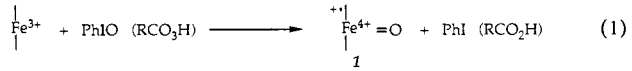
Homolytic and Heterolytic Scission of Organic Hydroperoxides by (*meso*-Tetraphenylporphinato)iron(III) and Its Relation to Olefin Epoxidation

Regine Labeque and Lawrence J. Marnett*

Contribution from the Department of Chemistry, Wayne State University, Detroit, Michigan 48202. *Received October 7, 1988*

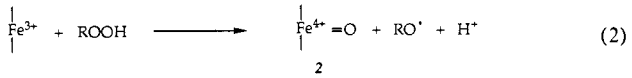
Abstract: Reaction of 10-hydroperoxyoctadeca-8,12-dienoic acid (10-OOH-18:2) with chloro(*meso*-tetraphenylporphinato)iron(III) (Fe^{3+}-TPP) in CH_2Cl_2 produces two products identified as 10-oxodec-8-enoic acid (10-oxo-10:1) and 10-oxooctadeca-8,12-dienoic acid (10-oxo-18:2). These compounds are derived from an alkoxyl radical intermediate generated by homolytic cleavage of 10-OOH-18:2. Only a trace of the heterolytic cleavage product 10-hydroxyoctadeca-8,12-dienoic acid (10-OH-18:2) is observed. Inclusion of imidazole alters the product profile so that 10-OH-18:2 is the major product (62%). Reaction of 10-OOH-18:2 or *t*-BuOOH (0.05 M) with Fe^{3+}-TPP (0.001 M) and *cis*-stilbene (5 M) produces *trans*-stilbene oxide as the only oxidation product (55% based on hydroperoxide). Oxidation in the presence of imidazole (0.3 M) produces approximately equal amounts of *cis*-stilbene oxide (22%) and *trans*-stilbene oxide (23%). Butylated hydroxytoluene inhibits formation of both epoxides from *cis*-stilbene whereas *p*-methoxyanisole selectively inhibits formation of *cis*-stilbene oxide. The data indicate that two separate oxidizing agents are produced in the reaction of hydroperoxides with Fe^{3+}-TPP. In the absence of imidazole, Fe^{3+}-TPP cleaves 10-OOH-18:2 and *t*-BuOOH homolytically to alkoxyl radicals and Fe^{4+}=O·TPP. Both species oxidize the hydroperoxide to peroxyl radicals that epoxidize *cis*-stilbene nonstereospecifically. In the presence of imidazole, Fe^{3+}-TPP also cleaves the hydroperoxides heterolytically to alcohol and Fe^{4+}=O·$TPP^{\cdot+}$, which epoxidizes *cis*-stilbene stereospecifically. The results reconcile apparently conflicting observations in the literature and suggest that the pathway of hydroperoxide cleavage by heme complexes and heme proteins is a sensitive function of the environment of the heme.

Studies of the reactions of metalloporphyrins with iodosylbenzene (PhIO), peracids, and hydroperoxides have provided important insights into the nature of the oxidizing agents produced from hemeproteins such as peroxidases, catalase, and cytochrome P-450. The consensus of these studies is that PhIO and peracids transfer an oxygen atom with two oxidizing equivalents (the result of heterolytic cleavage) to form a high valent iron–oxo complex (eq 1) capable of epoxidizing olefins.[1] In the case of ferric



(1)

1



(2)

2

porphyrins, the iron–oxo complex (*1*) contains a ferryl iron and a radical cation of the porphyrin.[1a,2] These complexes are electronically analogous to the initial oxidizing agents (compounds I) produced during the catalytic turnover of peroxidases.[3]

Although PhIO and peracids are useful mechanistic tools, they are of limited biological relevance because they do not occur in plant or animal metabolism. In contrast, hydrogen peroxide and fatty acid hydroperoxides are widely distributed in the plant and animal kingdom, and they support a variety of oxidations by hemeproteins. Studies of their reactions with heme complexes and hemeproteins can provide direct information about the nature of the oxidants in many biological oxidations and highlight differences in mechanism induced by the protein component of hemeproteins. Despite intensive investigation of heme–hydroperoxide reactions, a clear mechanistic consensus has not evolved. Several studies of hydroperoxide-dependent oxidations by model ferric porphyrins suggest the heme group reacts with hydroperoxides by a different mechanism than it reacts with PhIO and peracids.[1c,d,4] Hemes appear to cleave the hydroperoxide bond homolytically to produce an alkoxyl radical and a ferryl–oxo complex (**2**) that contains only one of the oxidizing equivalents of the hydroperoxide (eq 2). The ferryl–oxo complex (**2**) is electronically analogous to compound II of peroxidases.[2c,3a,5] In contrast to complexes such as **1**, complexes analogous to **2** do not appear to catalyze stereospecific olefin epoxidation.[6] Recently, Traylor and Xu reported that hydroperoxides are oxidized to peroxyl radicals during reactions with heme complexes and suggested hydroperoxide scavenging of **1** competes with its reaction with olefins (eq 3).[7] They offered a unifying hypothesis that

$$1 + ROOH \rightarrow 2 + ROO^{\cdot} + H^{+} \qquad (3)$$

heme–hydroperoxide reactions proceed by heterolytic scission analogous to their peracid counterparts but that secondary reaction with hydroperoxide consumes **1**.

One can estimate the extent of heterolytic hydroperoxide cleavage using stereospecific olefin epoxidation to detect **1**.[2a] The absence of molecules that react with ferryl–oxo complexes **2** in a diagnostic fashion limits one's ability to quantitate homolytic

* Address correspondence to L.J.M. at Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232.

(1) (a) Groves, J. T.; Nemo, T. E.; Myers, R. S. *J. Am. Chem. Soc.* **1979**, *101*, 1032–1033. (b) Groves, J. T.; Nemo, T. E. *J. Am. Chem. Soc.* **1983**, *105*, 5786–5791. (c) Lee, W. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1985**, *107*, 513–514. (d) Lee, W. A.; Yuan, L.-C.; Bruice, T. C. *J. Am. Chem. Soc.* **1988**, *110*, 4277–4283. (e) Collman, J. P.; Kodadek, T.; Brauman, J. I. *J. Am. Chem. Soc.* **1986**, *108*, 2588–2594.

(2) (a) Groves, J. T.; Watanabe, Y. *J. Am. Chem. Soc.* **1986**, *108*, 507–508. (b) Groves, J. T.; Hauslater, R. C.; Nakamura, M.; Nemo, T. E.; Evans, B. J. *J. Am. Chem. Soc.* **1981**, *103*, 2884–2886. (c) Balch, A. L.; Latos-Grazynski, L.; Renner, M. W. *J. Am. Chem. Soc.* **1985**, *107*, 2983–3002.

(3) (a) Dunford, H. B.; Stillman, J. S. *Coord. Chem. Rev.* **1976**, *19*, 187–251. (b) Roberts, J. E.; Hoffman, B. M.; Rutter, R.; Hager, L. P. *J. Biol. Chem.* **1981**, *256*, 2118–2121. (c) Dolphin, D.; Forman, A.; Borg, D. C.; Fajer, J.; Felton, R. H. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 614–618. (d) La Mar, G. N.; deRopp, J. S. *J. Am. Chem. Soc.* **1980**, *102*, 395–397. (e) *Cytochrome P-450: Structure, Mechanism and Biochemistry*; Ortiz de Montellano, P. R., Ed.; Plenum: New York, 1986. (f) Guengerich, F. E.; Macdonald, T. C. *Acc. Chem. Res.* **1984**, *17*, 9–16.

