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Chemical mechanisms for cytochrome P-450 oxidation: Spectral and catalytic properties of a manganese-substituted protein

(cytochrome P-450 mechanism/metalloporphyrin reconstitution/manganese(V)–oxo complex/olefin epoxidation)

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ABSTRACT **Bacterial cytochrome P-450 induced by camphor ($P\text{-}450_{cam}$) is reconstituted with manganese–protoporphyrin IX, yielding an enzyme that displays unique spectral properties relative to previously characterized manganese–porphyrin systems. The nitric oxide complex of the manganese(II)–protein shows a *hyper*-metalloporphyrin spectrum suggestive of thiolate ligation to the porphyrin-bound manganese ion. In the presence of iodosobenzene as a source of active oxygen, manganese-substituted cytochrome $P\text{-}450_{cam}$ serves as a catalyst for the epoxidation of an enzyme-bound olefin substrate. This reactivity proceeds through a spectrally detectable intermediate that resembles the manganese(V)–oxo complexes that have been well documented with model systems employing artificial manganese–metalloporphyrins in organic solution. Interestingly, manganese-substituted cytochrome-$P\text{-}450_{cam}$ shows no hydroxylation activity either in the reconstituted camphor hydroxylase system with pyridine nucleotide or in the presence of iodosobenzene and the Mn(III) form of the protein.**

A wide variety of biological oxidations are catalyzed by the cytochrome P-450 monoxygenase system whereby molecular dioxygen bond scission and two reducing equivalents are coupled to substrate oxygenation and water formation (1, 2). Bacterial cytochrome P-450 induced by camphor ($P\text{-}450_{cam}$), isolated from *Pseudomonas putida*, catalyzes both the 5-*exo*-hydroxylation of camphor and the *exo*-epoxidation of dehydrocamphor (3) in the presence of oxygen, NADH, and two additional protein components (putidaredoxin and NADH putidaredoxin reductase) that supply the P-450 with the necessary two electrons required for the overall reaction. One feature that distinguishes cytochrome P-450 from other *b*-type hemoproteins is the apparent presence of an axially bound thiolate ligand to the heme iron (4). Thiolate coordination is thought to be responsible for the unusual spectral properties of the P-450 enzymes in comparison to nitrogen-coordinated hemoproteins such as hemoglobin and horseradish peroxidase (5–9).

Recent investigations have focused on the complete elucidation of all of the chemical steps involved in substrate processing, oxygen—oxygen bond scission, and identification of the higher oxidation states of the heme iron reaction center. Model systems in organic solvents that employ artificial metalloporphyrins and a variety of oxidants have documented the existence and nature of some of these higher oxidation states. Chromium(III)- and manganese(III)-substituted porphyrins undergo reaction with oxidants such as iodosobenzene or *m*-chloroperbenzoic acid to form spectrally distinct intermediates that decompose in the presence of hydrocarbons to regenerate the metalloporphyrin in the 3^+ oxidation state with concomitant hydrocarbon oxygenation (10–12). For example, addition of iodosobenzene to chloroaquo(*meso*-tetraphenylporphinato)manganese(III) [Mn(III)Ph_4PorCl] in dichloromethane produces a strong oxidant capable of transferring oxygen to a hydrocarbon acceptor (11, 12). Physical studies including magnetic susceptibility (12) and ESR measurements (13) are consistent with a two electron oxidation of the metal:

$$\text{Mn(III)Ph}_4\text{PorCl} + \text{iodosobenzene} \rightarrow \text{O=Mn(V)Ph}_4\text{PorCl} + \text{iodobenzene}$$

$$\text{O=Mn(V)Ph}_4\text{PorCl} + \text{R} \rightarrow \text{Mn(III)Ph}_4\text{PorCl} + \text{RO}.$$

Transfer of oxygen to substrate (R) has been written as a single step but it is likely to be composed of at least two distinct reactions (11, 12). The Fe(III)Ph_4PorCl/iodosobenzene system is also reported to be effective in catalyzing hydrocarbon oxygenation reactions (14, 15). Recently, the higher oxidation states of iron metalloporphyrins have been partially characterized, although it remains to be established whether the oxidation equivalents in the iron-oxo species reside on the metal, the porphyrin, or both (16). These oxygenating intermediates of the iron systems are considerably more reactive than the chromium or manganese analogs, with chemical characterization requiring experimentation at very low temperature.

