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Chemistry

Chemical mechanisms for cytochrome *P*-450 hydroxylation: Evidence for acylation of heme-bound dioxygen

(cytochrome *P*-450 mechanism/acyl peroxide intermediate/oxygen-18/camphor hydroxylation)

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ABSTRACT **Using isotopic tracer methods, we have shown that dihydrolipoic acid (2,3-thioctic acid) acylates the distal oxygen of ferrous oxygenated *Pseudomonas* cytochrome *P*-450, forming a transient acyl peroxide intermediate that facilitates oxygen–oxygen bond cleavage. Single-turnover studies with $^{18}O_2$ indicate one oxygen-18 atom incorporated into the carboxylate group of lipoic acid for each oxygen-18 inserted into the substrate, camphor, forming the product, *exo*-5-hydroxycamphor. Such a branching ratio for label indicates that water is initially released from an unlabeled position and illustrates that the general *P*-450 mixed-function oxidase stoichiometry generates $H_2{}^{18}O$ from $^{18}O_2$ only after multiple-turnover equilibration with the acylating carboxylate oxygen. Formation of an acyl peroxide state is a natural intermediate in peracid, "oxene," or radical mechanisms for methylene carbon oxygenation.**

One of the most critical metabolic pathways for xenobiotics, toxins, and carcinogens is catalyzed by the cytochrome *P*-450 mixed-function oxidases and involves the two-electron reductive cleavage of atmospheric dioxygen with concomitant substrate level hydroxylation (1–7). The *P*-450 hemoprotein, consisting of a single polypeptide chain of approximately 50,000 daltons, with protoporphyrin IX as a prosthetic group, is found ubiquitously in bacterial and eukaryotic organisms in both synthetic pathways, such as adrenal steroid formation, and degradative pathways, such as hepatic biotransformation reaction and camphor catabolism in *Pseudomonas*.

The chemistry of hydroxylation involves heme-iron and oxygen adducts of various redox and oxygen atom stoichiometries. A coherent picture of *P*-450-catalyzed mixed-function oxidation can be presented by integrating the known and postulated *P*-450 intermediates with the detailed elements of peroxidase chemistry (8–14). Focusing on the iron–oxygen states, one can elaborate the coordinated scheme shown in Fig. 1 for both the peroxidases and monooxygenases. Beginning with Fe^{3+} heme, the monoxygenase pathway has sequential steps of ferric–ferrous reduction; $Fe^{3+} + e^- \rightarrow Fe^{2+}$, followed by dioxygen binding to yield the intermediate $[Fe^{2+}O_2]$. This state is analogous to oxygenated hemoglobin and myoglobin, and its existence has been clearly demonstrated in the bacterial *P*-450 system by both rapid reaction methodologies and low-temperature cryoenzymology (15–19). The oxygenated intermediate in hepatic *P*-450 systems was also resolved with intermediates described in both stopped-flow and steady-state experiments (19–22). Difficulty in trapping $[Fe^{2+}O_2]$ in the liver *P*-450 is perhaps due to the increased autoxidative rates and loose coupling in the hepatic systems, resulting in an apparently greater lability of the oxygenated intermediate. The stabilized oxyheme complex of the camphor hydroxylase has been analyzed in detail by numerous methodologies (17, 23–26). In particular, Mössbauer spectroscopy has demonstrated that the iron center in this diamagnetic state has quadrupole splitting and isomer shift values that strongly resemble those of ferric iron (23). For these reasons, the oxygenated intermediate in Fig. 1 is indicated with a superoxide–ferric iron charge distribution.

