Communications to the Editor

Transient Heme N-Alkylation of Chloroperoxidase by Terminal Alkenes and Alkynes

Annette F. Dexter and Lowell P. Hager*

Department of Biochemistry
University of Illinois, Urbana, Illinois 61801

Received October 7, 1994

Chloroperoxidase (CPO) from the mold *Caldariomyces fumago* is a heme enzyme possessing a broad range of oxidative activities, spanning those characteristic of heme peroxidases (one- and two-electron peroxidations; organic halogenation[1a]), catalase (dismutation of hydrogen peroxide, oxidation of simple alcohols[1]), and cytochrome P-450 (heteroatom dealkylation,[2] oxygen transfer to alkenes,[3] sulfides,[4] and arylamines[5]). Extensive spectral evidence indicates that CPO shares cysteine thiolate heme ligation with the cytochrome P-450 monooxygenase family,[6] while possessing a polar active site milieu similar to those of the heme peroxidases and catalase.[7] Functionally and structurally, it thus occupies a bridging position between cytochrome P-450 and the peroxidase class of heme enzymes. We here report a novel P-450 type reaction of CPO: suicidal formation of *N*-alkylporphyrins during the oxidation of terminal alkenes and alkynes. The inactivated CPO formed in these reactions undergoes spontaneous loss of the heme alkyl moiety with partial restoration of enzymatic activity. The corresponding dealkylation reaction has not been reported for cytochrome P-450.

In the presence of hydrogen peroxide and allylbenzene,[8] native CPO was rapidly and quantitatively converted to a green species which lacked enzymatic activity and exhibited visible absorption maxima at 313 (sh), 419, 549 (sh), 607, and 648 nm (Figure 1a). Very similar spectra were observed when allylbenzene was replaced with 4-pentenoic acid, allyl bromide, or allyl alcohol, suggesting that the terminal unsaturated bond

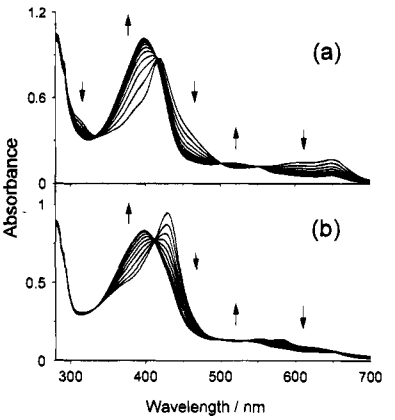

Figure 1. Absorption spectra of CPO after treatment with unsaturated substrates. (a) Spectral changes in CPO following inactivation with allylbenzene. Spectra were recorded at 2 h intervals over 0–24 h after the inactivation reaction. (b) Spectral changes in CPO following inactivation with 1-hexyne. Spectra were recorded at 3 h intervals over 0–36 h after the inactivation reaction. The final spectra in each case are similar but not identical to those of native chloroperoxidase.

mediated the inactivation. The reaction of CPO with allylbenzene was accompanied by formation of free allylbenzene oxide (80 equiv, 35% ee); however, the enzyme was not inactivated by stirring with epoxide alone. On standing, the allylbenzene-modified species gradually changed to a second species with absorption maxima at 398, 514, 547 (sh), 589 (sh), and 649 nm, similar to those of native CPO (Figure 1a). In parallel with the spectral changes, a first-order increase in enzymatic chlorination activity was observed, to a maximum of 80% of the original activity. At 25 °C, the half-life for both the spectral changes and the restoration of activity was 5.8 h. The reactivation half-life was strongly sensitive to temperature, but was not sensitive to pH in the range (pH 4.5–6.5) in which the enzyme is stable. CPO modified with allylbenzene could be processed through multiple cycles of inactivation and reactivation with a similar half-life and recovery of activity on each cycle.

Suicide inactivation of microsomal cytochrome P-450 during the oxidation of terminal alkenes and alkynes has been studied extensively by other workers[10,11] and typically results in N-alkylation of the prosthetic heme by addition of the unsaturated substrate plus an oxygen atom. We thus investigated the possible formation of heme adducts in inactivated CPO, using electrospray mass spectrometric analysis of the whole protein.[12] In mass spectral analyses performed immediately following the reaction of CPO with allylbenzene, heme b (*m/z* 616) was not observed. Instead, a new species was present consistent with addition of allylbenzene plus an oxygen atom to the heme (*m/z*

(1) (a) Thomas, J. A.; Morris, D. R.; Hager, L. P. *J. Biol. Chem.* **1970**, *245*, 3135–3142. (b) Geigert, J.; Dalietos, D. J.; Neidleman, S. L.; Lee, T. D.; Wadsworth, J. *Biochem. Biophys. Res. Commun.* **1983**, *114*, 1101–1108.