Attempts to observe a higher valence state of the cytochrome P-450 hemoprotein so far have been unsuccessful, although a variety of studies suggests intermediates on the reaction path to substrate oxygenation (17–21). A possible link between the studies with model porphyrin systems and the native cytochromes P-450 comes from the observation that a variety of exogenous oxidants, such as iodosobenzene, can effectively mimic the oxygen-dependent reaction by supplying the enzyme with an oxygen atom and the proper number of electrons in a single step (22, 23). Several investigators have proposed that a metal-oxo species similar to that found in chromium and manganese model systems may be involved in cytochrome P-450-dependent reactions.

In this investigation we report the preparation of manganese-substituted cytochrome $P\text{-}450_{cam}$ (Mn-P-450) using the previously reported technology for heme removal and reconstitution of P-450 (24, 25). Characterization of Mn-P-450 has revealed unique spectral properties compared to other manganese-substituted hemoproteins implicating thiolate ligation to the manganese metal. Furthermore, Mn-P-450 forms an easily observable spectral intermediate on reaction with iodosobenzene which shows striking similarity to previously reported Mn(V)–oxo complexes. Interestingly, Mn-P-450 is found to catalyze exog-

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Abbreviations: $P\text{-}450_{cam}$, cytochrome P-450 induced by camphor; Mn-P-450, manganese-substituted cytochrome $P\text{-}450_{cam}$; Mn(III)Ph_4PorCl, chloroaquo(*meso*-tetraphenylporphinato)manganese(III).

enous oxidant-dependent epoxidation but not hydroxylation reactions.

MATERIALS AND METHODS

$P\text{-}450_{cam}$, putidaredoxin, and NADH putidaredoxin reductase were purified as described from 2 kg of frozen cell paste (*P. putida* ATCC 29607) (26). Protoporphyrin IX was prepared by dehydration of commercially available hematoporphyrin IX hydrochloride (Sigma) in refluxing dimethylformamide (27) and metallated with manganese(II) acetate monohydrate by the general procedure of Adler *et al.* (28). The crude metalloporphyrin was purified by chromatography on polyamid resin (Machery and Nagel, 0.07 mm, Brinkmann) by using benzene/methanol/90% formic acid, 110:30:1 (vol/vol), as the developing solvent (27). $Mn(III)Ph_4PorCl$ was prepared by metallating *meso*-tetraphenylporphine (Aldrich) with manganese(II) chloride tetrahydrate in dimethylformamide and purified by column chromatography on basic alumina (28). Dehydrocamphor was kindly provided by Pentti Mälkönen. Iodosobenzene was prepared as described (21). Nitric oxide (Linde) was passed through 1 M KOH to remove higher oxidation products.

Apoprotein $P\text{-}450_{cam}$ was prepared by the acidified acetone procedure of Yu and Gunsalus (24). Twenty-five milliliters of reagent-grade acetone was acidified by adding 50 μl of 12 M HCl. The solution was placed in a Potter–Elvehjem homogenizer and cooled to below −20°C by immersion in a bath of dry ice/ethanol. One milliliter of holoenzyme solution (1.4 mM) in 50 mM KP_i, pH 7.0/200 μM camphor was added with constant stirring. Homogenation was continued for 2 min as the protein solution became turbid. The mixture was transferred to a stainless steel tube and centrifuged at 17,000 × *g* for 10 min at −15°C. The red supernatant was decanted and the white protein pellet was resuspended in 25 ml of precooled acetone and recentrifuged as above. After decanting, the tube was evacuated to remove remaining acetone and the pellet was dissolved in degassed 0.06 M NH_4OH (5 ml) at 4°C. All steps involving acetone must be carried out below −20°C and as quickly as possible. Apoprotein was dialyzed against water for 6 hr at 4°C under argon and then overnight against 50 mM KP_i, pH 7.0/20% (vol/vol) glycerol. Reconstitution reactions were carried out on 0.1 mM apoprotein samples in 50 mM KP_i, pH 7.0/20 mM dithiothreitol/20% (vol/vol) glycerol and initiated by the addition of 1.5 equivalents of Mn(III)protoporphyrin IX acetate. A few milligrams of the metalloporphyrin was dissolved in 50 μl of 0.1 M KOH, diluted to 0.5 ml with water, and added to the reconstitution reaction over a period of 10 min. The vessel was gassed with argon and gently agitated at 20°C for 24 hr. Reconstituted protein was purified by the procedure of Wagner *et al.* (25). Total protein was measured by using a modified Lowry assay (29), residual holo-P-450 by carbon monoxide difference spectroscopy (26), and manganese content by atomic absorption spectroscopy. Apoprotein $P\text{-}450_{cam}$ was quantitated from the absorbance at 280 nm by using an extinction coefficient of 48 $mM^{-1}cm^{-1}$ (25).