(4) (a) Mansuy, D.; Bartoli, J.-F.; Momenteau, M. *Tetrahedron Lett.* **1983**, *23*, 2781–2784. (b) Lindsay-Smith, J. R.; Mortimer, D. N. *J. Chem. Soc., Perkin Trans. 2* **1986**, 1743–1749. (c) Koola, J. D.; Kochi, J. K. *J. Org. Chem.* **1987**, *52*, 4545–4553. (d) Mansuy, D. *Pure. Appl. Chem.* **1987**, *59*, 759–770.

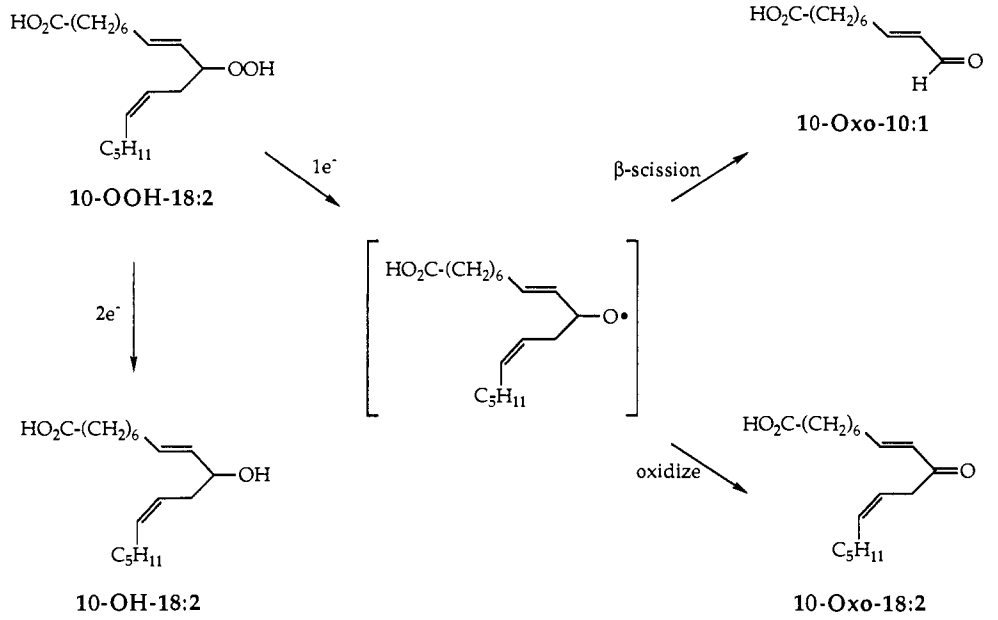
(5) Balch, A. L.; Chan, Y.-W.; Cheng, R.-J.; La Mar, G. N.; Latos-Grazynski, L.; Renner, M. W. *J. Am. Chem. Soc.* **1984**, *106*, 7779–7785.

(6) (a) Mansuy, D.; Battioni, P.; Renaud, J. P. *J. Chem. Soc., Chem. Commun.* **1984**, 1255–1257. (b) Chin, D.; La Mar, G. N.; Balch, A. L. *J. Am. Chem. Soc.* **1980**, *102*, 5945–5947.

(7) Traylor, T. G.; Xu, F. *J. Am. Chem. Soc.* **1987**, *109*, 6201–6202.

0002-7863/89/1511-6621$01.50/0

Scheme I



hydroperoxide scission. This highlights the need for novel approaches that provide a complete picture of the chemistry of hydroperoxide cleavage by metal complexes and metalloproteins. We recently described a fatty acid hydroperoxide, 10-hydroperoxyoctadeca-8,12-dienoic acid (10-OOH-18:2), that is capable of simultaneously differentiating homolytic and heterolytic scission of the hydroperoxide bond (Scheme I).[8a] Heterolytic scission produces 10-hydroxyoctadeca-8,12-dienoic acid (10-OH-18:2) whereas homolytic scission produces an alkoxyl radical that undergoes β-scission to 10-oxodec-8-enoic acid (10-oxo-10:1) or oxidation to 10-oxooctadeca-8,12-dienoic acid (10-oxo-18:2). 10-OOH-18:2 is readily synthesized from linoleic acid, can be produced with radioactive label for sensitive product detection, and exhibits solubility properties that enable it to be used in aqueous or organic solvents. We have used 10-OOH-18:2 to demonstrate that ferric bleomycin catalyzes homolytic hydroperoxide cleavage and prostaglandin H synthase catalyzes heterolytic hydroperoxide cleavage.[9]

The importance of defining the mechanism of hydroperoxide reduction by model heme complexes prompted us to investigate the reaction of 10-OOH-18:2 with chloro(*meso*-tetraphenylporphinato)iron(III) (Fe^{3+}-TPP) in CH_2Cl_2. We have also conducted experiments to relate the pathway of hydroperoxide cleavage to the oxidants produced that epoxidize olefins. Our results provide strong evidence for homolytic scission of the peroxide bond and explain discrepancies between previous studies that employed olefin epoxidation to infer the identity of the oxidant produced in heme–hydroperoxide reactions.

Experimental Procedures

Materials. *cis*-Stilbene, *trans*-stilbene, Fe^{3+}-TPP, *tert*-butyl hydroperoxide (*t*-BuOOH, 90%), and iodosobenzene diacetate were purchased from Aldrich (Milwaukee, WI) and butylated hydroxytoluene was from Sigma (St. Louis, MO). Phenol (Fisher) was distilled prior to use. Silica gel was from Merck (Rahway, NJ), and 3% OV-17 on 100/120 Chromosorb Q was obtained from Anspec (Ann Arbor, MI). [1-^{14}C]10-OOH-18:2 was synthesized as previously described.[8b] PhIO was prepared according to the method described by Lucas and Kennedy.[10] *p*-Methoxythioanisole was a gift from Patrick Plë, Wayne State University. Solvents used for chromatography were HPLC grade. Other chemicals were reagent grade.

Purification of *cis*-Stilbene. Commercially available *cis*-stilbene was contaminated with 2–3% *trans*-stilbene. Therefore *cis*-stilbene was purified by medium-pressure liquid chromatography on a silica gel column eluted with hexane. The fractions eluting from the column were assayed by gas chromatography (GC) using a program that was isothermal at 80 °C for 3 min followed by a linear gradient to 240 °C at 8 °C/min. The retention times of *cis*- and *trans*-stilbene were 11.5 and 14.1 min, respectively. Fractions of *cis*-stilbene containing less than 0.2% *trans*-stilbene were combined and concentrated. The purified *cis*-stilbene was 99.8% pure.

Instrumentation. HPLC was performed with an LDC pump connected to an Alltech Partisil 10 column (10-μm silica, 4.6 × 250 mm). Radioactivity eluting from the column was quantitated in a continuous fashion with a Radiomatic Flo-One HP radioactivity flow detector using Scinti-Verse LC, premixed scintillant, from Fisher. GC analysis was performed on a Varian Model 3700 with flame ionization detection using a 2-ft column packed with 3% OV-17 on Chromosorb Q. Product quantitation was performed by integrating the GC signal with a Hewlett Packard HP 3380A integrator.

Reaction of 10-OOH-18:2 with Fe^{3+}-TPP. Fe^{3+}-TPP (100 μM) was dissolved in CH_2Cl_2. Reactions were initiated by addition of 5 mM [1-^{14}C]10-OOH-18:2 (final concentration) and allowed to continue at room temperature for 30 min with stirring. An aliquot of the reaction mixture was analyzed by normal-phase HPLC (hexane/tetrahydrofuran/acetic acid:895/100/5). Imidazole, or phenol, when present, was added to the solution of Fe^{3+}-TPP 2 min prior to the addition of 10-OOH-18:2.

