. 0.4 units, parallel experiments in H_2O buffer solutions at pH 5.9 were also performed to account for any pH influence on the apparent rates of disappearance. Table 2 shows the pH and solvent kinetic isotope effects on reactions with constant substrate (1.0 mM), HRP (78 nM), and hydrogen peroxide (3.0 mM) concentrations but varying oxygen concentrations. Regardless of the oxygen concentration, reactions of **1b** with HRP are considerably slower in D_2O buffers compared with H_2O. This difference indicates that there is a solvent isotope effect on a rate-limiting step in turnover. The product ratio is markedly affected as well (*10*). In reactions in which both **2b** and **3b** are generated (HRP/H_2O_2/air), the product ratio (**2b**/**3b**) changes from 70:30 in H_2O buffers to 85:15 in D_2O buffers. The decrease in yield of **3b** relative to **2b** is consistent with a partial rate limitation by an obligatory protonation step in the formation of **3b** but not **2b** (see Scheme 2).

Differences in the rates of **1b** disappearance under anaerobic, peroxide-free conditions in H_2O vs D_2O were even more pronounced (156 vs 28.6 μM^{-1} min^{-1}, respectively) resulting in an apparent solvent isotope effect k_H/k_D of 5.7. The large magnitude of this effect suggests that the protonation step in Scheme 2 is substantially rate-limiting in turnover. The lower isotope effects observed in the presence of hydrogen peroxide suggest that other solvent-independent steps may also be partially rate-limiting in turnover, and in fact, hydrogen peroxide significantly retards turnover of **1b**−**e** (*10*).

Scheme 3



*Oxidation of Methyl Esters of **4a**, **4b**, and **4f** with HRP*. To evaluate the role of the ionized carboxyl group in substrates **1a**−**e**, the methyl esters **4a**, **4b**, and **4f** were synthesized (*14*) and submitted to HRP oxidation. Under peroxidatic conditions, the methyl esters were oxidized by HRP with apparent zero-order rate constants of 16, 45, and 146 μM^{-1} min^{-1}, respectively. Both **4b** and **4f** reacted exclusively via loss of the ester side chain to generate **2b** and aniline, respectively, suggesting that after formation of the aminium ion, the most acidic α hydrogen is lost generating a carbon radical on the ester side chain. A second one-electron oxidation followed by hydrolysis releases the ester side chain as methyl glyoxylate (**6** in Scheme 3), the formation of which was confirmed by DNPH trapping of quenched reactions, analysis by HPLC, and GC/EIMS in comparison to a synthetic standard.

The mode of reactivity of the cyclopropyl-containing ester **4a** was quite different from **4b** and **4f**. The products generated from oxidation of **4a** with HRP under peroxidatic conditions include **4f** (trace), aniline (**2f**, 10 mol %), methyl glyoxylate (**6**; 18 mol %) and quinolinum compound **7** (81 mol %), identified by HPLC and FAB-MS comparison to a synthetic standard (*10*). In the absence of a reactive carboxylate group to serve as an electrofuge, the cyclopropyl group responds to the formation of the aminium ion by undergoing a carbon−carbon bond fragmentation generating a distonic cation radical (Scheme 4). Intramolecular cyclization of the distonic radical onto the aromatic ring followed by deprotonation and further oxidation generates the quinolinium species **7**. In a competing pathway, the distonic cation radical may react with molecular oxygen and liberate a three-carbon moiety to generate **4f**, which in turn is rapidly oxidized by HRP to **2f** and methyl glyoxylate (**6**, Scheme 3). Using ^{13}C NMR and specifically enriched substrates, Shaffer et al. (*11*) identified the three-carbon moiety derived from the cyclopropyl group of a related compound (**3a**) during oxidation by HRP as 3-hydroxypropionic acid. Considering the similarity in metabolites generated from **4a** and **3a** with HRP, 3-hydroxypropionic acid is also likely to be the ultimate fate of the three-carbon moiety released in conversion of **4a** to **4f**, although this was not specifically investigated in this study.