Reduced Mn(II)-P-450 was prepared either by reduction with sodium dithionite or by photoreduction (30). The reduced NO complex of Mn-P-450 was prepared by adding NO gas to a Thundberg cuvette under rigorously anaerobic conditions followed by photoreduction of the mixture. Samples for ESR studies were prepared by dithionite reduction as monitored optically *in situ*. Redox potentials were measured at 20°C as reported (31) on 5 μM samples of Mn-P-450 in 1 ml of 100 mM KP_i, pH 7.0/50 mM EDTA/200 μM camphor/8 μM safranine-T (−289 mV). Fractions of oxidized protein and dye were determined by absorbance measurements at 410 and 540 nm, respectively. NADH-driven turnover reactions were carried out in 1 ml of 100 mM KP_i, pH 7.0/200 μM camphor/2.5 μM reductase/3.7 μM putidaredoxin/100–200 nmol of NADH and initiated by the addition of the P-450 component. Iodosobenzene-dependent turnovers were carried out in 1 ml of 100 mM KP_i at pH 7.0 containing 5 μM cytochrome P-450 and 200 μM substrate (camphor or dehydrocamphor) and initiated by the addition of 10 μl of 100 mM iodosobenzene in methanol. After 1 hr, excess iodosobenzene was destroyed by the addition of a small amount of solid sodium bisulfite and 10 nmol of camphoroquinone (Aldrich) was added as an internal standard. The mixture was extracted three times with chloroform, concentrated, dried, and analyzed for product by gas chromatography on a 6-ft (1.8 m) OV-17 column (Supelco) at 160°C. All optical spectra were recorded on a Cary 219 spectrophotometer either at 20°C or at −20°C with a thermoelectric cooling apparatus (32). ESR spectra were obtained on a Varian E-4 instrument fitted with an Air Products nitrogen gas cooler.

RESULTS AND DISCUSSION

Reconstitution of apoprotein $P\text{-}450_{cam}$ with aquo(acetato)-manganese(III) protoporphyrin IX followed by purification resulted in a 20% overall yield of Mn-P-450. Because manganese-substituted hemoproteins, including Mn-P-450, do not bind carbon monoxide (33), the amount of native cytochrome-P-450 remaining in the Mn-P-450 preparation was measured by CO difference spectroscopy and constituted only 1–2% of the total protein. The manganese/protein ratio of purified Mn-P-450 was determined from a combination of atomic absorption and protein measurements and found to be 0.91 ± 0.05 (±SEM). The purified protein had an absorbance ratio (A_{378}/A_{280}) (±SEM) of 1.0 ± 0.06 and this value was used to monitor the purity of the enzyme. Atomic absorption analysis of a protein solution of known absorbance yielded an extinction coefficient (±SEM) of 103 ± 8 $mM^{-1}cm^{-1}$ at 378 nm.

The optical spectrum of Mn(III)-P-450 is shown in Fig. 1 along with a spectrum of aquo(acetato)manganese(III) protoporphyrin IX under identical conditions. The Soret band of the protein is red shifted from that of free heme (378 vs. 370 nm) and significant differences in the 450- to 650-nm regions of the spectra are also observed. Absorption spectra of high spin manganese(III) porphyrins are unusual, relative to most metalloporphyrin spectra, containing a strong band near 470 nm. Boucher (34) has assigned the band near 370 nm to the Soret transition, the bands in the 500- to 625-nm region to the α- and β-transitions, and the strong band near 470 nm to a ligand-to-metal charge transfer. The spectrum of Mn(III)-P-450 has no band near 470 nm but shows three bands at 496, 516, and 560 nm. Although absolute assignments cannot be made, this is consistent with a shift in the charge transfer band from the free porphyrin value of 465 nm to higher wavelength. The spectrum of the reduced protein obtained by dithionite addition to Mn(III)-P-450 is given in Fig. 1. Several minutes were required for complete reduction, in contrast to the rapid reduction of native $P\text{-}450_{cam}$. The Soret maximum of the reduced protein is blue shifted compared to the manganese(III)-free heme Soret band (412 vs. 420 nm) and only two bands are observed in the 450- to 650-nm region.

