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Current Topics

Microbial Metabolism of Aliphatic Alkenes†

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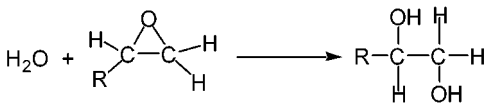
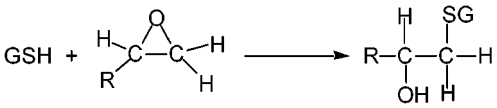
Our rapid transition to a highly industrialized society has resulted in increased atmospheric concentrations of a variety of carbon-based gases, including CO_2, CO, aliphatic and aromatic hydrocarbons, and halocarbons. While many of these gases are produced as natural intermediates or end products of bacterial, plant, and mammalian metabolism, the increased concentrations of these gases in the atmosphere pose human health risks and threaten to destabilize our ecosystems through a variety of mechanisms. Accordingly, there is keen interest in the potential of microorganisms to transform these gases into less environmentally detrimental compounds. In addition to the importance of identifying and exploiting bacteria capable of metabolizing xenobiotics, there is also a need to understand the fundamental biological mechanisms the bacteria employ to metabolize these compounds. Short-chain aliphatic alkenes (olefins) are one class of hydrocarbons of concern as potential human health hazards and that are capable of supporting the growth of a number of bacteria (Figure 1). Studies of bacterial alkene metabolism have revealed the involvement of previously unrecognized metabolic pathways, new and surprising types of enzymes, and atypical cofactors. These recent developments in the study of bacterial alkene metabolism are the topic of this paper.

Biological Reactivity of Alkenes. The toxicity of aliphatic alkenes arises from their ability to serve as substrates for broad-substrate specificity oxygenases (e.g., cytochrome P450 enzymes and others), resulting in the formation of highly reactive electrophilic epoxides (Figure 2) (*1*, *2*). Depending upon the organism, epoxides formed in this manner can undergo one of several fates (Figure 2). Epoxides may react abiotically with cellular nucleophiles (e.g., proteins and DNA) to form covalent adducts, a process that accounts for the toxic properties of these compounds (*3*). To counteract this, some organisms contain detoxification enzymes, specifically glutathione-*S*-transferases (GSTs)[1] and epoxide hydrolases, that use glutathione or water, respectively, as nucleophiles to attack and open the oxirane ring as shown in eqs 1 and 2 (*4*, *5*).



Equation 1



Equation 2

These adducts may then be excreted or further metabolized. Alkene-oxidizing bacteria are unique in being able to carry out the productive catabolism of aliphatic epoxides by their conversion to central metabolites that meet the carbon and energy needs of the bacteria.

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[1] Abbreviations: 2-hydroxypropyl-CoM, 2-[2-hydroxypropylthio]-ethanesulfonate; 2-KPCC, NADPH:2-ketopropyl-CoM oxidoreductase/carboxylase; 2-ketopropyl-CoM, (2-[2-ketopropylthio]ethanesulfonate); AMO, alkene monooxygenase; CoM, coenzyme M (2-mercaptoethanesulfonate); DSOR, NADPH:disulfide oxidoreductase; EXAFS, extended X-ray absorption fine structure; GST, glutathione-*S*-transferase; HGMB, 1-hydroxy-2-glutathionyl-2-methyl-3-butene; HPCDH, hydroxypropyl-CoM dehydrogenase; MetE, cobalamin-independent methionine synthase; MMO, methane monooxygenase; SDR, short-chain dehydrogenase/reductase; THF, tetrahydrofolate; TR-I and TR-II, tropinone reductase-I and -II.

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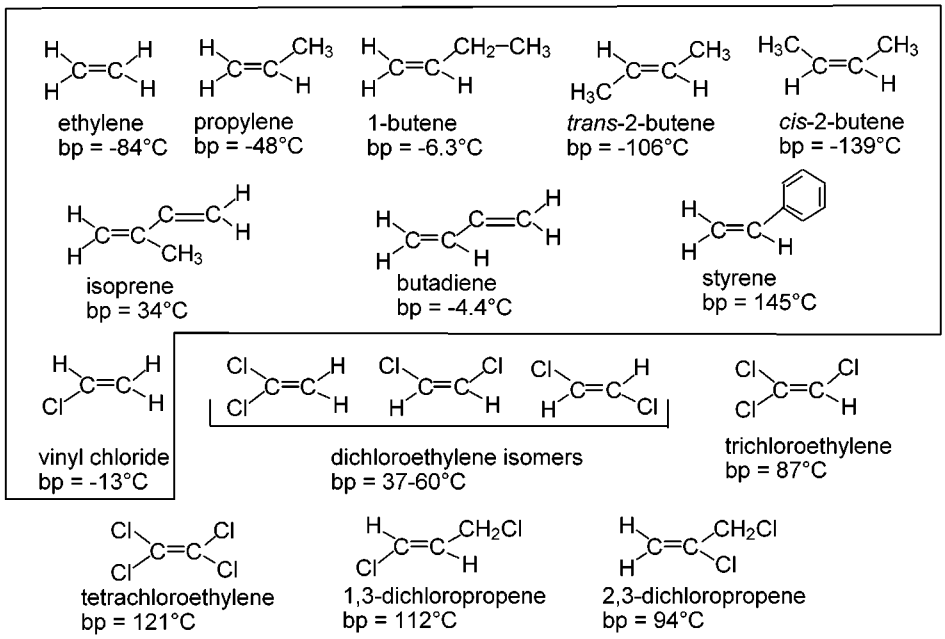


FIGURE 1: Structures of selected short-chain aliphatic, aromatic, and chlorinated alkenes. The alkenes that are boxed are capable of supporting growth of certain aerobic bacteria. Those not boxed, with the exception of tetrachloroethylene, are cometabolized by certain alkene-oxidizing and other aerobic bacteria. Tetrachloroethylene does not undergo aerobic oxidation but is subject to reductive dechlorination by certain anaerobic bacteria.