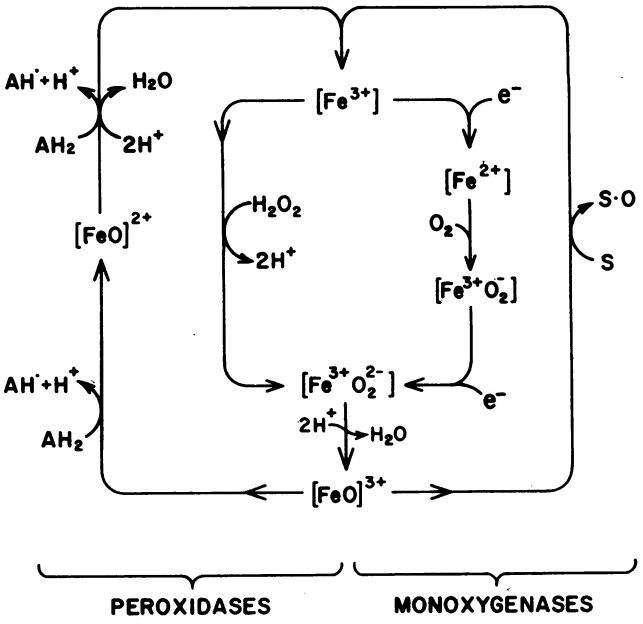

FIG. 1. Iron–oxygen states of *P*-450 and peroxidases.

Events pertaining to the input of the second electron required for oxygen bond cleavage are less well defined. For simplicity in electron and oxygen counting, the hypothetical state $[Fe^{3+}O_2^{2-}]$ represents a precursor that has all the electron equivalents needed for methylene carbon hydroxylation, water production, and regeneration of the ferric *P*-450 resting state. In one proposed scheme, the next step in the catalytic cycle following $[Fe^{3+}O_2^{2-}]$ can be written formally as a loss of water to yield $[FeO]^{3+}$, which can be viewed as an oxene oxygen atom (six valence electrons) bound to ferric heme iron, analogous in terms of redox stoichiometry to the compound I intermediate of peroxidase (25, 27). Considering ferric heme as +1 and water −2, this state has a total formal charge of 3+. Much evidence has been accumulated in recent years concerning the actual distribution of charge in compound I of peroxidases, and a feryl iron with a porphyrin cation radical is favored by some workers (27–30). For the purposes of this paper compound I and the oxene intermediate will be referred to as $[FeO]^{3+}$.

Moving from this intermediate counterclockwise around the scheme in Fig. 1, the ferric resting state of *P*-450 can be regenerated by writing the chemistry of methylene carbon hydroxylation as the transfer of the iron-bound oxene of $[FeO]^{3+}$

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Abbreviation: GC, gas chromatography.

to the carbon–hydrogen bond of the substrate, a process mechanistically analogous to known carbene and nitrene insertion reactions (31). This proposed scheme for *P*-450 oxygenases will be herein termed the "oxene mechanism."

Peroxidases possess many of the same reaction intermediates in regard to overall redox and oxygen-atom stoichiometries as the mixed-function oxidases. For peroxidases, the reaction of ferric heme with hydrogen peroxide conceptually yields $[Fe^{3+}O_2^{2-}]$ directly (32), because peroxide directly carries both the dioxygen and the two electrons needed for the monooxygenase cycle. Production of $[FeO]^{3+}$ again results from water release, whereas reduction back to the $[Fe^{3+}]$ state is accomplished through single-electron reactions with donor molecules through compound II, or $[FeO]^{2+}$. The similarities between the monoxygenase and peroxidase reaction states presented in Fig. 1 imply the possibility of supporting substrate hydroxylation with ferric *P*-450 by using peroxides (or, alternatively, peracids) to donate both oxygen and reducing equivalents. A significant step in the understanding of mixed-function oxidase chemistry occurred when several investigators (8–14, 33, 34) reported the generation of oxygenated substrates under anaerobic conditions with ferric *P*-450 and exogenously supplied oxidants. Subsequent labeling studies using oxygen-18 (10) confirmed that the oxygen atom incorporated into the substrate originated from the peroxide rather than water.

Alternative models to the proposed oxene mechanism for oxygen cleavage during *P*-450-catalyzed monoxygenation can be envisioned that provide chemically definable structures for the active oxygen precursor. Hamilton (35, 36) has suggested that an enzyme-bound peracid or peramide, generated through acylation of bound atmospheric dioxygen, is a likely candidate for the oxenoid precursor involved in direct methylene carbon attack. The role of peracids in supporting anaerobic *P*-450 hydroxylation could thus be due to their direct participation with an activated enzyme–substrate complex, although the general low reactivity of peracids toward carbon–hydrogen bonds indicates that further substrate activation is probably required. Conversely, acylation of bound dioxygen with the formation of an acyl peroxide intermediate can also account for the generation of the $[FeO]^{3+}$ oxene intermediate, through heterolytic cleavage of the oxygen–oxygen bond as illustrated schematically in Fig. 2 (8). The distinguishing feature between the two mechanisms is the actual position of bond rupture. Heterolytically cleaving the acyl peroxide intermediate between the atoms of atmospheric dioxygen (position B in Fig. 2) generates the oxene intermediate with regeneration of the carboxylic acid, whereas breaking the iron-proximal oxygen atom bond (position A in Fig. 2) results in formation of the Hamilton peracid hydroxylating species. Additionally, the acyl peroxide intermediate may be cleaved, with subsequent catalytic hydrogen abstraction from the substrate methylene carbon leading to an "oxygen rebound" mechanism for cytochrome *P*-450 (37).