These spectral data for Mn(III)-P-450 and Mn(II)-P-450 together with previously published data for various manganese-substituted hemoproteins are summarized in Table 1. Mn-P-450 is clearly unique because all other manganese(III)-substituted hemoproteins show an intense charge transfer band near 470 nm. Although azide is known to alter the spectrum of some manganese(III)-substituted hemoproteins by coordinating to the metal ion (35, 38), no spectral change occurred with Mn(III)-

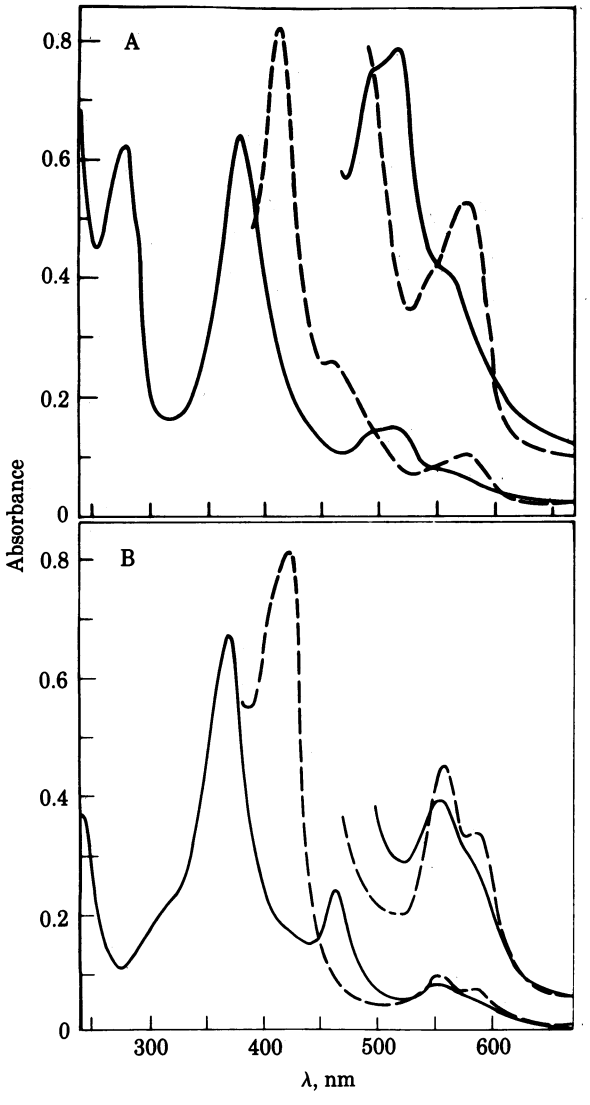

FIG. 1. Optical absorption spectrum of (*A*) 6 μM Mn(III)-P-450 in 100 mM KP_i at pH 7.0 (solid line) and Mn(II)-P-450 (dashed line) and (*B*) aquo(acetato)manganese(III) protoporphyrin IX (solid line) and the Mn(II) porphyrin (dashed line). Spectra were obtained as described.

P-450 in the presence of 10 mM sodium azide. Addition of camphor to Mn(III)-P-450 yielded no observable spectral change, although substrate binding to the native Fe(III)-protein is known to produce significant spectral changes correlated with a low-spin to high-spin transition of the heme iron (31). Manganese(III) porphyrins are high spin even in ligand fields of moderately high strength and one might not expect a change in spin state upon substrate binding. Mn(II)-P-450 fails to form a spectrally detectable complex with atmospheric oxygen but oxidizes directly to Mn(III)-P-450. Oxygenation of an anaerobic solution of the reduced protein at −20°C had little effect on the spectrum, but rapid oxidation back to Mn(III)-P-450 was observed on warming. Manganese-substituted hemoglobin and myoglobin also do not form stable complexes with oxygen (39) and it has been suggested that the required displacement of the axially coordinated sixth ligand to the porphyrin-bound metal may not be favored (39).