(2) (a) Kedderis, G. L.; Koop, D. R.; Hollenberg, P. F. *J. Biol. Chem.* **1980**, *255*, 10174–10182. (b) Kedderis, G. L.; Hollenberg, P. F. *J. Biol. Chem.* **1984**, *259*, 3663–3668.

(3) (a) Geigert, J.; Lee, T. D.; Dalietos, D. J.; Hirano, D. S.; Neidleman, S. L. *Biochem. Biophys. Res. Commun.* **1986**, *136*, 778–782. (b) Allain, E. J.; Hager, L. P.; Deng, L.; Jacobsen, E. N. *J. Am. Chem. Soc.* **1993**, *115*, 4415–4416. (c) Colonna, S.; Gaggero, N.; Casella, L.; Carrea, G.; Pasta, P. *Tetrahedron: Asymmetry* **1993**, *4*, 1325–1330.

(4) (a) Kobayashi, S.; Nakano, M.; Goto, T.; Kimura, T.; Schaap, A. P. *Biochem. Biophys. Res. Commun.* **1986**, *135*, 166–171. (b) Colonna, S.; Gaggero, N.; Manfredi, A.; Casella, L.; Gullotti, M.; Carrea, G.; Pasta, P. *Biochemistry* **1990**, *29*, 10465–10468. (c) Colonna, S.; Gaggero, N.; Casella, L.; Carrea, G.; Pasta, P. *Tetrahedron: Asymmetry* **1992**, *3*, 95–106.

(5) (a) Kedderis, G. L.; Rickert, D. E.; Pandey, R. N.; Hollenberg, P. F. *J. Biol. Chem.* **1986**, *261*, 15910–15914. (b) Doerge, D. R.; Corbett, M. D. *Chem. Res. Toxicol.* **1991**, *4*, 556–560.

(6) (a) Dawson, J. H.; Sono, M. *Chem. Rev.* **1987**, *87*, 1255–1276. (b) Sono, M.; Hager, L. P.; Dawson, J. H. *Biochim. Biophys. Acta* **1991**, *1078*, 351–359.

(7) (a) Dugad, L. B.; Wang, X.; Wang, C.-C.; Lukat, G. S.; Goff, H. M. *Biochemistry* **1992**, *31*, 1651–1655. (b) Hu, S.; Kincaid, J. R. *J. Biol. Chem.* **1993**, *268*, 6189–6193.

(8) Allylbenzene- or 1-hexyne-modified chloroperoxidase was prepared by slow (0.01 mL/min) addition of 2M H_2O_2 to a rapidly stirred suspension (5 mL) of CPO (0.6 mg/mL) and allylbenzene or 1-hexyne (0.1% w/v) in 20 mM sodium acetate, pH 5.2. For electrospray MS analyses, reactions were carried out in distilled water. Inactivation was generally complete within 60 s. Concentrations of native CPO were determined using an extinction coefficient of 91 200 M^{-1} cm^{-1} at 400 nm. Enzyme activity was determined using the MCD chlorination assay.[9]

(9) Morris, D. R.; Hager, L. P. *J. Biol. Chem.* **1966**, *241*, 1763–1777.

(10) *Cytochrome P-450. Structure, Mechanism, and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1986; Chapter 8, pp 273–314.

(11) Lavallee, D. K. *The Chemistry and Biochemistry of N-Substituted Porphyrins*; VCH Publishers: New York, 1987; Chapter 3, pp 41–102; Chapter 7, pp 209–260.

(12) Positive ion electrospray mass spectra were obtained in the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois, using a Fisons Instruments VG Quattro quadrupole–hexapole–quadrupole mass spectrometer purchased in part with a grant from the Division of Research Resources, NIH (RR 07141). Samples containing 10–20 pmol/mL enzyme were denatured by addition of 25% (v/v) acetonitrile 1–48 h prior to analysis and acidified with 0.1% (v/v) formic acid immediately before injection. A flow system of 1:1 acetonitrile/water was employed. Peaks in the range of interest were averaged over a period of 6 min following injection.

0002-7863/95/1517-0817$09.00/0

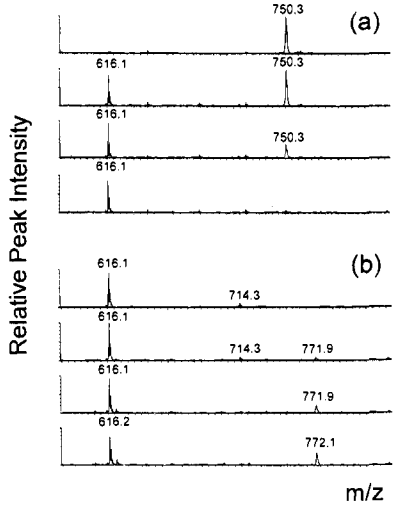

Figure 2. Electrospray mass spectral analysis (600–800 *m/z* range) of CPO whole protein after treatment with unsaturated substrates. (a) Changes in the porphyrin composition following inactivation of CPO with allylbenzene. Samples were taken (from top) at 0, 6, 15, and 48 h after the inactivation reaction. (b) Changes in the porphyrin composition following inactivation of CPO with 1-hexyne. Samples were taken (from top) at 0, 8, 21, and 50 h after inactivation.