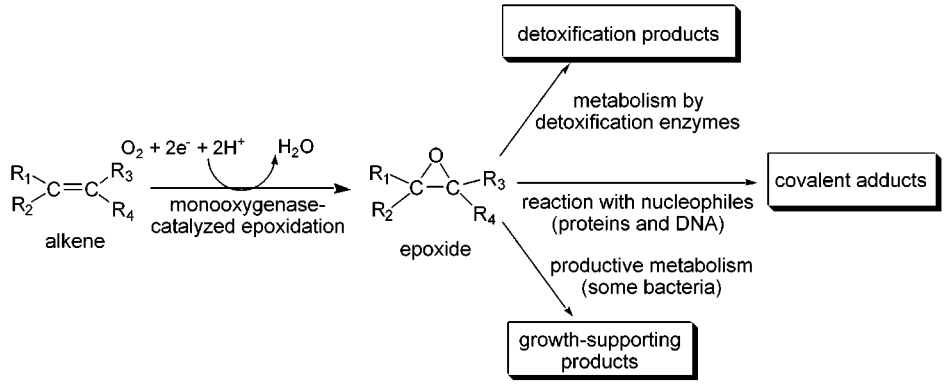


FIGURE 2: Biological reactivity of aliphatic alkenes.

Alkene-Oxidizing Bacteria. Diverse bacteria, including various strains of *Rhodococcus*, *Mycobacterium*, *Nocardia*, *Xanthobacter*, *Alcaligenes*, *Pseudomonas*, and *Corynebacterium*, are capable of aerobic growth with short-chain aliphatic alkenes (C2 to C6) as the sole added carbon source (*6*). While some alkene-oxidizing bacteria are restricted to growth on a single alkene, e.g., ethylene or isoprene (2-methylbutadiene), others exhibit less selectivity for the growth substrate. For example, *Xanthobacter* strain Py2, a bacterium isolated with propylene, can also grow using ethylene, 1-butylene, 2-butylene, 1-pentene, and 1-hexene as carbon sources (*7*, *8*). An interesting feature of alkene-oxidizing bacteria is the diversity of additional growth substrates that the bacteria are capable of using. For example, some of the alternative growth substrates for *Xanthobacter* strain Py2 include glucose, fructose, organic acids, C-1 to C-4 alcohols, acetone, 1,2-propanediol, and H_2 plus CO_2 (*8*). When growing the strains with alternate carbon sources, the enzymes responsible for alkene oxidation are not synthesized, but are rapidly expressed upon transfer to media containing the growth-supporting alkene (*9*). This metabolic diversity and enzyme induction distinguishes alkene-oxidizing bacteria from some other hydrocarbon-oxidizing bacteria. For example, obligate methane-oxidizing bacteria are restricted to using methane and methane oxidation intermediates (e.g., methanol and formate) as their only growth-supporting substrates (*10*).

Alkene Monooxygenases. All of the alkene-oxidizing bacteria isolated to date are aerobic bacteria that initiate alkene oxidation by O_2- and reductant-dependent monooxygenase reactions. Alkene monooxygenases (AMOs) have been purified and characterized from two propylene-oxidizing bacteria: *R. rhodochrous* strain B276 (*11*) and *Xanthobacter* strain Py2 (*12*). Both enzymes are NADH-dependent, multicomponent enzymes that catalyze alkene epoxidation according to the reaction shown in eq 3:

$$H_2C{=}CH{-}R + O_2 + NADH + H^+ \longrightarrow H_2C\overset{O}{\diagup\diagdown}CH{-}R + NAD^+ + H_2O \qquad \textit{Equation 3}$$

AMO from *R. rhodochrous* is a three-component enzyme system composed of the epoxygenase, which consists of 53- and 35-kDa subunits arranged in an $\alpha\beta$ quaternary structure

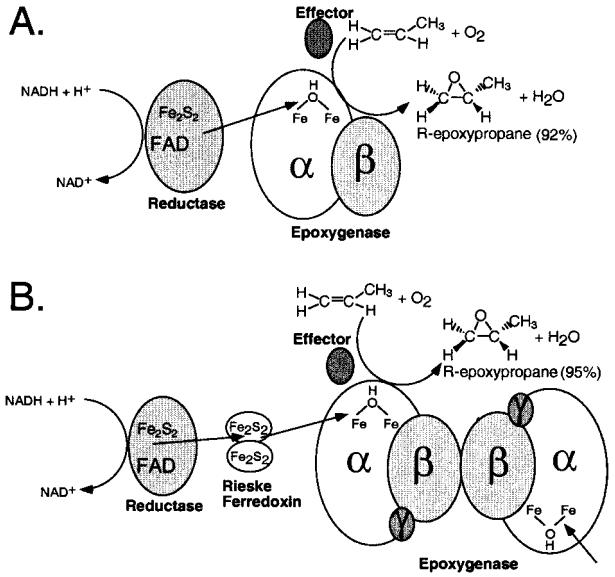


FIGURE 3: Comparison of alkene monooxygenases from (A) *R. rhodochrous* B276 and (B) *Xanthobacter* sp. strain Py2.

and containing two moles of nonheme iron, a 40-kDa monomeric reductase that contains FAD and a 2Fe-2S cluster, and a 14-kDa effector protein (Figure 3A) (*11*). The biochemical, spectroscopic, and genetic characterization of *R. rhodochrous* AMO (*13*−*15*) suggests that it belongs to the family of diiron oxygenases of which soluble methane monooxygenase (MMO) is the best-characterized example (*16*). As determined from the crystal structure of the hydroxylase component of MMO, these enzymes contain a binuclear iron center with two terminal His and Glu residues, two bridging Glu residues, and water molecule(s) forming a bis-μ-hydroxo-bridged "diamond-core" structure (*17*, *18*). The positions of the His and Glu residues that form the diiron binding site are highly conserved among this family of enzymes, a feature that is exploited to deduce the presence of the cluster in enzymes that have not been structurally characterized (*19*, *20*). The binuclear iron center serves as the site of O_2 binding and activation for the oxygenation of the organic substrate, i.e., hydroxylation of methane for MMO and epoxidation of propylene for AMO (*16*, *21*, *22*). It should be noted that MMO and other diiron hydroxylases are capable of catalyzing the epoxidation of short-chain alkenes, as well as various other monooxygenation reactions, in place of the hydroxylation of their natural substrates (*23*, *24*). In contrast, AMO is more restricted in its substrate specificity and catalyzes organic hydroxylation reactions only at very low rates and with a limited range of substrates (*25*). Thus, alkene epoxidation appears to be a ubiquitous capability of diiron monooxygenases, while hydroxylation is a more restricted function.