Addition of nitric oxide had no effect on the spectrum of Mn(III)-P-450, although the reduced form did react rapidly with NO to form a spectrally distinct complex (Fig. 2). This optical spectrum of the NO complex is very similar to the CO complex of ferrous P-450 (6), showing an apparent hyperspectrum with a red-shifted Soret band at 447 nm and a near-UV band at 364 nm (Table 1). In contrast, nitric oxide complexes of manganese(II)-horseradish or cytochrome *c* peroxidase, hemoglobin, and myoglobin (37) all show a normal Soret absorbance near 430 nm. A molecular orbital model for the origin of the carboxycytochrome P-450 hyperspectrum has been presented and demonstrates the critical role of thiolate coordination to the metal ion in determining the optical properties of the complex (6). The nitric oxide complex of ferrous cytochrome P-450 has also been shown to display a hyperspectrum (40), and model studies with porphyrins in solution have confirmed that ligation of a thiolate anion, rather than a thiol, to the heme iron is necessary to mimic the observed optical properties of nitrosyl cytochrome P-450 (41).

These results suggest that the observed hyperspectrum for NO–Mn(II)-P-450 is a result of thiolate ligation to the porphyrin-bound manganese. The ESR spectrum of Mn(II)-P-450 was obtained to compare the results with previously reported spectra for other manganese-substituted hemoproteins. X-band spectra recorded on 300-μM protein samples at 130 K were almost featureless (spectra not shown), presumably as a result of extensive line broadening. Such broadening could be due to a highly anisotropic zero-field contribution to the spin hamiltonian for the high spin d^5 system. Highly broadened ESR spectra have been observed with Mn(II) phosphoglucomutase complexes (42) making their ESR spectra difficult to observe even with millimolar amounts of protein. Such spectra differ markedly from the ESR spectra of manganese-substituted hemoglobin and peroxidase (43), which show X-band spectra similar to their native high spin ferric proteins.

Table 1. Light absorption maxima of manganese protoporphyrin IX-substituted hemoproteins

Protein	Absorption maxima, nm		Ref.
	Soret	Additional bands	
Mn(III)-P-450	378	496, 516, 560	This work
Mn(III)-cytochrome *c* peroxidase	377	483, 568, 605	35
Mn(III)-horseradish peroxidase	373	482, 564, 584	35
Mn(III)-myoglobin	373–377	471, 556	35, 36
Mn(III)-protoporphyrin IX	370	465, 556	35; this work
Mn(II)-P-450	412	548, 576	This work
Mn(II)-cytochrome *c* peroxidase	439	563, 595	35
Mn(II)-horseradish peroxidase	412	509, 624	35
Mn(II)-myoglobin	438–440	560, 595	35, 36
Mn(II)-protoporphyrin IX	420	547–556, 577–588	35; this work
Mn(II)-NO-P-450	447	364, 558, 588	This work
Mn(II)-NO-cytochrome *c* peroxidase	427	543, 572	37
Mn(II)-NO-hemoglobin	433	538, 580	37

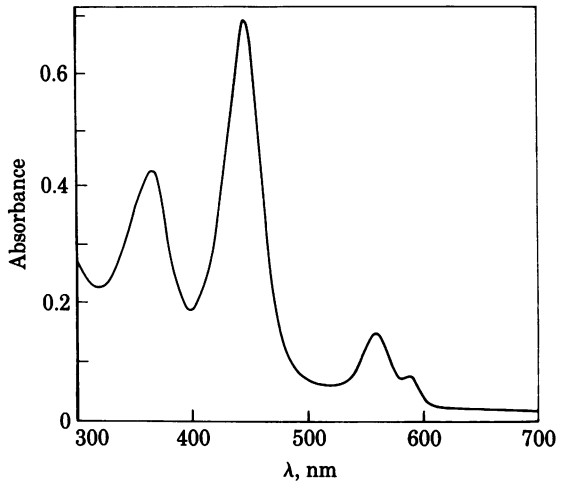

FIG. 2. Optical absorption spectrum of the nitric oxide complex of Mn(II)-P-450 (6.4 μM).

