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Characterization of Propane Monooxygenase: Initial Mechanistic Studies

Pat J. Unkefer*, Marc A. Alvarez, and Clifford J. Unkefer

Abstract

Extensive industrial and DOE use of chlorinated hydrocarbons has resulted in widespread soil and ground-water contamination. Bioremediation is a potential remedy because various bacterial strains degrade chlorinated compounds, including trichloroethylene (TCE). Previous reports indicated that the propane monooxygenase (PMO) enzyme from *Mycobacterium vaccae* degraded TCE. These reports included additional substrates and resulting products that were inconsistent with PMO forming an epoxide intermediate; thus PMO appeared to be an attractive alternative to the epoxide-forming methane monooxygenase (MMO) enzyme. PMO had not been isolated and was largely uncharacterized. This project characterized PMO and its mechanism. PMO had a multi-component quaternary structure that was remarkably similar to that of MMO. The products formed by PMO from two key substrates were not as previously reported and were precisely those predicted by an epoxide intermediate. The observed lack of unique character of PMO has caused us to forgo further study.

Background and Research Objectives

Biochemical Mechanisms for Dechlorination --To date, three general mechanisms for enzymatic dehalogenation have been identified: reductive dehalogenation, nucleophilic substitution, and oxygenation (Figure 1). Certain microbes cleave halides reductively from polychlorinated hydrocarbons. While these enzymes have not been characterized, reduced pyridine nucleotides (NADH) are thought to provide the hydride equivalent displacing chloride. A number of biochemical substitution reactions have been identified that dehalogenate. For example, the hydrolytic dehalogenases use a water molecule and catalyze an Sn2-type substitution of hydroxide for the halide on primary alkyl halides. Intramolecular substitutions in α-chloro-β-hydroxy compounds yield epoxides. Dichloromethane dehalogenase uses a thiolate from glutathione to displace chloride. As discussed below, the mixed function monooxygenases that effect the hydroxylation of hydrocarbons can act on chlorinated solvents to generate intermediate chlorohydrins which decompose spontaneously. As discussed below, in studies using whole cell, PMO has been shown to dehalogenate a variety of chlorinated solvents. The

substrate specificity and product distribution of these dechlorination reaction are not consistent with any known biochemical mechanism.

Monooxygenases -- For many years it has been known that bacteria are able to grow using simple hydrocarbons as their sole source of carbon. Two classes of organisms have been identified based on their ability to grow aerobically on either methane or propane. The first step in their catabolic pathway is the oxidation of either methane to methanol or propane to isopropanol. The enzyme that catalyzes the oxidation of methane, methane monooxygenase (MMO) is remarkable. This enzyme activates dioxygen and inserts an atom of oxygen selectively into a C-H bond. In the absence of the enzyme, this process involves an activation barrier of more than 100 Kcal/mole. MMO is one of a general class of enzymes called mixed function monooxygenases which also include the P450 enzymes. Mixed function monooxygenases hydroxylate substrates using molecular oxygen and a reductant, usually NADH, in the following reaction:

The soluble form of MMO has been isolated from a number of organisms and is now reasonably well characterized. MMO contains three components including a regulatory component, a reductase, which serves to transfer reducing equivalents from NADH, and a hydroxylase component. The hydroxylase contains a unique non-heme dinuclear iron center which serves as the oxygen binding site. This iron center has only oxygen and nitrogen ligands donated by amino acid side chains and a μ-oxo bridge. The details of the reaction mechanism are the subject of intense investigation in several laboratories. The specificity of MMO has been studied in detail. Bacterial P450's are Fe-heme enzymes and are also well characterized

Except for our initial purification, PMO from *M. vaccae* remains largely uncharacterized. Substrate specificity data described here is derived from whole cell studies. The similarity in function suggests that the PMO from *M. vaccae* is also a mixed function monooxygenase. However, differences in regioselectivity described below suggest the detailed chemical mechanism by which PMO hydroxylates substrates is distinct from MMO and the P450's.

Substrate Specificity -- Shown in Table 1 is a partial list of the substrates that MMO and PMO can oxidize (3,4,5). Clearly both enzymes have very broad ranges of

specificity. In addition to methane, MMO will hydroxylate longer straight-chain alkanes, at primarily C-1 and, to a lesser extent, at C-2, and aromatic compounds including benzene and naphthalene. PMO is distinct from MMO in that it will not oxidize methane. Further, PMO is more regioselective; longer chain hydrocarbons are oxidized exclusively at C-2. The fact that PMO hydroxylates exclusively at the secondary position of propane or butane suggests a radical mechanism. A detailed understanding of PMO's mechanism for hydroxylation of alkanes would open the possibility of designing more effective chemical catalysts for this difficult reaction.