Like AMO from *R. rhodochrous*, AMO from *Xanthobacter* Py2 is a multicomponent diiron enzyme; however, the enzyme has a fundamentally different composition. *Xanthobacter* Py2 AMO is composed of four rather than three components: an epoxygenase, which consists of 58, 38, and 10 kDa subunits arranged in an $\alpha_2\beta_2\gamma_2$ quaternary structure and that contains 4 mol of nonheme iron, a 35-kDa monomeric reductase that contains FAD and a 2Fe-2S cluster, a homodimeric (13-kDa subunit) ferredoxin containing two Rieske-type 2Fe-2S clusters, and an 11-kDa effector protein (Figure 3B) (*12*). Like *R. rhodochrous* AMO, biochemical and genetic characterization suggests that the iron is arranged in a binuclear MMO-like cluster, with the alpha subunits forming the cluster-binding domains (one cluster per each alpha subunit) (*12*, *25*).

No other alkene monooxygenases have, to date, been purified to homogeneity, although the available evidence suggests that some share common features with those of *Xanthobacter* Py2 and *R. rhodochrous.* AMO from an ethylene-oxidizing bacterium, *Mycobacterium* sp. strain E3, was resolved from cell extracts into two fractions that could be recombined with restoration of AMO activity (*26*). The active component from one of these fractions was purified and shown to be a 56-kDa NADH reductase containing FAD and a 2Fe-2S cluster (*27*). An 8.5-kb DNA fragment was cloned from the isoprene-oxidizing bacterium *Rhodococcus* sp. strain AD45 and shown to contain six genes encoding a putative four-component isoprene monooxygenase with high similarity to the four-component alkene monooxygenase from *Xanthobacter* Py2 (*28*). Finally, FAD-dependent styrene monooxygenase activity has been detected in cell extracts of various bacterial isolates capable of growth using styrene as the carbon source (*29*). While the styrene monooxygenase has not been further characterized biochemically, the gene cluster encoding styrene monooxygenase and related enzymes has been cloned from several styrene-oxidizing *Pseudomonas* strains (*30*−*32*). From the analysis of this gene cluster, two genes have been identified, *styA* and *styB*, that apparently encode an FAD-dependent styrene monooxygenase and an FMN oxidoreductase, respectively (*32*, *33*). These results suggest that styrene monooxygenase is a flavin-dependent two-component system with similarity to other flavin-dependent monooxygenases, including some bacterial aromatic hydroxylases (*30*). It should be noted that the aromatic ring of styrene distinguishes it from the purely aliphatic alkenes, so it is perhaps not surprising that styrene monooxygenase belongs to a fundamentally different class of oxygenase than the diiron AMOs. Additionally, not all styrene-oxidizing bacteria initiate styrene oxidation via epoxidation of the vinyl side chain; for example, in the styrene-oxidizer *Rhodococcus rhodochrous* strain NCIMB 13259, styrene catabolism is initiated by dioxygenation of the aromatic ring to form styrene-*cis*-glycol (*34*).

An interesting and distinguishing feature of AMOs is the high degree of stereoselectivity they exhibit for alkene epoxidation. For example, the purified AMOs from *Xanthobacter* Py2 and *R. rhodochrous* catalyze the oxidation of propylene with 95 and 92% yields, respectively, of R-epoxypropane (*13*, *35*). Styrene monooxygenase (StyA and StyB) from *Pseudomonas* sp. Strain VLB120, when expressed in *Escherichia coli*, oxidizes styrene with a 99.5% yield of *S*-phenylepoxyethane (*S*-styrene oxide) (*32*). In contrast, broad-substrate specificity oxygenases that catalyze alkene epoxidations fortuitously typically do so with little or no enrichment for a particular enantiomer, although there are some exceptions to this generalization (*24*, *36*, *37*).

Coenzyme M-Dependent Pathway of Epoxypropane Carboxylation. The best-characterized pathway of aliphatic alkene oxidation is that used for propylene metabolism in *Xanthobacter* Py2 and *R. rhodochrous* B276 (*35*, *38*−*43*). As shown in Figure 4, epoxypropane is converted to