Mn-P-450 was not active in the promotion of NADH oxidation in the reconstituted hydroxylation system consisting of flavoprotein, putidaredoxin, NADH, and camphor. If the redox potential for the Mn(III)/Mn(II) couple is too negative, electron transfer from reduced putidaredoxin to Mn(III)-P-450 could be unfavorable, preventing coupled NADH oxidation. This possibility seems likely because high spin manganese-substituted porphyrins are known to reduce less easily than the corresponding high spin ferric porphyrins (44). To ascertain the validity of this interpretation, the redox potential of Mn-P-450 was measured. The value observed from standard Nernst analysis, −260 mV, is considerably more negative than the Fe-P-450 system with camphor bound [−173 mV (31)]. The redox potential of putidaredoxin, when bound to the P-450 macromolecule, is −196 mV (31) and hence, electron flow from reduced putidaredoxin to Fe-P-450 is much more favored than transfer to Mn-P-450, and these simple redox potential differences could account for the observed lack of pyridine nucleotide oxidation.

It has been well documented that addition of exogenous oxidants such as iodosobenzene to Mn(III)Ph_4PorCl leads to the formation of a Mn(V)–oxo metalloporphyrin complex characterized by dramatic optical changes (11, 12). Therefore, we investigated the reaction of Mn(III)-P-450 with iodosobenzene to determine whether such a system is catalytically active towards hydroxylation or epoxidation (or both) of enzyme-bound substrates. Upon addition of iodosobenzene to a solution of Mn(III)-P-450, a new spectral species immediately formed (Fig. 3). This new species is characterized by a red shift in the Soret band from 378 to 410 nm and a broadening of the bands in the 450- to 600-nm region, very similar to that described for the Mn(V)Ph_4Por–oxo complex, which displays a Soret maximum at 420 nm and broadening of the visible bands (11, 12) (Fig. 3). It seems likely that oxygen atom transfer from iodosobenzene to manganese has occurred to generate a protein-bound Mn(V)–oxo complex. Reaction between Mn(III)-P-450 and iodosobenzene only occurred when excess oxidant was added, and under these conditions rapid porphyrin destruction occurred, presumably as a result of iodosobenzene-dependent porphyrin oxidation.

Fig. 3 also suggests that a small amount of free manganese porphyrin is released from the protein after reaction with iodosobenzene, because two small absorbances at 370 and 465 nm were observed in the spectrum. Oxidation of Mn(III)Ph_4PorCl with iodosobenzene can also lead to irreversible heme degradation (11), although the reaction is much less rapid than that

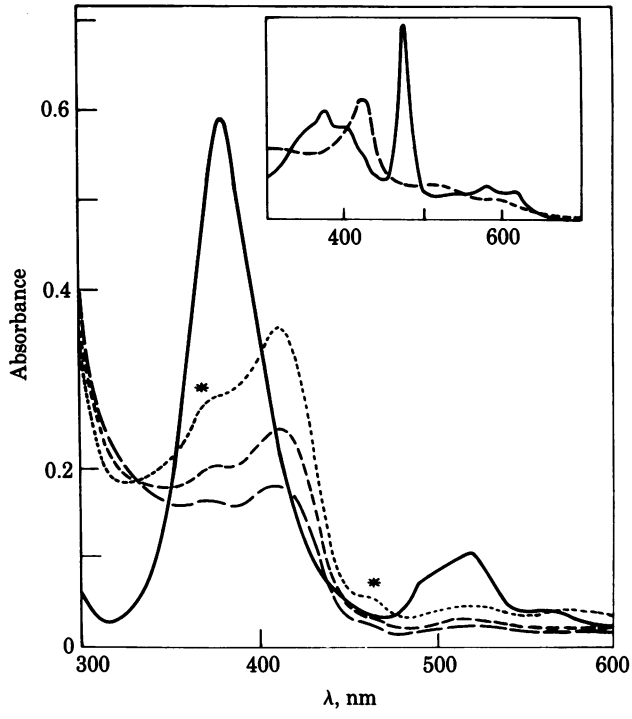

FIG. 3. Addition of iodosobenzene to a 5.7 μM solution of Mn(III)-P-450 (solid line) in 100 mM KP_i at pH 7.0 to produce the Mn(V)–oxo complex (dashed line). Three spectra of the oxo complex taken at 1-min intervals are shown to illustrate the porphyrin destruction caused by the iodosobenzene-dependent oxidation of the protoporphyrin IX ligand. Peaks marked with an asterisk refer to the small amount of free manganese porphyrin generated during the reaction. (*Inset*) The absorbance spectrum of Mn(III)Ph_4PorCl (solid line) in dichloromethane and of the Mn(V)–oxo complex (dashed line) formed by the addition of iodosobenzene.