Scheme 4

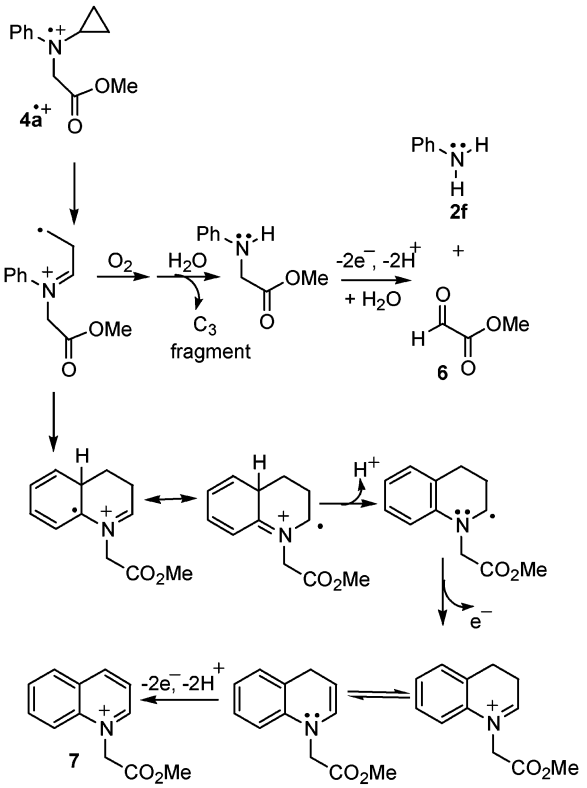


Table 3: Effect of Oxygen on Oxidation of **4a** by HRP[a]

	product yield (mol %)		
atmosphere	**2f**	**6**	**7**
O_2	32	70	30
air	15	21	80
N_2	0	0	100

[a] Initial conditions: 1 mM substrate, 3 mM H_2O_2, HRP 39−78 nM in 0.4 M potassium phosphate buffer, pH 5.5.

The oxygen-sensitive step in Scheme 4 was further investigated by oxidizing **4a** with HRP under varying atmospheric oxygen concentrations. Table 3 shows that in the absence of molecular oxygen to trap the carbon radical generated after the fragmentation of the cyclopropyl ring, quinolinium compound **7** becomes the only metabolite formed (i.e., 100% yield, 100% conversion), while under 100% oxygen atmosphere methyl glyoxylate becomes the major metabolite (70 mol %), the balance being converted to **7**.

Thus, when the reactivity of the carboxylate group of **1** is blocked by methylation, the compound responds to the formation of the aminium ion via reaction of the most sensitive group remaining in the molecule, that is, fragmentation of the cyclopropyl group if present or deprotonation at the most acidic site, C-α of the ester side chain. It is important to note that methyl esters **4a**, **4b**, and **4f** do not react with HRP if hydrogen peroxide is not added to the incubation. In this regard, the *N*-alkyl-*N*-phenylglycine esters **4** behave like ordinary tertiary aniline derivatives, which are substrates only for the active intermediates of HRP (i.e., compounds I and II) generated during the peroxidatic cycle of eqs 1−4; they do not react with ferric HRP alone.

Oxidation Potentials of Glycine Derivatives and Their Metabolites. Since compound I of HRP is an SET oxidant, differences in the oxidation potentials of substrates will determine their susceptibility to HRP oxidation. For example, HRP readily oxidizes *N*,*N*-dimethylaniline ($E_{1/2}$ = 0.84 ± 0.01 V) but does not oxidize a related compound *p*-nitro-*N*,*N*-dimethylaniline ($E_{1/2}$ = 1.28 ± 0.02 V) (*18*). Therefore, the $E_{1/2}$ values for the compounds in Chart 1 were measured using cyclic voltammetry. In all cases, the electrochemical oxidations observed were irreversible, even at scan speeds as high as 10 000 V/min. A list of the $E_{1/2}$ values obtained is presented in Table 4.