***cis*-Stilbene Oxidation.** *cis*-Stilbene (3–5 M) and Fe^{3+}-TPP (1 mM) were dissolved in CH_2Cl_2, and hydroperoxide (*t*-BuOOH or 10-OOH-18:2) was added to initiate the reaction. The PhIO oxidation of *cis*-stilbene was performed as follows: *cis*-stilbene (250 mM) and Fe^{3+}-TPP (7 mM) were dissolved in CH_2Cl_2, and PhIO was slowly added. The reaction mixtures were assayed by GC using a linear gradient from 80 to 240 °C at 3 °C/min. The retention times of *cis*-stilbene, *cis*-stilbene oxide, *trans*-stilbene, and *trans*-stilbene oxide were 12.7, 16.5, 19.3, and 20.5 min, respectively.

Results

Products of the Reaction of 10-OOH-18:2 with Fe^{3+}-TPP. When 10-OOH-18:2 (5 mM) was reacted with Fe^{3+}-TPP (100 μM) in CH_2Cl_2, two products resulted. These compounds were identified as 10-oxo-18:2 and 10-oxo-10:1. They exhibited identical chromatographic and mass spectral properties as reported previously.[8b] 10-Oxo-18:2 and 10-oxo-10:1 are derived from an alkoxyl radical formed by homolytic scission of 10-OOH-18:2 by Fe^{3+}-TPP. Only trace amounts of the heterolytic scission product, 10-OH-18:2, were detected. The time course of reaction of 10-OOH-18:2 with Fe^{3+}-TPP showed complete consumption of the hydroperoxide after 20 min (data not shown). The ratio of the yields of 10-oxo-18:2 to 10-oxo-10:1 was constant with time. No

(8) (a) Labeque, R.; Marnett, L. J. *J. Am. Chem. Soc.* **1987**, *109*, 2828–2829. (b) Labeque, R.; Marnett, L. J. *Biochemistry* **1988**, *27*, 7060–7070.

(9) (a) Padbury, G.; Sligar, S. C.; Labeque, R.; Marnett, L. J. *Biochemistry* **1988**, *27*, 7846–7852. (b) Marnett, L. J.; Chen, Y.-N. P.; Maddipati, K. R.; Plë, P.; Labeque, R. *J. Biol. Chem.* **1988**, *263*, 16532–16535.

(10) Lucas, H. J.; Kennedy, E. R. *Org. Synth.* **1955**, *3*, 482–483.

Table I. Reaction of 10-Hydroperoxy-8,12-Octadecadienoic Acid with Fe^{3+}–TPP[a]

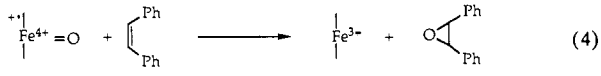
	10-oxo-18:2, %	10-oxo-10:1, %	10-OH-18:2, %
Fe^{3+}–TPP	24.2 ± 0.8	72.1 ± 0.5	<1
Fe^{3+}–TPP, phenol	24.5 ± 0.5	71.7 ± 0.1	<1
Fe^{3+}–TPP, imidazole, phenol	9.5 ± 0.6	28.3 ± 0.4	62.1 ± 0.3

[a] Reactions were performed at 30 °C with 5 mM [1-^{14}C]10-OOH-18:2, 100 μM Fe^{3+}–TPP in CH_2Cl_2 for 30 min. The concentrations of phenol and imidazole were 30 mM and 60 mM, respectively. The yields are the mean of triplicates ± standard deviation.

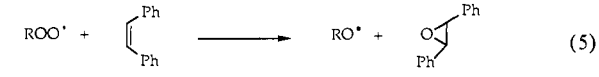
alteration of the product profile was observed when reactions were conducted in the presence of 30 mM phenol (Table I). Phenol was added to reduce any metal–oxo intermediates and oxy radicals. Reduction of metal–oxo intermediates accelerates catalytic turnover, protects the catalyst from oxidative inactivation, and prevents oxidation of the hydroperoxide to peroxyl radicals. The product profile determined in the presence of phenol or other reducing agents reflects the chemistry of the initial heme–hydroperoxide reaction. In the case of the reaction of Fe^{3+}–TPP with 10-OOH-18:2, the products result from homolytic cleavage of the hydroperoxide.

Mansuy and co-workers have reported that in the presence of imidazole, Fe^{3+}–TPP catalyzes epoxidation of alkenes by cumene hydroperoxide.[6] The active epoxidizing agent is presumably a high-valent metal–oxo complex analogous to **1**, formed by heterolytic cleavage of the peroxide bond. Therefore, we conducted experiments to explore the effect of imidazole on the reaction of 10-OOH-18:2 with Fe^{3+}–TPP. Spectrophotometric titrations of CH_2Cl_2 solutions of Fe^{3+}–TPP (8×10^{-6} M) with imidazole decreased the shoulder at 375 nm and increased the maximum at 415 nm. The spectral changes were maximal at 5 mM imidazole so hydroperoxide reactions with 100 μM Fe^{3+}–TPP were carried out with 60 mM imidazole to maintain the same ratio of imidazole to heme. Phenol (30 mM) was included for the reasons described above. Figure 1 compares the HPLC elution profile of the products of reaction conducted in the absence (profile a) and presence of imidazole (profile b). Addition of imidazole caused a dramatic alteration in the product profile indicative of a change in the mechanism of hydroperoxide reduction. High yields (62%) of the heterolytic reduction product 10-OH-18:2 were observed (Table I). The presence of one-electron and two-electron reduction products (10-oxo-10:1/10-oxo-18:2 and 10-OH-18:2, respectively) in the reactions containing imidazole indicates the simultaneous operation of homolytic and heterolytic mechanisms of hydroperoxide cleavage. Reaction of 10-OOH-18:2 with Fe^{3+}–TPP and imidazole without phenol also produced 10-OH-18:2 but in somewhat reduced yield (~35%).

***cis*-Stilbene Oxidation.** The pathway of hydroperoxide cleavage by Fe^{3+}–TPP should be reflected in the oxidation products generated from *cis*-stilbene. Ferryl–oxo complexes formed by heterolytic hydroperoxide scission oxidize *cis*-stilbene stereospecifically to *cis*-stilbene oxide (eq 4) whereas ferryl–oxo com-



(4)



(5)

plexes formed by homolytic hydroperoxide scission do not.[1a,b,4d] Peroxyl radicals, which are also likely to be formed in heme hydroperoxide reactions, oxidize *cis*-stilbene to *trans*-stilbene oxide (eq 5).[11] Previous studies indicate that chloro(*meso*-tetramesitylporphinato)iron(III) (Fe^{3+}–TMP) and *t*-BuOOH epoxidize norbornene in CH_2Cl_2.[7] Concentrations of Fe^{3+}–TPP, *t*-BuOOH

(11) Catalano, C. E.; Ortiz de Montellano, P. R. *Biochemistry* **1987**, *26*, 8373–8380.

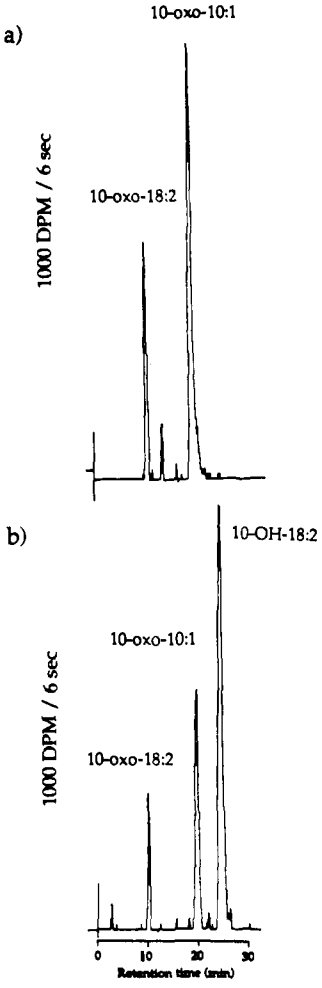


Figure 1. Radioactivity profile of the reaction products of 10-OOH-18:2 with Fe^{3+}–TPP: (a) 10-OOH-18:2 (5 mM) with Fe^{3+}–TPP (0.1 mM) in the presence of phenol (30 mM) for 30 min; (b) 10-OOH-18:2 (5 mM) with Fe^{3+}–TPP (0.1 mM) in the presence of phenol (30 mM) and imidazole (60 mM) for 30 min. Elution was performed with hexane/THF/acetic acid (885/100/5) at a flow rate of 1.3 mL/min using a Partisil 10 column (10 μm Silica, 4.6 × 250 mm).