of the protoporphyrin IX ligand due to the steric hindrance of oxidant approach by the bulky phenyl groups. Addition of iodosobenzene to a solution of aquo(acetato)manganese(III) protoporphyrin IX—under conditions identical to those used for Mn-P-450 oxidation—led to extremely rapid porphyrin destruction, although a small absorption shoulder was observed near 420 nm, suggestive of Mn(V)–oxo complex formation (spectrum not shown). Thus, it seems likely that the protein provides substantial protection to oxidative attack on the porphyrin ring.

The catalytic competency of the Mn(III)-P-450/iodosobenzene system was tested by forming the Mn(V)–oxo complex in the presence of substrate. Iodosobenzene was added to solutions of Mn(III)-P-450 in the presence of either camphor or dehydrocamphor and the formation of the Mn(V)–oxo complex was monitored by optical spectroscopy. After 1 hr, no porphyrin absorbance remained and the mixture was analyzed for product by gas chromatography (Table 2). Although the Fe(III)-P-450/iodosobenzene system supports both the 5-*exo*-hydroxylation

Table 2. Iodosobenzene-supported oxygenation reactions with $P\text{-}450_{cam}$

Enzyme	Substrate	Product yield, nmol/nmol of enzyme*
Fe(III)-P-450	Camphor	4.1
Fe(III)-P-450	Dehydrocamphor	4.3
Mn(III)-P-450	Camphor	<0.02
Mn(III)-P-450	Dehydrocamphor	2.3

* 5-*exo*-Hydroxycamphor and 5,6-*exo*-epoxycamphor formation with camphor and dehydrocamphor, respectively, as substrate.

of camphor (21) and the *exo*-epoxidation of dehydrocamphor (3), the Mn(III)-P-450/iodosobenzene system was active *only* in the epoxidation reaction. Epoxidation by direct transfer of oxygen from iodosobenzene to dehydrocamphor does not occur in control reactions in the absence of enzyme. We conclude that Mn-P-450 has some catalytic activity towards the epoxidation of an olefinic substrate although this reactivity is less than the analogous reactivity of native cytochrome P-450 (Table 2).

One possible explanation for the difference in epoxide product yield for the different enzymes comes from model studies in which it has been shown that the Mn(V)–oxo complexes of manganese porphyrins are much more stable than the oxo complexes with iron porphyrins (16). The sluggish reactivity of the Mn(V)–oxo complex of Mn-P-450 in oxygen transfer to substrate allows spectral observation of the higher valence state of the porphyrin, although diminished product yield may be a result of side porphyrin destruction reactions in the presence of oxidant. In the case of the Fe(III)-P-450/iodosobenzene system, no stable oxo complex formation is observed, although substrate oxygenation occurs to a greater extent during the time necessary to destroy the heme cofactor.

The reasons for the absence of a hydroxylation reactivity for Mn-P-450 are not clear from the present study. It is possible that the steps involved in oxygenation of a carbon—hydrogen bond are slow compared to the steps involved in oxygenation of an olefinic substrate, and, if the difference in these rates is of the same order as the cofactor destruction rate, no substrate hydroxylation will be observed. No products were formed when iodosobenzene was added to free Mn(III) protoporhyrin IX solutions in the presence of dehydrocamphor or camphor up to metalloporphyrin concentrations of 10 times that used in the enzyme studies. In this case, it is likely that the very rapid oxidation of the unprotected porphyrin prevents substrate oxygenation from occurring.

In summary, we have successfully reconstituted apoprotein $P\text{-}450_{cam}$ with manganese protoporphyrin IX. The unusual spectral properties of the manganese-substituted hemoprotein are consistent with thiolate ligation to the porphyrin-bound manganese. Our results suggest that Mn-P-450 reacts with iodosobenzene to form an easily observable Mn(V)–oxo complex, showing similar spectral properties to the previously characterized Mn(V)–oxo complex of tetraphenylporphyrin and that this higher valence state of the enzyme-bound cofactor can proceed through an epoxidation, but not a hydroxylation, reaction pathway. A similar higher valence state may be operating as well in native iron-bound cytochrome P-450.

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