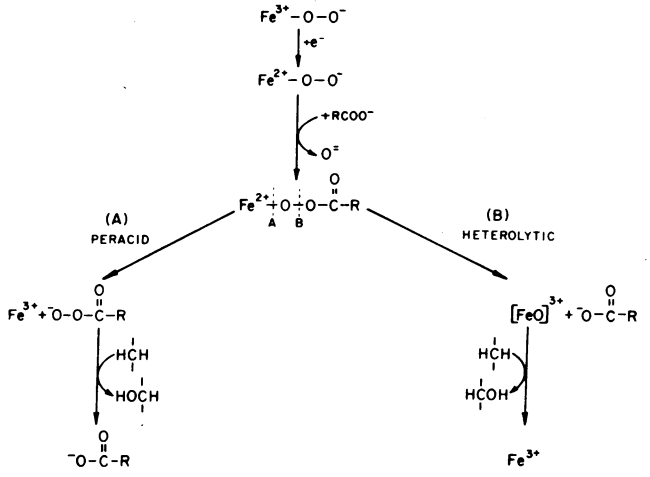

FIG. 2. Acylation of heme-bound dioxygen: Mechanisms for peracid generation and heterolytic O–O bond cleavage.

The mechanisms discussed above focus on the iron–oxygen redox states of monoxygenase catalysis. Substrate, however, may obviously also play a key role in either mechanism, or in fact, may activate the $[Fe^{2+}O_2^-]$ state directly in addition to any possible strain factors induced by substrate binding free energy. Mechanisms involving *solely* selective catalytic substrate attack with no oxygen activation are considered together for discussion in this presentation and will be termed "skeleton activation" mechanisms.

Any O_2 activation mechanism proposes a key role for a protein amino acid side chain or other effector molecule in the catalytic acylation process, with far-reaching implications in terms of accepted monoxygenase stoichiometry. This can be clearly seen by examining the ultimate distribution of labeled atmospheric dioxygen into product, protein, and water for each mechanism. A reaction scheme involving acylation of heme-bound dioxygen predicts that there should be an incorporation of oxygen-18 label into the protein fraction. In contrast, a skeleton activation mechanism partitions labeled oxygen equally between substrate and water, and therefore predicts that no ^{18}O label should be found in the protein or effector fraction. The salient features of the mechanism can be examined most clearly under single turnover conditions, in which it is possible to differentiate between acylation of bound dioxygen and skeleton activation by the use of $^{18}O_2$ tracer methods. Actual experimental methods, however, are far from trivial, because they involve the hydrolysis of the *P*-450 protein after a single turnover, isolation of the acylating fraction without oxygen exchange, and determination of oxygen isotopic ratios by using mass spectrometry. Although such investigations are appropriate for hepatic cytochrome *P*-450, a simpler system exists for demonstration of an acyl peroxide intermediate in *P*-450 mixed-function oxidation.

The bacterial camphor hydroxylase is distinct from the mammalian *P*-450 proteins in that previous mechanistic studies have shown an apparent requirement for a free acylating group in the catalytic cycle (8, 38). This residue is apparently provided in the native hydroxylase by the carboxy-terminal tryptophan or penultimate glutamine of putidaredoxin, which exists *in vivo* as a tight complex with the *P*-450 hemoprotein (38). Central to the mechanistic investigations reported in this manuscript is the fact that purified *P*-450 from *Pseudomonas* is unable to catalyze camphor hydroxylation without the presence of a suitable effector molecule (39). In the absence of the effector, the $[Fe^{3+}O_2^-]$ oxygenated intermediate decays by simple superoxide release without product formation (40). The precise chemistry of this effector–protein interaction has been elusive, inasmuch as several compounds have been shown to replace the *in vivo* effector, putidaredoxin, within a defined redox reaction (39). In particular, lipoic acid has been found to efficiently catalyze camphor hydroxylation from the oxygenated *P*-450 intermediate (39). We have examined the chemistry of this reaction by using $^{18}O_2$ and mass spectral studies of isotope distribution into product and effector after a single turnover of bacterial cytochrome *P*-450 in the presence of lipoic acid. The results were clearly consistent with a mechanism requiring that the effector carboxylate be subjected to a nucleophillic attack by bound dioxygen with the transient formation of an acyl peroxide intermediate in the oxygenation cycle. The re-