TABLE 1. Substrate Specificity of Bacterial Hydrocarbon Monooxygenases

Substrate	Methane Monooxygenase		Propane Monooxygenase	
	Relative Activity	Product	Relative Activity*	Product
Methane	100	Methanol	S	
Chloromethane	99	Formaldehyde		
Dichloromethane	97	Formate		
Carbon Tetrachloride	0		S	CO_2
Trichloroethylene		Glyoxylate	S	Dichloroethanol
1-chlorobutane			S	2-butanone
Propane	82	n- and 2-propanol	S	2-propanol
Propene	99	Propylene oxide	S	Propylene oxide
Butane	92	1- and 2-Butanol	S	2-Butanol
Pentane		1- and 2-Pentanol	S	2-Pentanol
Benzene	74	Phenol	S	Hydroquinone
Trinitrotoluene	0		S	Trinitrobenzylalcohol

*Propane Monooxygenase assays were carried out using whole cells; rates are estimated from growth rates.

Trichloroethylene Oxidation -- MMO and PMO share the property that they oxidize TCE. However, as indicated by the products of the reaction, the mechanisms by which MMO and PMO oxidize TCE are distinct. TCE oxidation by MMO is initiated by the formation of TCE epoxide which falls apart in non-enzymatic hydrolysis reactions (1). In contrast to MMO, PMO degradation of TCE is reported to form dichloroethanol. This observation is consistent with a P450-like mechanism for TCE degradation that does not involve TCE epoxidation (2). The epoxide intermediates formed by MMO react with

nucleophiles in the cell causing cytotoxicity and enzyme inactivation. In contrast, PMO is potentially advantageous for bioremediation of TCE because the oxidation of TCE may not involve an epoxide intermediate.

Dehalogenation of Primary Alkyl Halides --In whole cell studies, PMO has been implicated in the dehalogenation of primary chloroalkanes including 1-chloropropane and 1-chlorobutane. 2-Butanone was the only product isolated from *M. vaccae* cells incubated with 1-chloro-butane . This product is not consistent with the known biochemical mechanisms for dehalogenation listed in Figure 1. For example, the expected product is 1-butanone if the oxygenation mechanism was operative. If PMO-catalyzed hydroxylation at C2 was followed by an intramolecular substitution the product would be an epoxide.

Research Goals: The long term goal of this research project was to understand the mechanism by that PMO oxidized hydrocarbons and dehalogenates chlorinated solvents and to evaluate its potential for bioremediation. Fundamental to understanding the mechanism were characterization of the substrate specificity of PMO and the carbon products generated by its action upon these substrates. The second major key was determination of the general class of monooxygenase and the type of iron center present in PMO. Our specific objectives focused upon obtaining the data necessary to provide these two fundamentals.

Specific Objectives:

1. Complete the purification of PMO from *M. vaccae* by building upon our recent success in stabilizing and partially purifying PMO. Characterization and coning of the PMO genes.
2. Identify the type of iron center in PMO and prove that it is a mixed function monooxygenase

3. Using the purified PMO, examine the substrate specificity and products generated .

Importance to LANL's Science and Technology Base and National R&D Needs

Chlorinated organics make up the largest class of soil and ground water chemical pollutants with 200 million pounds released per year. Because they are toxic at very low

concentrations, chlorinated solvents are major risk drivers and high priorities for remediation. For example, TCE is an EPA priority pollutant and the most common contaminant found at the 517 National Priority List sites. *In situ* bioremediation of such sites is attractive because it is vastly cheaper than more invasive interventions. Identification of new, efficient enzyme catalysts for cleavage of carbon-halogen bonds is fundamentally important. In this regard, PMO was initially attractive because it did not appear to generate hazardous partial degradation products such as TCE epoxide or vinyl chloride.

This research effort that determined the quaternary structure and mechanism of action of a propane monooxygenase enzyme fits squarely within the laboratory's core competency in bioscience and within its programs in protein structure. The work provided strong scientific underpinning in the area of bioremediation, and addressed remediation of contaminants found at numerous DOE sites. It is particularly relevant because, all major activities funded by government agencies(particularly the Environmental Protection Agency) to address chlorinated hydrocarbons have essentially ceased, yet the extensive contamination remains without cost-effective remedies.

Scientific Approach and Accomplishments

Accomplishment 1. Complete the purification of PMO from M. vaccae. Conduct initial enzyme characterizations.

Purification: We purified PMO according to the procedure described below. This method produced a n enzyme preparation of moderate stability that allowed the appropriate examination of the products of the enzyme's action upon the various substrates.

Previously frozen cells are thawed in a water bath (35 degrees C), centrifuged (10,000xG, 10 min), and resuspended in 50mM Bis Tris Propane, pH 7.5, centrifuged and resuspended again in buffer (1:1 w/v). The cells were ruptured in a cold french pressure cell (20,000 psi) by passing the cell mixture through the cell twice. Dnase 1 was added to the cell material after the first pass through the french press to lessen problems associated with viscosity. The cell debris were removed by centrifugation (20,000 x, 25 min.)