750; Figure 2a). The *m/z* 750 species was a doubly charged ion, possibly due to the formation of oxo-bridged porphyrin dimers (P_2^{2+}). Sampling of the enzyme at later time intervals showed a decrease in intensity of the *m/z* 750 species and the reappearance of heme b, on a time scale consistent with the changes in visible spectra and activity for the same sample (Figure 2a). Denaturation of the modified enzyme with low concentrations of organic solvents prevented loss of the *m/z* 750 species and the formation of native heme, suggesting that dealkylation of the porphyrin depends on the presence of intact enzyme.

Extraction of the modified heme from CPO treated with allylbenzene, followed by acid demetalation, yielded a free base displaying an aetio-type spectrum with absorption maxima at 417, 512, 546, 594, and 652 nm, characteristic of N-alkylated porphyrins.[11] On addition of a methanolic solution of $ZnCl_2$, the zinc porphyrin was formed rapidly, with absorption maxima at 432, 546, 589, and 631 nm.

In light of the known suicide reactions of cytochrome P-450 with terminal alkynes as well as alkenes, we also investigated the reaction of 1-hexyne with CPO.[8] Treatment of the enzyme with 1-hexyne in the presence of H_2O_2 resulted in a rapid loss of enzyme activity and the formation of a yellow enzyme species with absorption maxima at 359 (sh), 430, 550, 580, and 624 (sh) nm (Figure 1b). On standing, this species also reverted to a native-like CPO with visible maxima at 397, 513 (sh), 548 (sh), 588 (sh), and 645 nm. The spectral changes were accompanied by a first-order increase in chlorination activity ($t_{1/2}$ = 10.9 h at 30 °C). However, the maximum activity attained was only 10% of that of untreated enzyme. Electrospray MS analysis revealed that CPO modified with 1-hexyne contained only a small proportion of the expected heme alkylation product (*m/z* 714; singly charged ion), with the remainder of the sample containing native heme (*m/z* 616, Figure 2b). It appears likely that the loss of enzyme activity in the fraction of the sample which is not heme-alkylated reflects a modification of active site amino acid residues. At later time intervals, the *m/z* 714 species disappeared and an unidentified doubly charged species appeared at *m/z* 772. Comparison of the peak intensities suggests that the *m/z* 772 species is not derived from the *m/z* 714 heme adduct, but may involve the transfer of an active site moiety to native heme. We are at present investigating possible amino acid modifications of chloroperoxidase following treatment with 1-hexyne.

Transient N-alkylation of synthetic hemins by alkenes under oxidizing conditions has been reported by other workers,[13] and mechanistic studies have been carried out on the metal-promoted nucleophilic dealkylation of *N*-alkylporphyrins.[14] While the mechanism of N-dealkylation for chloroperoxidase heme in the present work is not clear, it appears that the apoprotein plays a role in the reaction, as the isolated *N*-alkyl hemin does not revert to heme b at a significant rate. Despite extensive work on the suicidal heme N-alkylation reactions of microsomal cytochrome P-450, an equivalent dealkylation reaction has not been reported for this enzyme, and the accumulation of "green pigments" in the livers of rats treated with terminal alkenes and alkynes suggests that dealkylation is not significant *in vivo*. Further investigation of this apparent difference in the active site chemistry between cytochrome P-450 and chloroperoxidase may throw additional light on the control of catalytic activity in these versatile enzymes.

Acknowledgment. This work was supported by the National Institutes of Health (GM07768). A.F.D. is the recipient of a predoctoral fellowship from the Howard Hughes Medical Institute. We thank Brad Crawford for performing the chiral GC analyses.

JA943289R

(13) (a) Mashiko, T.; Dolphin, D.; Nakano, T.; Traylor, T. G. *J. Am. Chem. Soc.* **1985**, *107*, 3735–3736. (b) Traylor, T. G.; Nakano, T.; Miksztal, A. R.; Dunlap, B. E. *J. Am. Chem. Soc.* **1987**, *109*, 3625–3632.

(14) (a) Lavallee, D. K. *Inorg. Chem.* **1976**, *15*, 691–694. (b) Lavallee, D. K. *Inorg. Chem.* **1977**, *16*, 955–957. (c) Schauer, C. K.; Anderson, O. P.; Lavallee, D. K.; Battioni, J.-P.; Mansuy, D. *J. Am. Chem. Soc.* **1987**, *109*, 3922–3928. (d) Kuila, D.; Lavallee, D. K. *Inorg. Chem.* **1983**, *22*, 1095–1099. (e) Lavallee, D. K.; Kuila, D. *Inorg. Chem.* **1984**, *23*, 3987–3992.