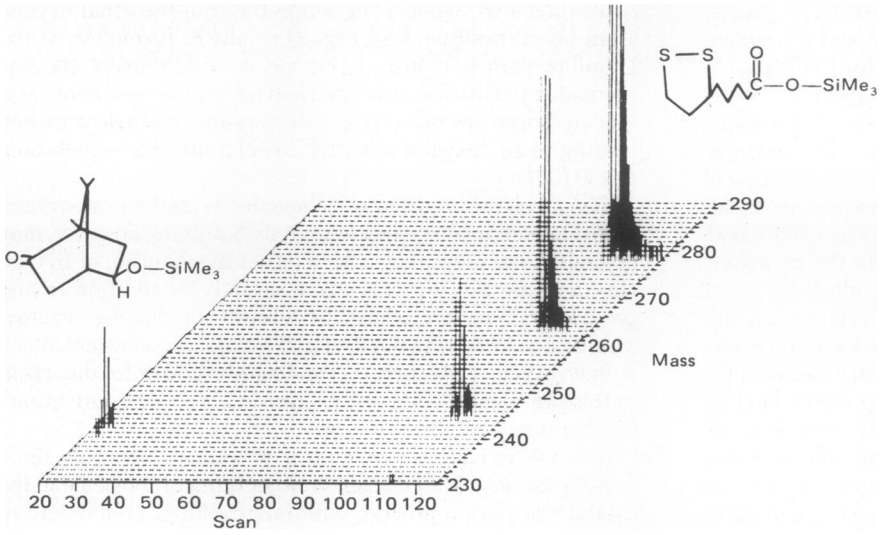

FIG. 3. Gas chromatography–mass spectrometry: Lipoate–*P*-450 products after a single turnover. The ion intensity (*z* axis) is plotted as a function of mass (*y* axis) and scan number proportional to GC retention time (*x* axis). The peaks at 240 and 242 mass units between scans 20 and 30 are [^{16}O]- and [^{18}O]hydroxycamphor, whereas the intensity between 110 and 120 scans represents part of the total lipoate fragmentations. The M^+ ions at 278 and 280 are the oxygen-16 and oxygen-18 isotopes incorporated into lipoate, the peaks at 263 and 265 are $M^+ - CH_3$, and 245 and 247 represent loss of an SH radical after ring opening.

sultant isotopic pattern requires that the oxygenation of camphor by the bacterial system using lipoic acid as the effector proceeds with OH^- release from an unlabeled position.

MATERIALS AND METHODS

Cytochrome *P*-450 was isolated from *Pseudomonas putida* strain PpG786 by the procedures described (41). Lipoic acid (Sigma) was reduced under nitrogen with sodium borohydride and brought to neutral pH by addition of 6 M HCl. The concentration of sulfhydryl groups was measured colorimetrically by the method of Ellman (42). All other chemicals were of the highest purity reagent grade obtained from standard suppliers.

Reaction mixtures (1 ml) typically contained between 5 and 200 μM cytochrome *P*-450, 1 mM *dl*-camphor (Eastman), 8 μM proflavin (Eastman), 8 mM EDTA, and 50 mM potassium phosphate buffer at pH 7.0. The mixtures were thoroughly deoxygenated and left on ice under nitrogen until used. Photoreduction of samples was accomplished by a 5-min exposure of the anaerobic mixture to white light from a 500-W xenon source. After photoreduction, the reaction mixture was oxygenated with either $^{18}O_2$ or $^{16}O_2$ and then dihydrolipoate was added by syringe to yield a final concentration of 1 mM. Reaction mixtures were incubated at room temperature for 15 min. Reaction completion was verified by following the 418-nm Soret band absorption maximum of the oxygenated cytochrome. The mixtures were then extracted with 10 ml of methylene chloride after the addition of 20 μg of benzylbenzoate (Aldrich) to each sample as an internal standard. The organic phase was removed and evaporated under nitrogen and the residue was treated with 100 μl of Regisil (Pierce Chemical) at 90°C for 12 hr to form trimethylsilyl (Me_3Si-) derivatives of hydroxycamphor and lipoic acid.