The nucleic acids were precipitated with 0.2% streptomycin sulfate (10 min in an ice bath) and removed by centrifugation (15,000 x G, 10 min). The supernatant containing the PMO activity could then be quick frozen using liquid nitrogen and stored at in a –80 degree C for 12-14 hours before use in either further purification work or assays. This crude enzyme preparation was thawed slowly at room temperature and allowed to contact an ion exchange resin, DEAE sepharose, in 50mM bis tris propane buffer pH 7.5 for 15 min; the resin was removed by centrifugation (5,000 x G, 5 min.) This preparation contains the reductase component or (Fraction 1). The ion exchange resin was then combined with 50mM bis tris propane, pH 7.5 containing 500mM LiCl and gently mixed (10-15 min.). The resin was removed by centrifugation (5,000 g, for 5 min). The supernatant contained the oxygenase component of the PMO. (Fraction 2). The fractions containing the reductase and oxygenase components could be recombined and the PMO activity measured using the assay for conversion of propylene to propylene oxide. The oxygenase component could be further purified with hydrophobic interaction chromatography by adsorbing the component onto a phenyl sepharose chromatography resin. Elution of the oxygenase component from this resin separated a third component, which was presumed to be a regulatory protein. This presumption was based upon the striking similarity of the PMO component structure and the characteristics of the enzyme during purification with those of MMO . Attempts to recombine the reductase component with the oxygenase component after the third component had been removed, no longer reconstituted PMO activity. This result is completely consistent with the results of similar reconstitution experiments with MMO.

Growth of Mycobacterium vaccae with Propane as the Carbon Source. The new method of culturing the organism was developed to produce the large quantities of the cells needed for the purification. In contrast to the published reports, the organism could be readily grown in large quantities with glucose as the carbon source, the media replaced with media without a carbon source and the cells then sparged with propane/air mixture to induce the PMO activity as the cells continued to grow.

Enzyme characterization:. As expected the purification work reveal important structural information about the native holoenzyme. The PMO is composed of at least three different polypeptide components-one is a reductase, the second is an oxygenase

and the third is presumed to be a regulatory protein. The weight of this holoenzyme was in excess of 600 kD.

Enzyme stability: The enzyme activity was extremely labile and numerous and detailed studies were conducted in attempts to stabilize the enzyme. The standard approaches of testing the effects of various protease inhibitors , numerous metal salts and chelators as well as sulfhydryl reagents, and alcohols were examined. No improvements were achieved with any of these tests.

Accomplishment 2. Using the purified PMO, the substrate specificity and products generated were re-examined. It was of primary importance to address the reported regioselectivity (oxidation at C-2 only) and stereoselectivity of the PMO reaction, as these characteristics were most indicative of a new and unusual mechanism of action for PMO as compared with other oxygenases. In sharp contrast to the reports in the literature, PMO did not produce the C-2 only oxidation for butane and pentane. The products were mixtures of 1- and 2-butanol and 1- and 2-pentanol, respectively. Thus, the products were those expected for a methane monooxygenase enzyme.

Accomplishment 3. Determined that PMO is actually an MMO. The findings of the first two accomplishments were much more characteristic of an MMO enzyme (1,6,7) than they appeared to support the reported hypothesis that PMO was an unusual enzyme. Additional information was sought to test if this is a propane oxidizing MMO. The most straight forward experiment was to answer two key questions: 1.) could the organism grow with methane as its sole source of carbon and 2.) if so, would growth on methane induce the propane oxidizing activity?

Growth of Mycobacterium vaccae with Methane as the Carbon Source. Unlike the previous reports, the organism grew readily with methane as its sole source of carbon, although it grew faster with propane as its carbon source. Methane grown cells contained substantial amounts of PMO activity.

Conclusions – The quaternary structure of the propane monooxygease from *Mycobacterium vaccae* was strikingly similar to that of methane monooxygenase. Each of these enzymes contain as separate polypeptides, a reductase, an oxygenase and a regulatory peptide. They each produce mixtures of 1- and 2- alcohols when acting on butane and propane, thus neither displays regioslectivity. The each oxidize methane to methanol and support the growth of their respective organisms when supplied with methane as the sole source of carbon. Thus, the putative uniqueness of PMO was not confirmed and the project was discontinued as it no longer provided the potential to improve bioremediation of chlorinated hydrocarbons.

Publications

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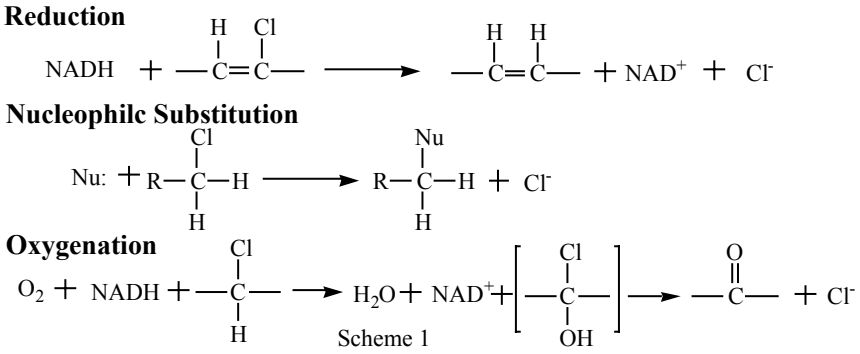
Figure 1: Biochemical Mechanisms for Dehalogenation



Scheme 1