or 10-OOH-18:2, and *cis*-stilbene identical with those published were employed to probe the nature of the oxidant generated. Under these conditions either 10-OOH-18:2 or *t*-BuOOH catalyzed the epoxidation of *cis*-stilbene. The major product was *trans*-stilbene oxide; only a trace of *cis*-stilbene oxide was detected. No oxidation products were generated when catalyst or hydroperoxide were omitted, and *cis*-stilbene oxide was stable to the reaction conditions. A trace amount of *trans*-stilbene was present in the starting material, and its concentration did not change during the course of the reaction. Thus, the *trans*-stilbene oxide produced in the presence of Fe^{3+}–TPP and *t*-BuOOH arose directly from *cis*-stilbene and did not arise from *trans*-stilbene formed by isomerization.

Figure 2 displays the dependence of *trans*-stilbene oxide yield on the concentration of *t*-BuOOH. The data are presented as yields based on *cis*-stilbene (panel A) or *t*-BuOOH (panel B). Both plots correspond closely to the yields reported for the epoxidation of norbornene by Fe^{3+}–TMP and *t*-BuOOH.[7] As reported previously, the yields of epoxide relative to *t*-BuOOH decreased as the concentration of hydroperoxide increased, although the yield of *trans*-stilbene oxide based on *cis*-stilbene increased. These data confirm the ability of Fe^{3+}–TPP/*t*-BuOOH to epoxidize olefins but suggest the oxidant is not the high-valent metal–oxo complex analogous to **1**.

Reaction of *cis*-stilbene with Fe^{3+}–TPP and PhIO resulted in exclusive formation of *cis*-stilbene oxide in 41% yield (Table II). Under these conditions *cis*-stilbene reacts 16 times faster than *trans*-stilbene. The stereospecificity of epoxidation and the higher

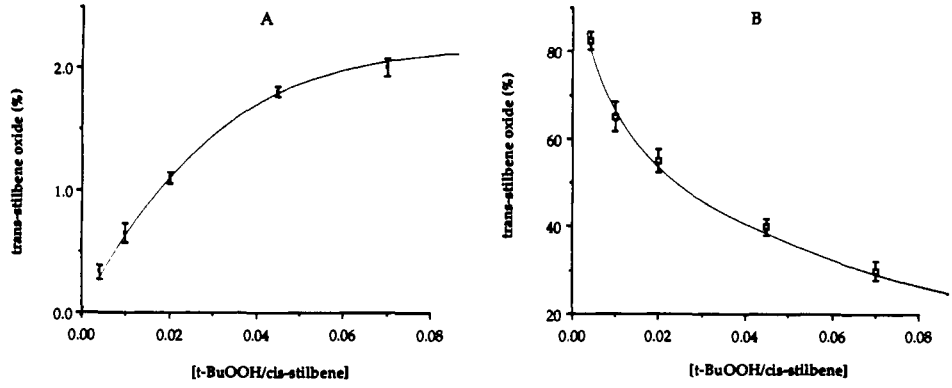


Figure 2. Effect of varying *t*-BuOOH concentration on *cis*-stilbene oxidation: Plots of yields of *trans*-stilbene oxide (based upon [*cis*-stilbene], closed squares, panel A) and yields of *trans*-stilbene oxide (based upon [*t*-BuOOH], opened squares, panel B) vs concentration of *t*-BuOOH and *cis*-stilbene. The reaction conditions were the following: 5 M *cis*-stilbene, 1 mM Fe^{3+}–TPP, and varying concentrations of *t*-BuOOH in dichloromethane at 30 °C.

Table II. Fe^{3+}-Catalyzed Epoxidation of *cis*-Stilbene[a]

catalyst	*cis*-Stilbene oxide,[a] %	*trans*-stilbene oxide,[a] %
Fe^{3+}–TPP; *t*-BuOOH	tr	55 ± 3
Fe^{3+}–TPP; PhIO	41 ± 2	tr
Fe^{3+}–TPP; *t*-BuOOH, imidazole	22 ± 2	23 ± 3

[a] Conditions for each reactions were as follows: 5 M *cis*-stilbene, 50 mM *t*-BuOOH, 1 mM Fe^{3+}–TPP in CH_2Cl_2; 250 mM *cis*-stilbene, 100 mM *t*-BuOOH, 7 mM Fe^{3+}-TPP in CH_2Cl_2; 5 M *cis*-stilbene, 50 mM *t*-BuOOH, 1 mM Fe^{3+}-TPP, 300 mM imidazole in CH_2Cl_2. Reactions were conducted with Fe^{3+}–TPP and *t*-BuOOH for 4 h. The other incubations were performed for 1–2 h. [a] The yields are reported based upon the oxidant and are the mean of triplicates ± standard deviation; tr: traces.

reactivity of *cis*-stilbene is consistent with previous reports of Fe^{3+}–TPP-catalyzed epoxidations by PhIO and peracids.[1a,b,12] In contrast *trans*-stilbene is slightly *more* reactive to the oxidant generated by reaction of Fe^{3+}–TPP with *t*-BuOOH (k_{cis}/k_{trans} = 0.8). The differences in stereochemistry and reactivity clearly indicate that the oxidizing agents generated by reaction of Fe^{3+}–TPP with PhIO and *t*-BuOOH are different.

As described above, reaction of 10-OOH-18:2 with Fe^{3+}–TPP in the presence of excess imidazole resulted in the formation of 10-OH-18:2 in 62% yield. If 10-OH-18:2 arises from a two-electron reduction mechanism, a stoichiometric amount of ferryl–oxo complex analogous to **1** will result from each heterolysis event. This is the same ferryl–oxo complex generated in the reaction of Fe^{3+}–TPP with PhIO, so significant amounts of *cis*-stilbene oxide should be produced under these conditions. When 5 M *cis*-stilbene was reacted with 1 mM Fe^{3+}–TPP, 300 mM imidazole, and 50 mM *t*-BuOOH, equal amounts of *cis*- and *trans*-stilbene oxide were detected (Table II). The rate of epoxidation in the presence of imidazole was faster than in its absence. In the presence of imidazole, epoxides were generated after 30 min–1 h, whereas in the absence of imidazole oxidation was only detected after 3–5 h. Similar results were obtained with 10-OOH-18:2.