After derivitization, the samples were analyzed by combined gas chromatography–mass spectrometry, using a Hewlett-Packard model 5985 gas chromatograph–quadrapole mass spectrometer equipped with a 60-cm, 0.32 cm inside diameter glass column packed with 3% OV-101 (Supelco). The column oven was temperature programmed from 130 to 200°C at 30°C/min after a 2.5-min isothermal period at 130°C to quantitate hydroxycamphor and was employed isothermally at 200°C to quantitate lipoic acid. Ion source temperature was 150°C, ionization current was 300 μA, and the electron energy was 70 eV. Retention times for the Me_3Si- derivatives of benzylbenzoate and lipoate at 200°C were 1.4 and 2.3 min, respectively, whereas the retention time of the Me_3Si- derivative of hydroxycamphor at 120°C was 4.25 min. Examination of the complete mass spectrum of the components of the reaction mixture revealed that there were no gas chromatographic peaks containing mass ions indicative of a sulfoxide-lipoate derivative. Selected ion monitoring was employed to selectively follow the molecular ions of lipoate-$SiMe_3$ at mass-to-charge ratio, m/e, = 278 and hydroxycamphor-$SiMe_3$ at m/e = 240 as well as their mass M + 2 ions, which reflect the incorporation of ^{18}O. The M − 15 ion of lipoic acid-$SiMe_3$, which is characteristic of the loss of a methyl group, and the ^{18}O-enriched counterpart were also monitored. Peak areas were integrated for each ion by using a Hewlett-Packard 21 MX-E series computer. Standard curves for lipoic acid and for hydroxycamphor were used to calculate the amount of each compound present. For lipoic acid, the number of nmol of [^{18}O]lipoate was calculated from the percent increase in the ratio of 280/278. In all experiments, approximately 7% of the total derivitized lipoic acid existed as a tri-$SiMe_3$ derivative with a correspondingly longer gas chromatography (GC) retention time. Final incorporation ratios included quantitation of this minor component at m/e = 278 and m/e = 280. It was assumed as a first approximation that because the fragmentation patterns for the mono- and tri-$SiMe_3$ derivatives were similar except in the region of m/e >400, the

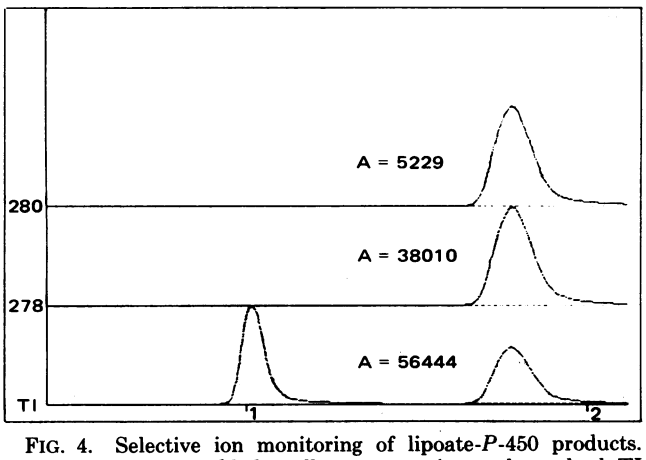

FIG. 4. Selective ion monitoring of lipoate-*P*-450 products. Benzylbenzoate was added in all cases as an internal standard. TI indicates total ion current. Ion intensities are plotted as a function of GC retention time in minutes. A refers to the area in arbitrary units.

