J|A|C|S
COMMUNICATIONS

Published on Web 08/01/2002

Non-Oxidative Decarboxylation of Glycine Derivatives by a Peroxidase

Rheem A. Totah and Robert P. Hanzlik*

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045-7582

Received April 19, 2002

Heme-containing peroxidases are widely distributed in nature and serve an equally wide variety of biological roles, including biosynthesis of prostaglandins,[1,2] activation of anti-tubercular drugs,[3] and degradation of compost and wood.[4] One of the best studied and most readily available peroxidases is horseradish peroxidase (HRP).[5,6] Its crystal structure[7] shows that each protein monomer contains a single heme, the iron of which is ligated from the distal side by a histidine nitrogen. On the proximal side are several amino acids that catalyze the reaction of the ferric enzyme with H_2O_2 to form water and a two-electron-oxidized form of the enzyme (compound I) containing a porphyrin cation radical/oxoiron(IV) complex (eq 1). Compound I, a moderately strong oxidant, oxidizes substrates by single-electron transfer (SET), giving compound II and a substrate-derived radical ($SH^{+\bullet}$ in eq 2). Compound II is a less potent oxidant but also oxidizes substrates by SET in returning to the ferric form (eq 3). The initially formed radical products undergo follow-up reactions such as disproportionation or reaction with molecular oxygen en route to forming stable products (eq 4).

Cytochrome P450 heme thiolate monooxygenases are also

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

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

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

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

thought to generate a compound I-like intermediate[8] which oxidizes most substrates by transferring an oxygen atom to them rather than by abstracting an electron from them. One possible exception is the P450-catalyzed *N*-dealkylation of amines, where carbinolamine formation has been suggested to be initiated by an SET event.[9−11] In the course of our studies of *N*-dealkylation mechanisms, HRP has been useful as an unambiguous model for exploring the SET reactivity of radical-clock-type substrates.[12,13] For example, we recently found that, upon SET oxidation by HRP, *N*-cyclopropyl-*N*-phenylglycine undergoes oxidative decarboxylation *faster* than cyclopropyl ring-opening.[14] In extending this work, we made the unprecedented observation that, in addition to catalyzing the oxidative decarboxylation of *N*-alkyl-*N*-phenylglycines, HRP also catalyzes their *non-oxidative* decarboxylation under *anaerobic, peroxide-free* conditions. In this Communication, we describe our observations in this area and propose a novel redox cycle for HRP to explain them.

The reactions of *N*-isopropyl-*N*-phenylglycine (NIPG, **1**) described below are completely typical of its *N*-methyl, *N*-ethyl, and *N*-(*n*-propyl) congeners, except that the latter are oxidized somewhat faster overall. Under typical *peroxidatic* conditions,[15] NIPG oxidation follows strict zero-order kinetics through >95% consumption in ca. 20 min (Table 1, line 1). *N*-Isopropylaniline (NIA, **2**) is formed in 70−75% yield, but an additional product, subsequently shown[16] to be *N*-isopropyl-*N*-methylaniline (NINMA, **3**), is also formed in 25−30% yield. Compared to the rate of NIPG consumption, the oxidation of NINMA to NIA is relatively slow and follows first-order kinetics through >95% consumption in 40 min (k_{app} = 0.1 min^{-1}); *N*-methylaniline and acetone are not formed. Furthermore, if NIPG is present, NINMA is not oxidized until NIPG has been consumed. At long reaction times, the yield of formaldehyde[17] is 93−95% of theory (eq 5); glyoxylic acid is not detected, although if added it is stable and detectable in reaction mixtures.

$$\mathbf{1} + \text{H}_2\text{O} \rightarrow 2\text{e}^- + 2\text{H}^+ + \text{Ph(iPr)NH} + \text{CO}_2 + \text{CH}_2\text{O} \quad (5)$$

$$\mathbf{1} \rightarrow \text{Ph(iPr)NCH}_3 + \text{CO}_2 \quad (6)$$

The formation of NINMA, a product of *non-oxidative* decarboxylation of NIPG (eq 6), was completely unexpected. To investigate its formation, we kept peroxide, HRP, and NIPG concentrations constant but varied the pH and oxygen availability. Decreasing the pH from 5.5 to 5.0 accelerates turnover and increases the ratio of non-oxidative vs oxidative product (i.e., **3**:**2**), while increasing the pH from 5.5 to 7.0 has exactly the opposite effect (Table 1, lines 1−3). Surprisingly, excluding air *dramatically* accelerates turnover and *completely* eliminates the oxidative pathway, despite the presence of an ample excess of hydrogen peroxide (line 4). Conversely, replacing air with a 100% oxygen atmosphere retards turnover and completely prevents NINMA formation (line 5).

When H_2O is replaced by D_2O, GC/MS examination of the NINMA produced reveals the incorporation of one and only one atom of deuterium specifically into its methyl group; neither unreacted NIPG nor NIA product is found to contain any deuterium.