The mixture of *cis*- and *trans*-stilbene oxide produced in the oxidation of *cis*-stilbene by Fe^{3+}–TPP with hydroperoxides in the presence of imidazole is consistent with the products of 10-OOH-18:2 reduction under comparable conditions. The results are most easily interpreted by postulating the simultaneous operation of homolytic and heterolytic pathways for hydroperoxide scission. In an attempt to differentiate the oxidants responsible for formation of the isomeric epoxides, *cis*-stilbene oxidation was conducted with Fe^{3+}–TPP, *t*-BuOOH, and imidazole in the presence of potential inhibitors. Addition of the phenolic anti-

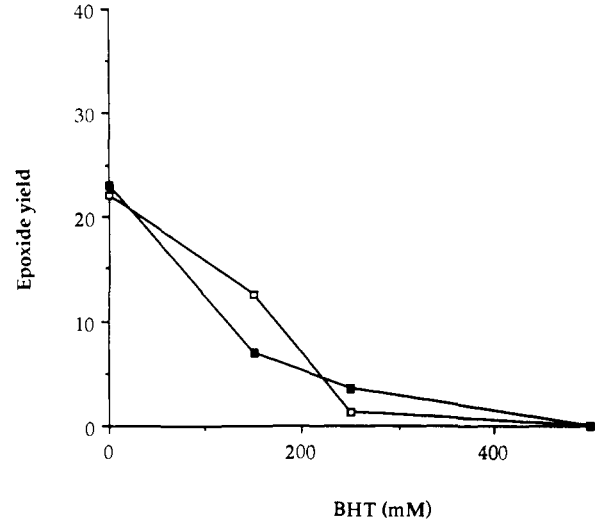


Figure 3. Inhibition of *cis*-stilbene epoxidation by butylated hydroxytoluene. Yields of *cis*-stilbene oxide (opened squares) and *trans*-stilbene (closed squares) are reported. The reaction mixtures contained *cis*-stilbene (5 M), Fe^{3+}–TPP (1 mM), imidazole (300 mM), and varying concentrations of butylated hydroxytoluene. The reactions were initiated by addition of *t*-BuOOH (50 mM). Yields are reported based upon the oxidant.

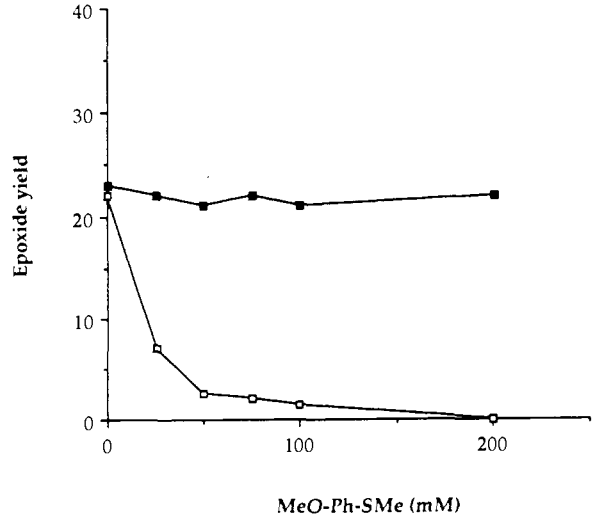


Figure 4. Inhibition of *cis*-stilbene epoxidation by *p*-methoxythioanisole. Yields of *cis*-stilbene oxide (opened squares) and *trans*-stilbene (closed squares) are reported. The reaction mixtures contained *cis*-stilbene (5 M), Fe^{3+}–TPP (1 mM) imidazole (300 mM), and varying concentrations of *p*-methoxythioanisole. The reactions were initiated by addition of *t*-BuOOH (50 mM). Yields are reported based upon the oxidant.

(12) (a) Castellino, A. J.; Bruice, T. C. *J. Am. Chem. Soc.* **1988**, *110*, 158–162. (b) Traylor, T. G.; Xu, F. *J. Am. Chem. Soc.* **1988**, *110*, 1953–1958.

oxidant butylated hydroxytoluene inhibited formation of both epoxide products (Figure 3) whereas addition of *p*-methoxythioanisole selectively inhibited production of *cis*-stilbene oxide (Figure 4).

Discussion

Oxidants generated in the reaction of heme complexes with PhIO, peracids, and hydroperoxides are extremely reactive and difficult to characterize spectroscopically. This necessitates the use of indirect approaches for detection and quantitation of reactive intermediates such as ferryl–oxo complexes, peroxyl radicals, and alkoxyl radicals. The most common approach is to trap the oxidant with a molecule diagnostic for reaction with a particular species. Stereospecific epoxidation of olefins is very useful for detection of ferryl–oxo complexes such as **1**, but unfortunately a battery of diagnostic traps is not available for the other oxidants produced. A complementary approach is to employ hydroperoxides that undergo reactions characteristic of the intermediates producted.[8a,13] For this purpose, 10-OOH-18:2 is quite useful because it yields a simple product profile diagnostic of one- or two-electron reduction or one-electron oxidation.[8]

Previous experiments with 10-OOH-18:2 indicate that Fe^{3+}–protoporphyrin IX (hematin) cleaves fatty acid hydroperoxides homolytically when reactions are performed in detergent-containing buffers.[8b] Detergent is required in aqueous buffers to solubilize the reagents and to stimulate the catalytic activity of hematin. The heterogeneous nature of the model system makes it difficult to compare findings obtained with it to studies conducted in pure organic solvents. Therefore, in the present report we describe a study of the products of reaction of 10-OOH-18:2 with Fe^{3+}–TPP in CH_2Cl_2. The results strongly indicate that Fe^{3+}–TPP catalyzes homolytic scission of hydroperoxides. In fact, only trace amounts of the heterolytic scission product, 10-OH-18:2, were detected.

Addition of 10-OOH-18:2 to Fe^{3+}–TPP in CH_2Cl_2 generates 10-oxo-10:1 and 10-oxo-18:2 in a ratio of 3:1. 10-Oxo-10:1 is produced by β-scission of an intermediate alkoxyl radical, and we have proposed that 10-oxo-18:2 is produced via oxidation of the alkoxyl radical by ferryl–oxo complex **2**.[8b] An alternative pathway of formation of 10-oxo-18:2 is concerted dehydration via a four-centered transition state. The available evidence favors stepwise formation of 10-oxo-18:2 via oxidation of the alkoxyl radical. The 3:1 ratio of 10-oxo-10:1 to 10-oxo-18:2 is constant thoughout the time course of reaction and *is unchanged by the addition of imidazole*. In addition, the ratio of aldehyde to ketone is dependent on the presence of the 12–13 double bond. 10-Hydroperoxyoctadec-8-enoic acid, which lacks this double bond, reacts with hematin at a rate comparable to 10-OOH-18:2 to produce 10-oxo-10:1 and 10-oxooctadec-8-enoic acid in a ratio of 0.045:1. The dramatically enhanced yield of 10-oxo-10:1 from 10-OOH-18:2 is most easily explained by allylic stabilization driving the β-scission of an alkoxyl radical that is a common intermediate in the formation of 10-oxo-10:1 and 10-oxo-18:2. The sensitivity of ketone formation to the presence of a remote double bond (Δ^{12-13}) is inconsistent with a concerted mechanism for ketone formation. Since 10-oxo-10:1 and 10-oxo-18:2 arise via homolytic cleavage of 10-OOH-18:2, it is clear that one-electron reduction of 10-OOH-18:2 by Fe^{3+}–TPP is nearly quantitative (Table I).

If Fe^{3+}–TPP reduces hydroperoxides homolytically, the initial oxidants are the ferryl–oxo complex analogous to **2** and the alkoxyl radical derived from the hydroperoxide. Neither of these species effects stereospecific olefin epoxidation and, indeed, none is observed when *cis*-stilbene is included in the reaction mixture. What is observed are high yields (based on oxidant) of *trans*-stilbene oxide. A likely candidate for the oxidizing agent responsible for *trans*-stilbene oxide formation is a peroxyl radical produced by reactions 6 or 7.[4c,7,14] If both reactions occur quantitatively, the

$$2 + \mathrm{ROOH} \longrightarrow \overset{|}{\underset{|}{\mathrm{Fe^{3+}}}} + \mathrm{ROO^{\cdot}} + \mathrm{H^{\cdot}} \qquad (6)$$

$$\mathrm{RO^{\cdot}} + \mathrm{ROOH} \longrightarrow \mathrm{ROH} + \mathrm{ROO^{\cdot}} \qquad (7)$$

minimum yield of peroxyl radicals is 67% of the starting hydroperoxide concentration. This is precisely the yield reported by Traylor and Xu for the epoxidation of norbornene by Fe^{3+}–TMP/*t*-BuOOH at limiting hydroperoxide concentrations.[7] In our study, the maximum yield of *trans*-stilbene oxide was 82% at low hydroperoxide concentration. This may indicate other pathways exist for peroxyl radical generation or that other epoxidizing agents are produced. Epoxidation of an olefin by a peroxyl radical releases an alkoxyl radical (eq 5) that can oxidize another molecule of hydroperoxide. Hydroperoxide oxidation forms another molecule of peroxyl radical and increases the yield of epoxide above 67%. Recent work by Groves and colleagues suggests that ferryl–oxo complexes such as **2** catalyze the *nonstereospecific* epoxidation of *cis*-stilbene to *trans*-stilbene oxide.[15] However, experiments by Balch and co-workers indicate **2** derived from (*meso*-tetra-*p*-tolylporphinato)iron(III) is less reactive to olefins than aryl sulfides, which means that *p*-methoxythioanisole should inhibit *trans*-stilbene oxide formation, which it did not.[16] Thus, it seems likely that peroxyl radicals are responsible for most of the oxidation of *cis*-stilbene to *trans*-stilbene oxide.