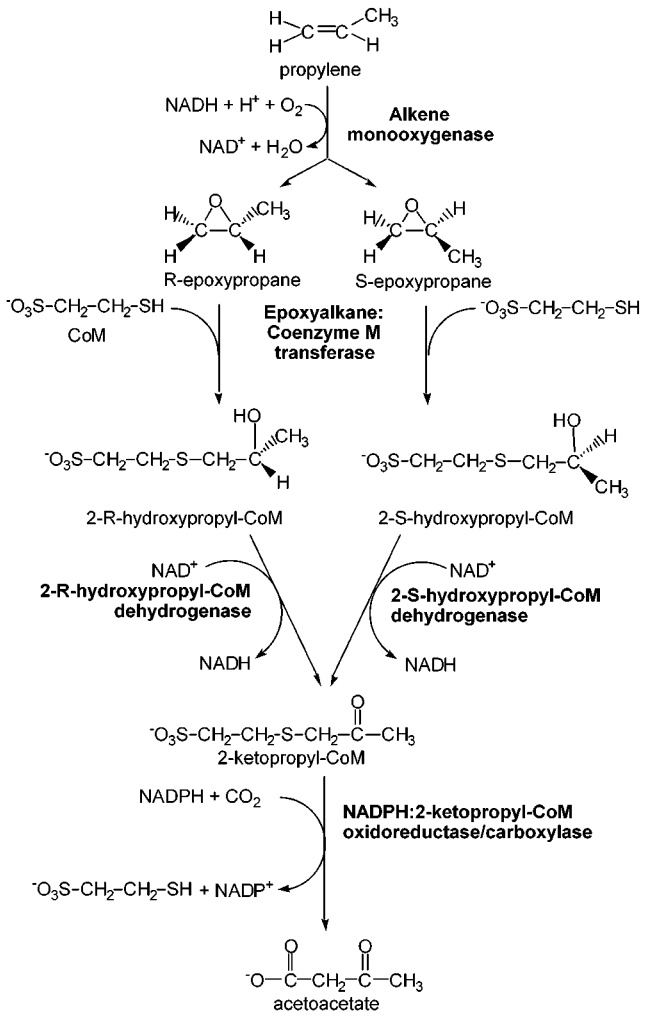


FIGURE 4: CoM-dependent pathway of propylene metabolism utilized by *Xanthobacter* sp. strain Py2 and *R. rhodochrous* strain B276.

acetoacetate in a three-step sequence of reactions that requires four enzymes, CO_2, NAD^+, NADPH, and an unusual cofactor, coenzyme M (CoM; 2-mercaptoethanesulfonic acid). While the transformation of an epoxide to a β-ketoacid is itself an unprecedented metabolic conversion, even more surprising is the discovery that CoM is the cofactor that facilitates this transformation (*35*). CoM was identified in 1974 as a central cofactor in the process of methanogenesis (*44*, *45*). In the methanogenic Archaea, a methyl group is transferred to CoM to form a methyl thioether that is subsequently reduced to methane (*45*, *46*). Prior to the identification of CoM as the cofactor of epoxypropane carboxylation, its role in methanogenesis was the only known function for this cofactor.

In the initial step of epoxypropane carboxylation, a CoM transferase catalyzes the reaction of CoM and epoxypropane to form a 2-hydroxypropylthioether conjugate (Figure 4) (*35*). This transferase, discussed in more detail below, uses either the *R*- or *S*-enantiomer of epoxypropane as substrate, forming the corresponding *R*- or *S*-2-hydroxypropylthioether (Figure 4). Two dehydrogenases that exhibit a high degree of stereoselectivity for their respective substrates catalyze the NAD^+-dependent oxidation of the hydroxypropylthioether conjugates to form the achiral 2-ketopropyl-CoM (*35*, *43*). Finally, a previously unknown type of carboxylase catalyzes the NADPH-dependent reduction, cleavage, and carboxylation of 2-ketopropyl-CoM, forming acetoacetate and CoM (*47*). CoM is unchanged at the completion of the reactions and is recycled for additional rounds of catalysis.

As noted above, the AMOs of *Xanthobacter* Py2 and *R. rhodochrous* B276 exhibit a high degree of stereoselectivity, forming *R*-epoxypropane in 85−90% enantiomeric excess (*13*, *35*). This stereoselectivity is not a condition for further epoxide transformations, as both bacteria express the two dehydrogenases required for dealing with *R*- and *S*-2-hydroxypropyl-CoM (*40*, *43*). The levels of expression of the dehydrogenases are not correlated with the higher flux of propylene through *R*-epoxypropane, as both dehydrogenases are present at comparable levels (∼1% of soluble protein when grown on propylene) (*40*, *43*). It makes intuitive sense that the bacteria have evolved to deal with both enantiomers: aliphatic epoxides are sufficiently toxic that it would be unwise to have them accumulate to detectable levels within the cell. As long as AMO is not absolutely stereoselective, there remains a need to deal with the minor enantiomer. In addition, the formation of a chiral thiol adduct of the minor enantiomer that did not undergo further transformation, i.e., as a detoxification product, would be wasteful, as new cofactor would need to be synthesized to replace that which was tied up in the nonproductive complex. Given these considerations, it is curious that the AMOs of the propylene-oxidizers have evolved with significant selectivity for *R*-epoxypropane formation. The physiological significance of this phenomenon is not known at present.

In contrast to the AMOs of *Xanthobacter* Py2 and *R. rhodochrous*, which differ significantly in composition (Figure 3), the four enzymes of epoxypropane carboxylation are virtually indistinguishable from a biochemical standpoint (i.e., subunit composition, molecular weights, cofactors, specific activities, substrate range) in the two bacteria (*40*, *43*). To date, the genes encoding these four enzymes have been cloned and sequenced in *Xanthobacter* Py2 but not *R. rhodochrous* (*48*). The analysis of the amino acid sequences of the *Xanthobacter* enzymes, together with biochemical, spectroscopic, and kinetic studies of the purified enzymes, has been invaluable in formulating working models for the mechanisms of the enzymes. These models are summarized below.

Epoxyalkane:CoM Transferase: A Zinc Metalloenzyme and MetE Homolog. The chemistry of CoM addition to epoxypropane is very similar to that of glutathione-*S*-transferase: a thiolate serves as a nucleophile for attack on an electrophilic carbon of the oxirane ring, resulting in ring opening and formation of an alcoholic thioether. A key feature of these enzymes is the ability to deprotonate the thiol group at neutral pH, thereby activating it for nucleophilic attack. The three-dimensional structures of a number of soluble GSTs indicate that the pK_a of the thiol of glutathione is lowered by hydrogen bonding interactions at the active site of the enzyme (*49*, *50*). Specifically, an interaction of the thiolate with an active site tyrosine has been implicated in this stabilization (*49*, *50*).