If HRP is omitted, no reaction takes place. Likewise, heat-denatured HRP and free hemin are completely inactive as catalysts. On the other hand, omitting hydrogen peroxide has little effect on either turnover rate or product ratio (line 6 vs line 1). To examine this further, the experiments reported in lines 7−10 were performed with no hydrogen peroxide added. As in the experiments with hydrogen peroxide added, replacing air with oxygen retards the reaction slightly but completely stops NINMA formation (lines 1 vs 5; 6 vs 7), while replacing air with nitrogen stops NIA formation and accelerates turnover markedly (cf. lines 1 vs 4; 6 vs 8). Finally, while changing pH has a moderate effect on both turnover rate and product ratio under standard peroxidatic conditions (lines 1−3), under anaerobic peroxide-free conditions, where only one product is formed, changing pH has a more dramatic effect on turnover rate (lines 8−10).

To account for these observations, we propose the mechanisms of Scheme 1, which incorporates two unprecedented steps. The first

* Address correspondence to this author. E-mail: rhanzik@ukans.edu.

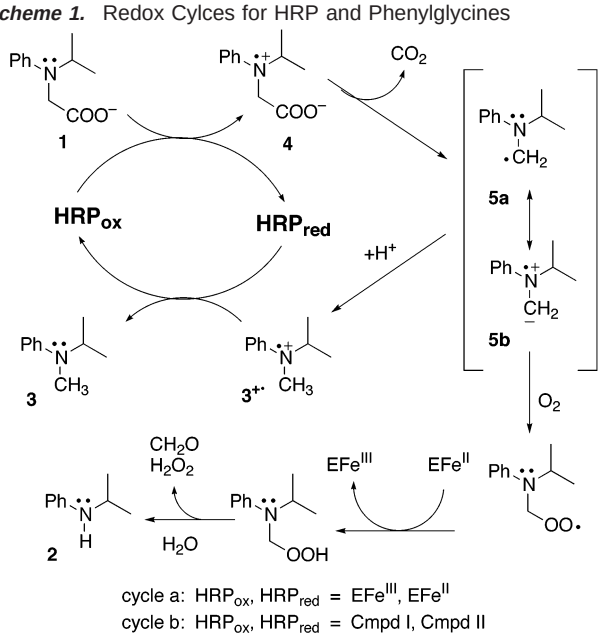
10.1021/ja020559u CCC: $22.00

Table 1. Effect of pH and Oxygen Partial Pressure on Turnover and Product Ratio

run no.	atmosphere	$[H_2O_2]$, mM	pH	TN,[a] min^{-1}	product ratio (3:2)
1[b]	air	3	5.5	52	30:70
2[b]	air	3	5.0	73	40:60
3[b]	air	3	7.0	23	10:90
4[c]	N_2	3	5.5	1190	100:0
5[b]	O_2	3	5.5	15	0:100
6[b]	air	0	5.5	40	30:70
7[b]	O_2	0	5.5	21	0:100
8[d]	N_2	0	5.5	3620	100:0
9[d]	N_2	0	7.0	487	100:0
10[d]	N_2	0	4.0	6440	100:0
11[c]	N_2	15	5.5	48	100:0

[a] Turnover number (moles of product per mole of HRP per minute). In all cases, [NIPG] is 1 mM and buffer is 0.4 M potassium phosphate. [b] [HRP] = 78 nM. [c] [HRP] = 39 nM. [d] [HRP] = 3.9 nM. HRP concentrations were determined spectrophotometrically using the extinction coeficient 102 mM^{-1} cm^{-1} at 402 nm (ref 6).

Scheme 1. Redox Cylces for HRP and Phenylglycines



is the SET oxidation of the anionic conjugate base of NIPG by ferric HRP (EFe^{III} in cycle a). Facile decarboxylation[14,18,19] of the resulting radical zwitterion **4** generates the neutral radical **5a**, whose radical ylide resonance form (**5b**) has carbanion character on its methyl carbon. Protonation of the latter, the second novel step in Scheme 1, accounts for both the pH-sensitive branching in product formation and the selective incorporation of a single deuterium from solvent to NINMA. However, C-protonation of **5b** generates $\mathbf{3}^{+\bullet}$ rather than **3** itself. Since **3** is a good substrate for HRP/H_2O_2, but is totally inert to HRP in the absence of peroxide (with or without oxygen present), it is reasonable to propose that $\mathbf{3}^{+\bullet}$ could reoxidize ferrous HRP (EFe^{II}), forming ferric HRP and NINMA to complete the cycle. Reaction of **5a** with oxygen accounts for the oxygen-sensitive branching in product formation. The rate retardation caused by oxygen may be due to the conversion of ferrous HRP to compound III, an inactive but reversibly formed derivative of HRP.[20] Finally, H_2O_2 itself inhibits turnover of NIPG by HRP (cf. lines 4, 8, and 11). We attribute this effect to the conversion of enzyme to forms other than EFe^{III}/EFe^{II} that are also capable of oxidizing **1** to **4** to initiate decarboxylation. Since reduction of compound I to compound II is reversible,[21] it is possible to envision a second catalytic cycle (Scheme 1, cycle b). Since EFe^{II} should be a much stronger reducing agent than compound II, the conversion of $\mathbf{3}^{+\bullet}$ to **3** could be rate limiting in cycle b (H_2O_2 present), whereas oxidation of **1** by ferric HRP could be the rate-limiting step in cycle a (H_2O_2 absent).