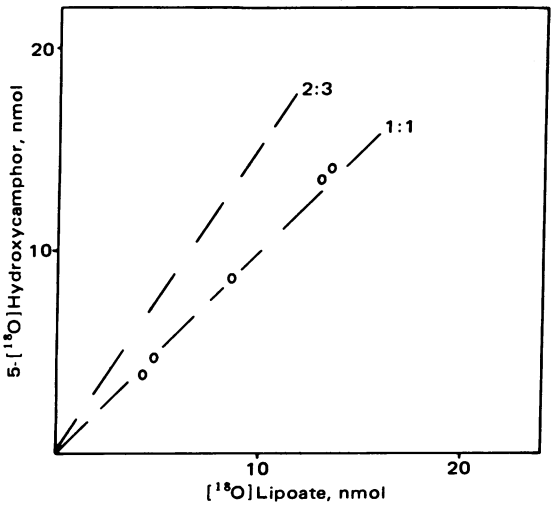

FIG. 5. Predicted and experimental (o) isotopic incorporation ratios. A 2:3 incorporation ratio is predicted if the oxygen–oxygen bond is broken prior to water release from a hypothetical tetrahedral intermediate. Because OH^- is a good leaving group, it may be expected to be released from an unlabeled position prior to formation of an acyl peroxide state, predicting an incorporation ratio of 1:1. Breaking the iron-proximal bond in a Hamilton peracid mechanism will always yield a 1:1 isotope distribution.

intensities of these ions would provide an initial estimate of the tri-$SiMe_3$ derivative present from the standard curves obtained for the mono-$SiMe_3$ lipoate.

RESULTS AND DISCUSSION

Fig. 3 schematically illustrates the observed ion fragmentation patterns observed for a single turnover of lipoate–*P*-450 in the presence of oxygen-18. Clearly evident at masses 240 and 242 are [^{16}O]hydroxycamphor and [^{18}O]hydroxycamphor, respectively, with the lipoate fragmentation pairs at 278 and 280, 263 and 265, and 245 and 247. For greater sensitivity, isotope incorporation ratios were calculated from data obtained in the selective ion monitoring mode as described in *Materials and Methods*. As an example, Fig. 4 represents the individual ion current chromatograms for lipoate and the internal standard benzylbenzoate as a function of time.

The data describing the concentration and enrichment ratios of oxygen isotope incorporation can be most simply presented by comparing the absolute nmol of ^{18}O found in lipoate to the absolute nmol of [^{18}O]hydroxycamphor molecules formed under single turnover conditions. Fig. 5 illustrates these data for various hydroxycamphor concentrations formed in several experiments. Also shown are the theoretical lines for 1:1 and 2:3 incorporation ratios. A homolytic or heterolytic reaction of the iron–acyl peroxide intermediate predicts that the proximal labeled oxygen atom is directly inserted into the substrate C–H bond, and isotope incorporation patterns into water and carboxylate depend on the fragmentation pathway. If O–O bond cleavage preceeds OH^- release, breakdown of a transient oxygenated carboxylate yields 33% of the label in water and 67% retained in the recycled carboxylate, predicting an [^{18}O]lipoate-to-[^{18}O]hydroxycamphor ratio of 2:3. However, the release of OH^- from an unlabeled position prior to oxygen bond cleavage would imply an incorporation ratio for lipoate and hydroxycamphor of 1:1. The peracid mechanism releases water from an unlabeled position after a single turnover, and hence also predicts a lipoate-to-product incorporation ratio of 1:1. The data presented in Fig. 5 show that the chemical mechanism of lipoate–*P*-450-catalyzed camphor hydroxylation clearly involves acylation of heme-bound atmospheric dioxygen by the carboxylate of lipoate and eliminates a skeletal activation mechanism as a possibility. The ratios calculated for [^{18}O]lipoate/[^{18}O]hydroxycamphor for all experiments were 0.92 or greater, unambiguously demonstrating that water production precedes O–O bond cleavage. Decision as to the mechanism of substrate methylene carbon attack is less certain, and must await further efforts. The observation of a transient spectral intermediate in peracid-*P*-450 camphor hydroxylation in both rapid-reaction (43) and low-temperature studies (8) may suggest an oxene transferase (44) or oxygen rebound (37) activity for cytochrome *P*-450, although substantial effort remains necessary in order to precisely identify the iron–oxygen–substrate states involved in this spectral species.

In summary, we have demonstrated the incorporation of isotopic oxygen from an atmospheric dioxygen precursor into an effector molecule for bacterial cytochrome *P*-450. Such a direct replacement reaction of a carboxylate oxygen directly implicates the existence of an acyl peroxide intermediate in the cleavage of the oxygen–oxygen bond in the *P*-450 mixed-function oxidases. Similar acylation mechanisms may be operative in other systems catalyzing oxygen cleavage.

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