The biochemical and genetic characterization of epoxyalkane:CoM transferase suggests that a fundamentally different mechanism is employed for activation of the thiol of CoM. As purified, epoxyalkane:CoM transferase is an α_6 hexameric protein that contains one zinc atom per subunit

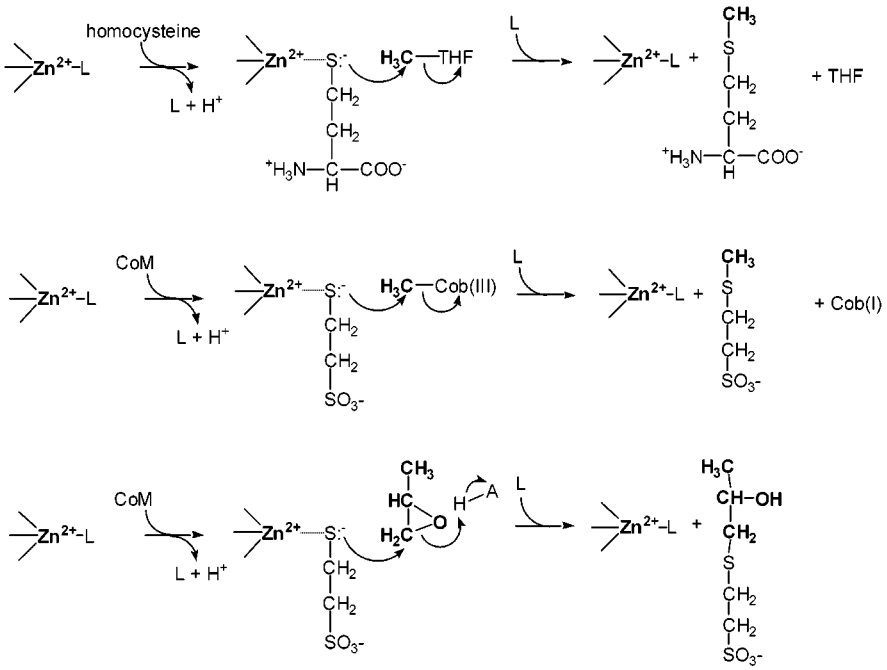


FIGURE 5: Mechanisms of MetE and homologues. (A) MetE; (B) epoxyalkane:CoM transferase; (C) methylcob(III)alamin:CoM methyltransferase.

(*40*, *51*). The amino acid sequence of the enzyme (*48*) shows sequence homology to another zinc protein, cobalamin-independent methionine synthase (MetE). MetE is one of a number of enzymes that have been shown or proposed to activate thiol groups by coordination to zinc (*52*, *53*). Other members of this enzyme family include farnesyl transferase (*54*, *55*), cobalamin-dependent methionine synthase (MetH) (*56*, *57*), the *E. coli* ADA protein (*58*, *59*), *S*-methylmethionine:homocysteine methyltransferase (*60*), betaine:homocysteine *S*-methyltransferase (*61*), and methylcob(III)alamin:CoM methyltransferase (MtaA) (*62*). In each enzyme, zinc is believed to coordinate the sulfur of the substrate, thereby lowering the pK_a of the thiol and activating it for alkylation. Several different zinc-binding motifs have been identified for this family of enzymes (*52*, *63*). In all cases, three permanent ligands are donated from Cys and His residues, with the fourth coordination site occupied by an exchangeable ligand that is removed upon thiol binding. In the case of epoxyalkane:CoM transferase, the putative zinc-binding motif is most like that of MetE, consisting of the consensus sequence **H**-X-**C**-X$_n$-D-**C**-G, with the permanent ligand residues highlighted in bold (*48*, *63*).

The zinc center of MetE has been extensively studied by biochemical techniques and EXAFS spectroscopy (*57*, *63*−*65*). In the resting state, zinc exists in a 2 S + 2 N/O tetrahedral coordination sphere (*57*). Upon binding homocysteine, the coordination changes to 3S + 1 N/O, as the exchangeable ligand, presumed to be water, dissociates and is replaced by homocysteine (Figure 5A) (*57*). Reaction with methyl-THF results in alkylation of homocysteine, dissociation of methionine from the enzyme, and reassociation of the exchangeable ligand (Figure 5A) (*57*).

EXAFS analysis of epoxyalkane:CoM transferase supports a similar mechanism for the alkylation of epoxypropane with CoM (*66*). In these studies, the addition of CoM resulted in a change of zinc environment from 2S + 2N/O to 3S + 1N/O (*66*). The addition of epoxypropane resulted in a return to a 2S + 2N/O environment, consistent with the mechanism shown in Figure 5C (*66*). Biochemical studies of MtaA, which has the same zinc-binding motif as MetE and epoxyalkane:CoM transferase, suggest a similar mechanism for the methylation of CoM (Figure 5B) (*62*). Thus, as summarized in Figure 5, the activation of CoM and homocysteine as nucleophiles is facilitated by coordination to a similar type of zinc center. MtaA and MetE share the additional common features that the alkylating agent is a methyl group donor (THF or cobalamin) that is itself a leaving group. In contrast, there is no leaving group in the alkylation of CoM by epoxyalkane:CoM transferase: ring opening leaves the entire epoxide unit bound to CoM as the secondary alcohol thioether adduct (Figure 5).

R- and S-2-Hydroxypropyl-CoM Dehydrogenases. The dehydrogenases that catalyze the oxidation of 2-hydroxypropyl-CoM enantiomers are highly specific for their respective substrates, exhibiting only 0.5 to 1% activity with the opposing enantiomer (*35*). Both dehydrogenases are homodimeric proteins, with subunit molecular weights of 25 000 and 26 000 for the S- and R-specific dehydrogenases, respectively (*40*, *43*). The amino acid sequences of the dehydrogenases reveal that they belong to the short-chain dehydrogenase/reductase (SDR) family of enzymes (*43*, *48*). The SDR family is composed of approximately 60 NAD^+ or $NADP^+$-dependent enzymes that are approximately 250 amino acids in length, active as dimers or tetramers, and carry out catalysis in the absence of a metal cofactor (*67*, *68*). The enzymes have three distinct domains: a conserved N-terminal NAD^+- (or $NADP^+$-) binding domain, a conserved central domain containing a catalytic triad of Ser, Tyr, and Lys, and a variable C-terminal domain that confers specificity for the specific enzyme substrate (*67*, *68*). Extensive structural, kinetic, and mechanistic characterization of SDR enzymes has allowed the formulation of a conserved mechanism of action (*67*, *68*). During catalysis, the Tyr residue of the catalytic triad is deprotonated and serves as a