An important question relevant to these observations is whether peroxyl radicals are responsible for the epoxidation of norbornene under similar conditions. The catalyst, oxidant, and *cis*-stilbene concentrations in the present study are identical with those reported to result in norbornene epoxidation.[7] Norbornene is a strained olefin that reacts with peroxyl radicals at 50 °C exclusively by addition to form epoxide.[17] Koola and Kochi recently reported that norbornene (1 M) is epoxidized by reaction with *t*-BuOOH (0.2 M) and Co^{2+} complexes (2 mM) in CH_2Cl_2 at 25 °C and that epoxidation is inhibited by the phenolic antioxidant BHT.[4c] Under similar conditions, cyclohexene forms an adduct between its allylic radical and a *tert*-butylperoxyl radical.[4c] These results indicate that olefins do react with peroxyl radicals in CH_2Cl_2 at 25 °C and that the products are those expected from earlier studies of the products of hydrocarbon autoxidation. Furthermore, reaction of norbornene (5 M) with *t*-BuOOH (50 mM) and the free-radical initiator azobisisobutyronitrile (50 mM) in CH_2Cl_2 at 25 °C for 24 h produces norbornene oxide in 64% yield based on *t*-BuOOH.[18] This verifies that peroxyl radicals generated independently of metal-catalyzed hydroperoxide reactions epoxidize norbornene at ambient temperatures.

Very recently, Traylor et al. found that chloro(*meso*-tetrakis(2,6-dichlorophenyl)porphinato)iron(III) catalyzes epoxidation of norbornene by *t*-BuOOH in 16% yield based on hydroperoxide.[19a] The ratio of exo to endo epoxide is diagnostic of oxidation by a metal–oxo complex analogous to **1**. Preliminary experiments in our laboratory indicate this octachloro iron porphyrin reduces 10-OOH-18:2 to the heterolytic scission product

(13) (a) White, R. E.; Sligar, S. G.; Coon, M. J. *J. Biol. Chem.* **1980**, *255*, 11108–11111. (b) McCarthy, M.-B.; White, R. E. *J. Biol. Chem.* **1983**, *258*, 9153–9158. (c) Wand, M. D.; Thompson, J. A. *J. Biol. Chem.* **1986**, *261*, 14049–14056. (d) Thompson, J. A.; Wand, M. D. *J. Biol. Chem.* **1985**, *260*, 10637–10644. (e) Yumibe, N. P.; Thompson, J. A. *Chem. Res. Tox.* **1988**, *1*, 385–390. (f) Vaz, A. D. N.; Coon, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1172–1176. (g) Dix, T. A.; Marnett, L. J. *J. Biol. Chem.* **1985**, *260*, 5351–5357.

(14) (a) Sheldon, R. A. *Recl. Trav. Chim. Pays-Bas* **1973**, *92*, 253–266. (b) Hiatt, R.; Irwin, K. C.; Gould, C. W. *J. Org. Chem.* **1968**, *33*, 1430–1435. (c) Gould, E. S.; Hiatt, R.; Irwin, K. C. *J. Am. Chem. Soc.* **1968**, *90*, 4573–4579. (d) Sharpless, K. B.; Verhoeven, T. R. *Aldrichimica Acta* **1979**, *12*, 63–74. (e) Balasubramanian. P. N.; Sinha, A.; Bruice, T. C. *J. Am. Chem. Soc.* **1987**, *109*, 1456–1462.

(15) Groves, J.; Stern, M. K. *J. Am. Chem. Soc.* **1988**, *110*, 8628–8638.

(16) Balch, A., personal communication.

(17) Mayo, F. *Acc. Chem. Res.* **1968**, *1*, 193–201.

(18) Crowley, J., unpublished result.

(19) (a) Traylor, T. G.; Fann, W.-P.; Bandyopadhyay, D. *J. Am. Chem. Soc.*, submitted for publication. (b) Labeque, R., unpublished result.

10-OH-18:2 in 20% yield.[19b] Comparison of this to the trace amount of 10-OH-18:2 produced by Fe^{3+}–TPP suggests the electronic structure of the porphyrin may contribute to the extent of homolytic vs heterolytic scission.

Addition of excess imidazole dramatically alters the chemistry of the Fe^{3+}–TPP/hydroperoxide reaction. The fifth and sixth ligands to the metal change, the metal changes from high spin to low spin, 10-OH-18:2 becomes a major product, and approximately equal amounts of *cis*- and *trans*-stilbene oxide are produced from *cis*-stilbene. These observations correspond to earlier reports of the effect of imidazole on olefin epoxidation and alkane hydroxylation by Fe^{3+}- and Mn^{3+}-porphyrins and hydroperoxides or hypochlorite.[6,20] The fact that an excess of imidazole to metal is required to effect the alterations in spectral and catalytic properties makes it difficult to assign the basis for the imidazole effect to a single cause. In addition to changing the metal spin state, additional imidazole molecules may hydrogen bond to an imidazole coordinated to the metal, thereby increasing the electron density and redox properties of the iron, or they may participate in general base–acid catalysis of the heterolytic cleavage of the hydroperoxide bond.[21]

Another possibility is that imidazole alters the fate of the alkoxyl radicals produced by homolytic scission. It seems unlikely that imidazole reduces alkoxyl radicals because the same concentration of phenol, which is a better reducing agent than imidazole, had no effect on the products of 10-OOH-18:2 cleavage. However, Bruice et al. have recently suggested that hydroxyl radicals may oxidize ferryl–oxo complex **2** to ferryl–oxo complex **1** (eq 8).[22]

$$\text{Fe}^{4+}{=}\text{O} + \text{HO}^{\bullet} \longrightarrow {}^{+\bullet}\text{Fe}^{4+}{=}\text{O} + \text{RO}^{-} \qquad (8)$$

If this happens, perhaps imidazole enhances the reaction, thereby producing 10-OH-18:2 and **1**, which then stereospecifically epoxidizes *cis*-stilbene. As reaction 8 is still hypothetical, any effects of imidazole on it are purely speculative.

The simplest interpretation of the imidazole effect is the conventional one—that imidazole ligates to Fe^{3+}–TPP and changes the chemistry of its reduction of the hydroperoxide bond from purely homolytic to a mixture of homolytic and heterolytic. If this is the basis for the imidazole effect, the results indicate that the chemistry of heme–hydroperoxide interactions is a sensitive function of the environment of the heme. This duality may be relevant to findings that liver microsomes, a rich source of cytochrome P-450, catalyze simultaneous homolytic and heterolytic cleavage of cumene hydroperoxide.[23] In addition, our results reconcile a considerable body of experimental data indicating that methemoglobin or metmyoglobin cleave hydroperoxides homolytically with a recent report by Ortiz de Montellano that methemoglobin catalyzes small amounts of olefin epoxidation by hydrogen peroxide in a process that requires heterolytic scission.[11] Furthermore, we have found that the heme peroxidase PGH synthase quantitatively reduces 10-OOH-18:2 to 10-OH-18:2 by what appears to be exclusively heterolytic scission.[9b] A histidine imidazole is the fifth ligand to the heme iron of PGH synthase.[24] All of these observations illustrate the versatility of heme complexes as hydroperoxide reductants that can be fine-tuned by ligands and other pendant groups in the vicinity of the heme. By proper choice of ligand it should be possible to successfully model the different reaction pathways of heme–hydroperoxide reactions.