Table 4: Apparent Oxidation Potentials of Aniline Derivatives **1a**−**4f**[a]

series	**1**	**2**	**3**	**4**
a, R = c-Pr	1093 ± 4	884 ± 6	901 ± 17	1072 ± 14
	1264 ± 8	1253 ± 8	1125 ± 19	1254 ± 11
b, R= i-Pr	985 ± 7	879 ± 8	824 ± 4	984 ± 5
c, R = n-Pr	982 ± 15	887 ± 13	824 ± 1	984 ± 5
d, R = Et	967 ± 10	893 ± 4	821 ± 3	
e, R = Me	990 ± 9	890 ± 18	843 ± 10	
f, R = H	1029 ± 17	1021 ± 21		

[a] $E_{1/2}$, mV vs SCE; mean ± SD, n = 3.

The data of Table 4 show several interesting features. First, the $E_{1/2}$ values for the tertiary *N*-cyclopropylanilines **1a**, **3a**, and **4a** are 77−108 mV more positive than their corresponding isopropyl analogues **1b**, **3b**, and **4b**. This is paralleled by the generally lower pK_a values of cyclopropyl- vs isopropylamines (*19*). Thus, while cyclopropyl substituents can be electron-donating via resonance, they can also be electron-withdrawing inductively by virtue of the increased *s* character of cyclopropane carbons compared to ordinary aliphatic carbons. The second interesting feature is that only the *N*-cyclopropyl derivatives show two distinct oxidation waves. The first wave undoubtedly represents generation of the aminium ion, which then undergoes rapid irreversible ring-opening to a primary carbon radical (Scheme 4). If oxidation of this primary radical to a primary carbenium ion were more difficult than the first oxidation, it would account for the appearance of the second wave at higher potential. With the other substrates, oxidation of the α-radical, generated by deprotonation or decarboxylation of the aminium ion (viz., **5** in Scheme 1) to form an iminium ion should be quite facile; hence only a single oxidation wave is seen. Finally, the oxidation potentials of the secondary anilines **2** are 50−70 mV higher than those of the tertiary anilines **3**, reflecting the inductive effect of the additional alkyl group compared to a hydrogen atom.

Within each class of substrate (aniline or glycine) differences in oxidation potential between the *N*-methyl and the *N*-isopropyl analogues are negligible and thus cannot explain the preference HRP has for smaller *N*-alkyl substituents as evident in the k_{app} rates in Table 1. Unlike cytochrome P450 enzymes, in which the apoprotein dictates the size and shape of the binding pocket, thereby controlling the binding and interaction of substrates with the reactive iron-oxo species (*20*), the heme in HRP is hindered on the distal side by protein residues, which prevents approach of organic substrates to the oxo-iron group of compound I (*21*). Thus, oxidation by HRP is thought to involve electron transfer upon approach of substrates from solution to the exposed edge of the heme porphyrin. The crystal structure of HRP (*21*) also reveals three peripheral phenylalanine residues (Phe142, 68,

and 179) surrounding the exposed heme edge. This may explain why HRP displays selectivity toward substrates having smaller *N*-alkyl substituents among a series of compounds having comparable $E_{1/2}$ values.

In summary, the decarboxylation of *N*-alkyl-*N*-phenylglycines catalyzed by HRP is a very fast and efficient process that requires neither the addition of hydrogen peroxide nor the presence of molecular oxygen. On the contrary, hydrogen peroxide and oxygen actually *impede* the rate of reaction (*10*). pH effects and solvent kinetic deuterium isotope effects for anaerobic, peroxide-free reactions indicate that proton transfer from solvent is a substantially rate-limiting step, whereas in the presence of hydrogen peroxide, multiple steps, some solvent-independent, limit the rate of the reaction. Among all the compounds studied, only **1a** caused irreversible inhibition of HRP, and although some turnover occurs under the normal peroxidatic conditions, no substrate disappearance or appearance of products occurs under anaerobic, peroxide-free conditions, indicating a very small partition ratio and efficient enzyme inactivation. The mechanism for this inactivation is currently under investigation.

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