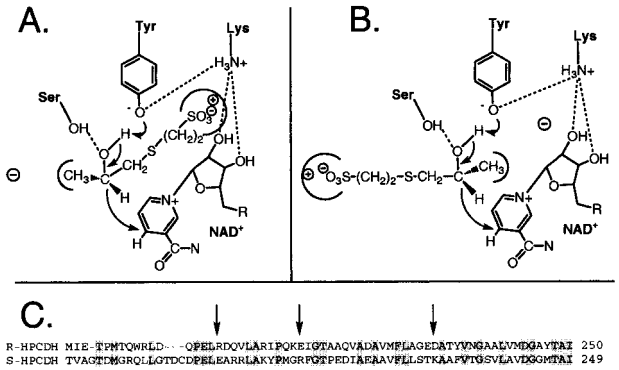
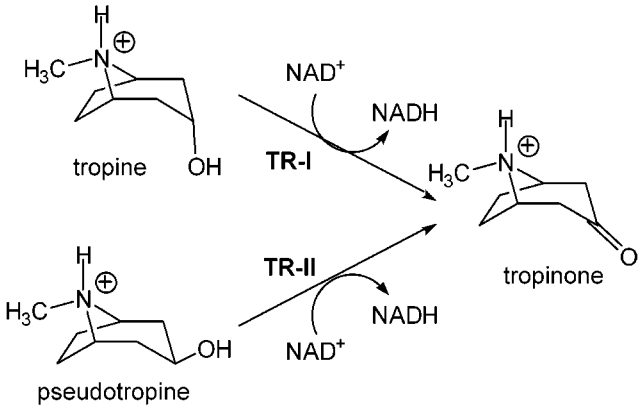


FIGURE 6: Stereospecific dehydrogenation of 2-hydroxypropyl-CoM enantiomers. (A) Model for substrate binding to S-HPCDH; (B) model for substrate binding to R-HPCDH; (C) global sequence alignment of the C-terminal domains of R- and S-HPCDHs. Nonidentities discussed in text are indicated by the arrows.

Scheme 1

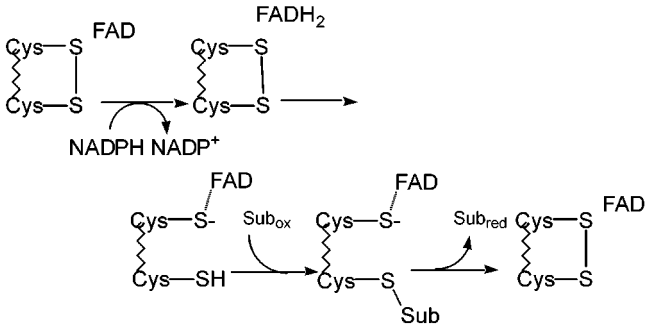


general base for abstraction of the proton from the hydroxyl group of the substrate. The Ser residue is believed to increase the acidity of the substrate hydroxyl through hydrogen bonding. The Lys residue hydrogen bonds to the ribose attached to the nicotinamide ring and stabilizes the deprotonated Tyr general base.

Figure 6 shows models for the binding and oxidation of *R*- and *S*-2-hydroxypropyl-CoM by their respective dehydrogenases. Since the positions of the hydroxyl and hydrogen groups on C2 must be oriented identically for catalysis to occur, the spatial orientation of the remaining groups, i.e., the methyl and methylene-CoM thioether moieties, will be reversed for the two enzymes (Figure 6). Presumably, differences in the C-terminal domains of the two SDRs are responsible for imparting specificity for the proper substrate. Electrostatic and/or hydrogen bonding interaction(s) with the sulfonate group of the CoM side chain are probably the primary determinant in substrate specificity, as there is little potential for specific interactions with the small, nonpolar methyl group (Figure 6). A relevant precedent for controlling specificity in this manner is found in the SDR enzymes tropinone reductase-I and II (TR-I and TR-II). TR-I and TR-II catalyze the reversible stereospecific reduction of the 3-keto group of tropinone to form the enantiomers tropine (α-hydroxy) and pseudotropine (β-hydroxy), respectively, as shown in Scheme 1 (*69*, *70*).

Electrostatic interactions between the positively charged ring nitrogen of the substrate and charged amino acid side chains control the active site orientation of the tropine

Scheme 2



stereoisomer so that the reacting hydroxyl is oriented properly with regard to the catalytic triad (*69*, *71*). For TR-II, Glu156 is proposed to form an electrostatic attraction that fixes the substrate in the proper orientation (*72*). In TR-I, His112 is proposed to repulse the positively charged nitrogen to facilitate the opposite binding orientation (*73*).

It is conceivable that a similar charge attraction/repulsion strategy, involving interactions with the negatively charged sulfonate moiety, controls proper binding of *R*- and *S*-2-hydroxypropyl-CoM to the proper SDRs. The C-terminal sequences of the dehydrogenases show similar sequence topologies interspersed with nonidentities (Figure 6C). Several of these nonidentities involve oppositely charged amino acid residues. For example, a positive Arg residue at position 203 in the R-HPCDH has a negatively charged Glu counterpart in S-HPCDH. The opposite change is present at position 214: a Glu in R-HPCDH has an Arg counterpart in S-HPCDH. Similarly, an Asp residue at position 232 in R-HPCDH has a lysine counterpart in S-HPCDH. It is tempting to speculate that the charge differences at one or more of these positions provide a charge attraction/repulsion mechanism for orientation of the proper enantiomer (Figure 6A and B). In recent studies of R-HPCDH, treatment of the enzyme with butanedione, an Arg-specific modifying agent, inactivated the enzyme for *R*-hydroxypropyl-CoM oxidation, but not for the oxidation of the smaller uncharged 2-propanol, which is a poor substrate for the enzyme (Clark and Ensign, unpublished results). *R*-hydroxypropyl-CoM protected against inactivation by butanedione, providing additional evidence that active site Arg residue(s) may be important to catalysis (Clark and Ensign, unpublished results). The crystallization and mutagenesis of the two HPCDHs are currently being pursued with the goal of elucidating the mechanisms of chiral discrimination.