In previous investigations, we were unable to absolutely rule out the possibility that alkoxyl radicals form via oxidation by ferryl–oxo complex **1** of the alkoxide or alcohol product of heterolytic cleavage (eq 9).[8b] Control experiments performed

$$\text{Fe}^{3+} + \text{ROOH} \longrightarrow {}^{+\bullet}\text{Fe}^{4+}{=}\text{O} + \text{ROH} \quad (\text{RO}^{-}) \longrightarrow \text{Fe}^{4+}{=}\text{O} + \text{RO}^{\bullet} \qquad (9)$$

with radioactively labeled 10-OH-18:2 and unlabeled 10-OOH-18:2 demonstrated the alcohol was not oxidized in the presence of the metal porphyrin, but it is possible exogenous alcohol does not penetrate the solvent cage. Our finding in the present study that Fe^{3+}-TPP catalyzes heterolytic scission of 10-OOH-18:2 to 10-OH-18:2 in the presence of imidazole establishes that alkoxide or alcohol generated in the solvent cage is not oxidized to alkoxyl radicals by **1**. This supports the conclusion that alkoxyl radicals that cleave to 10-oxo-10:1 arise by homolytic scission of the hydroperoxide bond catalyzed by the iron porphyrin.

The ability of *p*-methoxythioanisole to inhibit *cis*-stilbene oxide but not *trans*-stilbene oxide formation provides additional support for the simultaneous operation of two mechanisms of hydroperoxide reduction in the presence of imidazole. Oxidation of the sulfide, presumably to the sulfoxide, requires a two-electron oxidizing agent that may account for its preferential reaction with complexes analogous to **1**. This ferryl–oxo complex is a strong two-electron oxidant, whereas the ferryl–oxo complex analogous to **2** and peroxyl radicals are one-electron oxidants. The selectivity exhibited by *p*-methoxythioanisole suggests it may be useful as a diagnostic inhibitor of oxidations effected by ferryl–oxo complexes analogous to **1**.

In contrast to the selective inhibition of *cis*-stilbene oxide formation by *p*-methoxythioanisole, BHT inhibits *cis*-stilbene oxide and *trans*-stilbene oxide formation. Thus, BHT appears to react with both the ferryl–oxo complex **1** and peroxyl radicals. Reaction of BHT with peroxyl radicals is well-recognized as the basis for its chain-breaking antioxidant activity.[25] Phenols are also very reactive reductants of ferryl–oxo complexes analogous to **1** and **2** in proteins. For example, phenols react by one-electron reduction of the higher oxidation states of horseradish peroxidase that correspond to **1** and **2**.[26] Thus, it appears BHT scavenges the oxidants produced by homolytic and heterolytic cleavage of *t*-BuOOH, whereas *p*-methoxythioanisole only scavenges the oxidant produced by heterolytic cleavage.

The present results contribute to a growing consensus that model heme complexes catalyze homolytic cleavage of hydroperoxides. The evidence in favor of homolytic scission includes the types of products formed in hydroperoxide-dependent oxidations and the dependence of the kinetics of heme-catalyzed oxidations on the leaving group of the hydroperoxide.[4a,b,1c,d] With both types of experiments a clear differentiation is observed between the behavior of hydroperoxides and peracids or PhIO. Very recently, Balch and associates succeeded in recording the low-temperature NMR spectrum of the species formed in the reaction of Fe^{3+}–TMP and *t*-BuOOH.[16] Its spectral properties suggest it is the ferryl–oxo complex analogous to **2**, the product of homolytic hydroperoxide scission. Consistent with this, our results demonstrate that Fe^{3+}–TPP converts 10-OOH-18:2 to products derived exclusively from homolytic scission. However, our results also indicate that the balance between homolytic and heterolytic scission is a sensitive

(20) (a) Yuan, L.-C.; Bruice, T. C. *J. Am. Chem. Soc.* **1986**, *108*, 1643–1650. (b) Collman, J. P.; Brauman, J. I.; Meunier, B.; Hayashi, T.; Kodadek, T.; Raybuck, S. A. *J. Am. Chem. Soc.* **1985**, *107*, 2000–2005. (c) Collman, J. P.; Brauman, J. I.; Meunier, B.; Raybuck, S. A.; Kodadek, T. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 3245–3248.

(21) (a) Traylor, T. G.; Lee, W. A.; Stynes, D. V. *J. Am. Chem. Soc.* **1984**, *106*, 755–764. (b) Walker, F. A.; Lo, M-W.; Ree, M. T. *J. Am. Chem. Soc.* **1976**, *98*, 5552–5560. (c) Tondreau, G. A.; Sweigart, D. A. *Inorg. Chem.* **1984**, *23*, 1060–1065. (d) Doeff, M. M.; Sweigart, D. A. *Inorg. Chem.* **1982**, *21*, 3699–3705. (e) Harel, Y.; Felton, R. H. *J. Chem. Soc., Chem. Commun.* **1984**, 206–207.

(22) Bruice, T. C.; Balasubramanian, P. N.; Lee, R. W.; Lindsay Smith, J. R. *J. Am. Chem. Soc.* **1988**, *110*, 7890–7892.

(23) (a) Marnett, L. J.; Weller, P. E.; Battista, J. R. In *Cytochrome P-450*; Ortiz de Montelano, P. R., Ed.; Plenum: New York, 1986; pp 29–76. (b) Nordblom, G. D.; White, R. E.; Coon, M. J. *Arch. Biochem. Biophys.* **1976**, *175*, 524–533. (c) Hrycay, E. G.; O'Brien, P. J. *Arch. Biochem. Biophys.* **1971**, *147*, 28–35. (d) McMurry, T. J.; Groves, J. T. In *Cytochrome P-450*; Ortiz de Montelano, P. R., Ed.; Plenum: New York, 1986; pp 1–28. (e) Capdevilla, J.; Estabrook, R. W.; Prough, R. A. *Arch. Biochem. Biophys.* **1980**, *200*, 186–195.

(24) (a) Lambeir, A. M.; Markey, C. M.; Dunford, H. B.; Marnett, L. J. *J. Biol. Chem.* **1985**, *260*, 14894–14986. Kulmacz, R. J.; Tsai, A.-L.; Palmer, G. *J. Biol. Chem.* **1987**, *262*, 10524–10531.

(25) Ingold, K. U. *J. Phys. Chem.* **1960**, *64*, 1636–1642.

(26) (a) Job, D.; Dunford, H. B. *Can. J. Biochem.* **1977**, *55*, 804–811. (b) Critchlow, J. E.; Dunford, H. B. *J. Biol. Chem.* **1972**, *247*, 3702–3713. (c) Job, D.; Dunford, H. B. *Eur. J. Biochem.* **1976**, *66*, 607–614. (d) Dunford, H. B.; Adeniran, A. J. *Arch. Biochem. Biophys.* **1986**, *251*, 536–542.

function of the environment of the heme (ligand structure, electron density, acid-base properties, etc.). Therefore, it is not surprising that both pathways operate exclusively or simultaneously in a variety of hemeproteins.

Acknowledgment. This project was supported by a research grant from the National Institutes of Health (CA 47479). We are indebted to Jan Crowley for conducting some of the epoxidation experiments. NMR and mass spectra were obtained in the Central Instrument Facility of the Chemistry Department at Wayne State University. We thank John Groves, Alan Balch, and Teddy Traylor for communication of unpublished results.