Acknowledgment. We thank the NIH (Predoctoral Training Grant GM-08545) and the American Foundation for Pharmaceutical Education for fellowship support for R.A.T.

References

(1) Smith, W. L.; Garavito, R. M.; DeWitt, D. L. *J. Biol. Chem.* **1996**, *271*, 33157−33160.
(2) Smith, W. L.; Marnett, L. J. *Biochim. Biophys. Acta* **1991**, *1083*, 1−17.
(3) Wengenack, N. L.; Hoard, H. M.; Rusnak, F. *J. Am. Chem. Soc.* **1999**, *121*, 9748−9749.
(4) Kalyanaraman, B. *Xenobiotica* **1995**, *25*, 667−675.
(5) O'Brien, P. J. *Chem.-Biol. Interact.* **2000**, *129*, 113−139.
(6) Dunford, H. B. *Heme Peroxidases*; Wiley-VCH: New York, 1999.
(7) Gajhede, M.; Schuller, D. J.; Henriksen, A.; Smith, A. T.; Poulos, T. L. *Nat. Struct. Biol.* **1997**, *4*, 1032−1038.
(8) Kellner, D. G.; Hung, S.-C.; Weiss, K. E.; Sligar, S. C. *J. Biol. Chem.* **2002**, *277*, 9641−9644.
(9) Guengerich, F. P.; Yun, C. H.; Macdonald, T. L. *J. Biol. Chem.* **1996**, *271*, 27321−27239.
(10) Okazaki, O.; Guengerich, F. P. *J. Biol. Chem.* **1993**, *268*, 1546−1552.
(11) Zhao, A.; Mabic, S.; Kuttab, S.; Franot, C.; Castagnoli, K.; Castagnoli, N., Jr. *Bioorg. Med. Chem.* **1998**, *6*, 2531−2539.
(12) Chen, H.; deGroot, M. J.; Vermeulen, N. P. E.; Hanzlik, R. P. *J. Org. Chem.* **1997**, *62*, 8227−8230.
(13) Shaffer, C. L.; Morton, M. D.; Hanzlik, R. P. *J. Am. Chem. Soc.* **2001**, *123*, 8502−8508.
(14) Totah, R. A.; Hanzlik, R. P. *J. Am. Chem. Soc.* **2001**, *123*, 10107−10108.
(15) 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. Aliquots of quenched incubation mixtures (20 μL) were injected onto a Vydac C-18 column (5 μm, 4.6 mm × 150 mm) and 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.
(16) NINMA was synthesized (from *N*-methylaniline and acetone via Borch reduction) and fully characterized. The HPLC and GC retention times and MS fragmentation patterns of NINMA standard matched exactly those of the additional product in NIPG reactions with HRP.
(17) DNP reagent (0.15 M 2,4-dinitrophenylhydrazine dissolved in a mixture of concentrated H_2SO_4/water/absolute ethanol, 3:4:14, v/v/v) was extracted thoroughly with hexanes (to remove numerous interfering contaminants) immediately before use. After quenching a 1.0 mL incubation with 1.0 mL of MeCN, hexanes-extracted DNP reagent (15 μL) was added. The mixture was stirred at room temperature for 30 min and analyzed by HPLC. Standard solutions of formaldehyde and glyoxylic acid were prepared and analyzed similarly.
(18) Bonifacic, M.; Stefanic, I.; Hug, G. L.; Armstrong, D. A.; Asmus, K.-D. *J. Am. Chem. Soc.* **1998**, *120*, 9930−9940.
(19) Höbel, B.; von Sonntag, C. *J. Chem. Soc., Perkin Trans. 2* **1998**, 509−513.
(20) Metodiewa, D.; Dunford, H. B. *Arch. Biochem. Biophys.* **1989**, *272*, 245−253.
(21) Hayashi, Y.; Yamazaki, I. *J. Biol. Chem.* **1979**, *254*, 9101−9106.
(22) Gazaryan, I. G.; Lagrimini, L. M.; Ashby, G. A.; Thorneley, R. N. F. *Biochem. J.* **1996**, *313*, 841−847.
(23) Krylov, S. N.; Dunford, H. B. *Biophys. Chem.* **1996**, *58*, 325−334.
(24) Candeias, L. P.; Folkes, L. K.; Dennis, M. F.; Patel, K. B.; Everett, S. A. *J. Phys. Chem.* **1994**, *98*, 10131−10137.

JA020559U