NADPH:2-Ketopropyl-CoM Carboxylase/Oxidoreductase (2-KPCC). The final reaction of epoxypropane carboxylation is the CO_2- and NADPH-dependent cleavage of 2-ketoproyl-CoM to produce free CoM and acetoacetate (Figure 4). 2-KPCC is a homodimeric protein composed of 57-kDa subunits and containing 1 molecule of FAD per subunit (*41*, *74*, *75*). Sequence analysis of 2-KPCC showed the enzyme to be a member of the NADPH:disulfide oxidoreductase (DSOR) family of enzymes (*48*, *74*). Prominent members of this family include glutathione reductase, dihydrolipoamide dehydrogenase, and mercuric reductase (*76*). These enzymes are all NADPH-dependent, FAD-containing enzymes that contain a highly conserved redox active cysteine pair that participates in substrate reduction (*76*). A general mechanism for the DSOR enzymes is shown in Scheme 2,

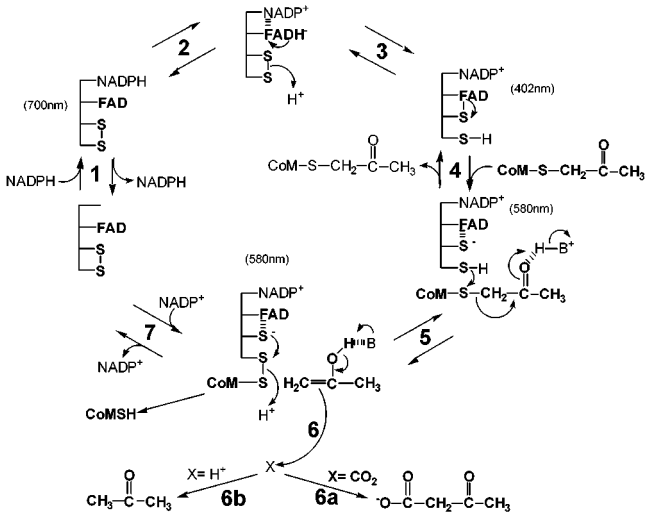


FIGURE 7: Proposed mechanism of reductive 2-ketopropyl-CoM cleavage and carboxylation by 2-KPCC. Step 1, binding of NADPH to oxidized enzyme; step 2, reduction of FAD; step 3, reduction of enzyme disulfide by $FADH_2$ with formation of interchange thiol-FAD covalent complex; step 4, electron transfer from FAD to interchange thiol to form charge-transfer complex; step 5, cleavage of thioether bond promoted by the formation of a heterodisulfide of proximal Cys and CoM; step 6, nucleophilic attack of enolacetone on CO_2 to form the product acetoacetate; step 7, reduction of heterodisulfide with release of CoM and formation of cysteine pair disulfide.

which highlights the roles of the cysteine pair in reducing the substrate molecule.

Kinetic and mechanistic studies of 2-KPCC (*47*, *75*) have allowed the formulation of a mechanism that uses this same general reaction scheme. As shown in Figure 7, the proximal cysteine is proposed to attack the sulfur atom of the substrate thioether linkage, resulting in the cleavage of the thioether bond and formation of a mixed disulfide between CoM and cysteine. The remaining portion of the substrate is released as the enolate of acetone. The attack of enolacetone on CO_2 results in the formation of the product acetoacetate. The mixed disulfide of CoM and cysteine is reduced by formation of the cystine disulfide pair to complete the catalytic cycle (Figure 7).

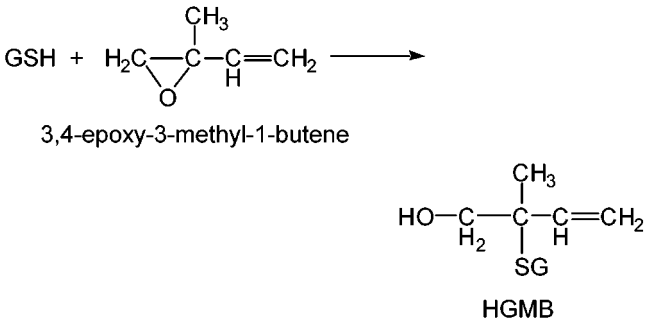
2-KPCC is unique in the DSOR family in two noteworthy regards. First, it is the only member of the family in which the substrate is a thioether. In all other cases but one, the substrate for DSOR enzymes contains a disulfide bond, e.g., oxidized glutathione or lipoamide, with reduction resulting in the formation of two reduced sulfhydryls (the exception is mercuric reductase, in which inorganic mercuric ion is reduced to elemental mercury). The second distinguishing feature of 2-KPCC is that it catalyzes the carboxylation of the product of reductive thioether bond cleavage. This product, enolacetone, is a highly reactive nucleophile that is extremely short-lived in aqueous solution. By some means, 2-KPCC is able to stabilize enolacetone for a sufficient duration to allow attack on CO_2. When CO_2 is excluded from assays, catalysis proceeds with the formation of acetone as a stoichiometric product of 2-ketopropyl-CoM cleavage due to a proton serving as an alternate electrophile for enolacetone (Figure 7, step 6b) (*47*). At present, it is not clear whether CO_2 or bicarbonate is the actual substrate for the enzyme. Recently, diffraction quality crystals have been obtained for 2-KPCC in the absence and presence of 2-ketopropyl-CoM and CoM (*77*). Interestingly, the presence of substrate or product provided the highest diffraction quality crystals (>2.0 Å resolution with 2-ketopropyl-CoM or CoM bound vs 2.8 Å for the free enzyme) (*77*). The structure of 2-KPCC is currently being solved for all three enzyme forms and will hopefully reveal insights into the novel features of this DSOR enzyme.