^{1}H NMR Spectra and Electron-Transfer Properties of Oxidized and Reduced [Fe_4Se_4] Derivatives of *Clostridium vinosum* High-Potential Iron Protein

M. Sola,† J. A. Cowan, and Harry B. Gray*

Contribution No. 7873 from the Arthur Amos Noyes Laboratory, California Institute of Technology, Pasadena, California 91125. *Received December 22, 1988*

Abstract: The high-potential iron protein (HiPIP) from *Clostridium vinosum* has been reconstituted with a [Fe_4Se_4] cluster. The modified protein has a reduction potential of 321 (7) mV vs NHE [μ 0.1 M phosphate; pH 7.0; 23 °C]. The upfield and downfield isotropically shifted resonances in the ^{1}H NMR spectra of both the oxidized and reduced proteins are assigned to β-CH_2 protons of coordinating cysteine residues and to neighboring aromatics. The variable-temperature behavior of each of these resonances is reported. Unlike the 2[Fe_4Se_4]Fd's from *C. pasteurianum* and *C. acidi-urici*, no evidence was found for the involvement of higher spin states. HiPIP self-exchange electron-transfer rate constants have been estimated from T_1 measurements: native, 1.7 (4) × 10^4; Se-modified, 7 (2) × 10^4 M^{-1} s^{-1} [μ 0.1 M phosphate; pH 7.0 (D_2O); 25 °C].

The EPR, Mossbauer, and NMR spectral features of the selenium-substituted ferredoxins (2[Fe_4Se_4]Fd's) from *C. pasteurianum* and *C. acidi-urici* have been interpreted in terms of a mixture of spin states ($S = {}^1/_2$, $S = {}^3/_2$, ${}^7/_2$) for each cluster, both in frozen solution and at ambient temperature.[1,2] Since only the $S = {}^1/_2$ state is observed for the modified [Fe_4Se_4]Fd from *Bacillus stearothermophilus*,[1] it has been inferred that the available spin states following substitution of sulfur by selenium are determined by the interaction between the cluster(s) and the polypeptide chain. This conclusion is generally supported by the close similarity of the EPR and NMR parameters for the synthetic analogues [$Fe_4X_4(SR)_4$]$^{2-,3-}$ (X = Se, S),[3,4] although recent work[5] has identified a $S = {}^3/_2$ ground state for [$Fe_4Se_4(SPh)_4$]$^{3-}$. It has been suggested that a similar type of cluster-polypeptide interaction is responsible for the different redox properties of [Fe_4S_4] low-potential and high-potential ferredoxins.[6-8] The substitution of selenium for sulfur might, therefore, be useful in determining the structural basis for differences in the physical properties of the cluster center between these two classes of proteins. In this paper, we report the ^{1}H NMR spectra and certain electron-transfer properties of oxidized and reduced [Fe_4Se_4] derivatives of *C. vinosum* high-potential iron protein (HiPIP). A study of the resonance Raman spectra of these Se derivatives already has appeared.[9]

Experimental Section

The preparation of the apoprotein and reconstitution with [Fe_4Se_4] were carried out using a modification of standard procedures.[10,11] A sample of HiPIP (20 mg) from *C. vinosum* [ATCC No. 17899 (strain D)] in μ 0.1 M Tris (pH 8, 7 mL) was added to a similar volume of trichloroacetic acid (32%) and stirred at 0 °C for 2 h under aerobic conditions. The precipitate was centrifuged (13 000 rpm, 10 min) and washed with trichloroacetic acid (5%). After dissolving in μ 0.1 M Tris (pH 9, 4 mL), the solution was dialyzed overnight against μ 0.1 M Tris (pH 8). Subsequent manipulations were carried out under an argon atmosphere. The solution of apoprotein (4 mL) was treated with a 50-fold excess of dithiothreitol (DTT) and left stirring for 30 min at room temperature. A 20-fold excess of Fe^{3+} (as a solution of $FeCl_3$) and Se^{2-} (prepared immediately before use)[11] was then added. After 1 h at ambient temperature, the solution was eluted through a DE-52 column equilibrated with μ 0.1 M Tris (pH 8), and the protein was eluted with the same buffer. A black band remained at the top of the column, while the reconstituted protein was eluted immediately following an orange band of inorganic reagents. Minor residual traces of these reagents were removed by three cycles of dilution/ultrafiltration with μ 0.1 M phosphate buffer (pH 7.0).

The electronic absorption spectrum of the Se-HiPIP is very similar to that of the native protein[12] in both the oxidized and reduced states [A_{red}(native) 283 and 388 nm, A_{red}(Se) 283 and 384 nm; A_{ox}(native) 283 nm, A_{ox}(Se) 283 nm]. In comparison with the selenium derivative of Fd from *C. pasteurianum*,[11] the Se-HiPIP is remarkably stable under aerobic conditions at ambient temperature. A half-life of 10 days was estimated by following the decrease in absorbance at 283 nm. The stability is greatly increased by maintaining the protein under an inert atmosphere (Ar) at 4 °C. Thus, the stability of the Se derivative does not differ significantly from that of the native species.

NMR samples were prepared by washing the protein several times with a μ 0.1 M phosphate buffer, made up in D_2O (pH 7.2, uncorrected

† Present address: Department of Chemistry, University of Modena, Via Campi 183, 41100 Modena, Italy.

(1) Gaillard, J.; Moulis, J.-M.; Auric, P.; Meyer, J. *Biochemistry* **1986**, *25*, 464-468.
(2) Gaillard, J.; Moulis, J.-M.; Meyer, J. *Inorg. Chem.* **1987**, *26*, 320-324.
(3) Bobrick, M. A.; Laskowski, E. J.; Johnson, R. W.; Gillum, W. O.; Berg, J. M.; Hodgson, K. O.; Holm, R. H. *Inorg. Chem.* **1978**, *17*, 1402-1410.
(4) Laskowski, E. J.; Reynolds, J. G.; Frankel, R. B.; Foner, S.; Papaefthymiou, G. C.; Holm, R. H. *J. Am. Chem. Soc.* **1979**, *101*, 6562-6570. Laskowski, E. J.; Frankel, R. B.; Gillum, W. O.; Papaefthymiou, G. C.; Renaud, J.; Ibers, J. A.; Holm, R. H. *J. Am. Chem. Soc.* **1978**, *100*, 5322-5337. Reynolds, J. G.; Laskowski, E. J.; Holm, R. H. *J. Am. Chem. Soc.* **1978**, *100*, 5315-5322.
(5) Carney, M. J.; Papaefthymiou, G. C.; Whitener, M. A.; Spartahan, K.; Frankel, R. B.; Holm, R. H. *Inorg. Chem.* **1988**, *27*, 346-352.
(6) Carter, C. W., Jr.; Kraut, J.; Freer, S. T.; Alden, R. A.; Sieker, L. C.; Adman, E.; Jensen, L. H. *Proc. Natl. Acad. Sci. U.S.A.* **1972**, *69*, 3526-3529.
(7) Palmer, G. In *The Enzymes*, 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1975; Vol XII, part B, pp 1-56.
(8) Sheridan, R. P.; Allen, L. C.; Carter, C. W., Jr. *J. Biol. Chem.* **1981**, *256*, 5052-5057.
(9) Moulis, J.-M.; Lutz, M.; Gaillard, J.; Noodleman, L. *Biochemistry* **1988**, *27*, 8712-8719.
(10) Rabinowitz, J. C. *Methods Enzymol.* **1972**, *24B*, 431-446.
(11) Moulis, J.-M.; Meyer, J. *Biochemistry* **1982**, *21*, 4762-4771.
(12) Bartsch, R. G. *Methods Enzymol.* **1978**, *53*, 329-340.

0002-7863/89/1511-6627$01.50/0