Genetic Characterization of Propylene Metabolism. The genes encoding AMO and the epoxyalkane carboxylation enzymes have been cloned and sequenced from *Xanthobacter* Py2 (*48*, *78*, *79*). The six genes encoding the AMO system are clustered in an operon, as are the four genes encoding the epoxide carboxylation enzymes. The AMO and epoxide carboxylation genes were cloned in separate studies, and it is not presently known how closely they reside to one another. Recently, all of the above genes were found to reside on a 320-kb linear megaplasmid in *Xanthobacter* Py2 (*80*). Repeated subculturing with glucose or acetate as the carbon source resulted in the spontaneous loss of the linear megaplasmid, demonstrating that selective pressure is required for its maintenance (*80*). Similarly, in *R. rhodochrous* B276, the genes encoding AMO were shown to reside on a 185-kb linear megaplasmid that was lost under nonselective growth conditions (*81*). The loss of the 320-kb plasmid in *Xanthobacter* Py2 correlates with a propylene-minus phenotype and inability to synthesize CoM, suggesting that the CoM biosynthetic genes are located on the plasmid as well (*80*). In support of this idea, a gene has been identified downstream of the epoxide carboxylase genes that encodes a homologue of a methanogen protein that may catalyze an initial step in CoM biosynthesis (*80*). However, this result remains to be confirmed biochemically and genetically.

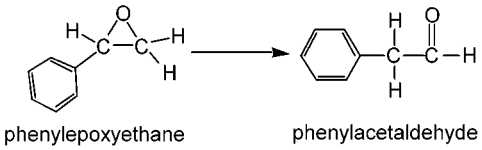
Other Pathways of Aliphatic Alkene Metabolism. To date, CoM has been identified as a cofactor of aliphatic alkene metabolism only for the two propylene-oxidizers discussed above. It remains to be determined whether other bacteria that metabolize short-chain alkenes by uncharacterized pathways, e.g., strains of *Mycobacterium* that grow on ethylene, use this cofactor as well. It is unclear why the propylene-oxidizers evolved to use CoM, an atypical cofactor, rather than a more conventional thiol cofactor such as glutathione. CoM is remarkable in its elegant simplicity: it is the world's smallest organic cofactor, consisting of sulhydryl and sulfonate functional groups linked by an ethane moiety. The only other organisms in which CoM has been found are the methanogens, which, as Archaea, are in a different domain of the phylogenetic tree. It is possible that the ability to synthesize CoM was acquired by hydrocarbon-oxidizing bacteria from methanogens through gene transfer. Alternatively, CoM may have evolved in eubacteria for specialized functions and been acquired by the methanogens at a later date. In any event, it is intriguing that this atypical cofactor has resurfaced in another pathway that involves microbial hydrocarbon metabolism.

Recent studies of isoprene and styrene metabolism suggest that they are oxidized by pathways that do not involve CoM. In the isoprene-oxidizing bacterium *Rhodococcus* sp. Strain AD45, the monoepoxide 1,2-epoxy-2-methylbutene was identified as the initial product of isoprene oxidation. As noted earlier, six genes encoding proteins with sequence identity to the AMO of *Xanthobacter* Py2 have been identified in this strain, suggesting the presence of a similar

Scheme 3



Scheme 4



four-component AMO (*28*). The monoepoxide is further metabolized by a glutathione-*S*-transferase with specificity for attack at the tertiary carbon atom, forming 1-hydroxy-2-glutathionyl-2-methyl-3-butene (HGMB) (*82*) as shown in Scheme 3.

Thus, in this bacterium, the more conventional glutathione rather than CoM is the nucleophile responsible for epoxide ring opening. HGMB is sequentially oxidized to the aldehyde and carboxylate by a single NAD^+-dependent dehydrogenase that is expressed at high levels (16% of soluble protein) in isoprene-grown cells (*82*). The HGMB dehydrogenase was purified and shown to be a homodimer of 25-kDa subunits (*82*). The N-terminal sequence of HGMB dehydrogenase suggests that it, like the dehydrogenases of epoxypropane metabolism, belongs to the SDR family of enzymes. The ultimate fate of the product of HGMB oxidation is not known at present, although it has been speculated that it may involve removal of glutathione and addition of CoA, forming an acyl-CoA thioester for entry into β-oxidation (*82*).

Finally, an entirely different strategy of epoxide conversion is found in styrene-oxidizing bacteria, where styrene oxide, the product of styrene epoxidation, is isomerized to phenylacetaldehyde by a styrene oxide isomerase (*32*, *83*, *84*) as shown in Scheme 4.

The gene encoding the styrene oxide isomerase, *styC*, was cloned and found to encode a 169-amino acid protein, but the enzyme itself has not, to date, been purified or characterized (*33*). The further metabolism of phenylacetaldehyde proceeds by oxidation to phenylacetate or reduction to phenylethanol followed by aromatic ring cleavage and further oxidation (*29*, *30*, *84*).

Summary. With the exception of methane, the detailed study of the genes, regulation, pathways, and enzymes of short-chain hydrocarbon metabolism has lagged behind that of more conventional metabolites. Short-chain aliphatic alkenes are now recognized as being important components of biogeochemical hydrocarbon cycling. For example, the combined yearly production of ethylene and isoprene by biological sources is estimated to be in the hundreds of millions of tons, an amount comparable to that of the annual production of methane (*85*). The studies summarized above demonstrate that bacterial hydrocarbon metabolism involves novel and surprising pathways, enzymes, and cofactors. The enzymes of bacterial propylene metabolism are especially illustrative for showing how common enzyme themes are applied to diverse metabolic transformations. The properties of alkene-oxidizing bacteria and their specialized enzymes suggest they could be valuable biotechnological tools for such important processes as bioremediation and the synthesis of enantiopure epoxides and biopolymers.

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