Carboxylases Involved in Microbial Acetone and Acetophenone Metabolism

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Carboxylases involved in microbial acetone and acetophenone metabolism

by

Ameya Mashruwala

A report submitted in partial fulfillment of the requirements for the degree of

Master of Science (Plan B)
in

Biochemistry

UTAH STATE UNIVERSITY
Logan, Utah

2010
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ABBREVIATIONS

AcCx
acetone carboxylase

ADP
adenosine 5'-diphosphate

AMP
adenosine 5'-monophosphate

ATP
adenosine 5'-triphosphate

CTP
cytidine 5'-triphosphate

EPR
electron paramagnetic resonance

GTP
guanosine 5'-triphosphate

ITP
inosine 5'-triphosphate

MgAMP
complex of Mg$^{2+}$-adenosine 5'-monophosphate

Rubisco
ribulose-1,5-bisphosphate carboxylase/oxygenase

TTP
thymidine 5'-monophosphate

XTP
xanthosine 5'-monophosphate

UTP
uridine 5'-monophosphate
A number of bacteria are capable of growth with acetone and acetophenone as their sole sources of carbon and/or energy. The pathways and enzymes involved in the transformation of these molecules into useable carbon and energy are unique. Among these are two novel enzymes, acetone carboxylase and acetophenone carboxylase, which represent a fundamentally novel classes of carboxylases.

The initial step in acetone metabolism, in *X. autotrophicus* st Py2, *R. capsulatus* st B10 and *R. rhodochrous*, is the thermodynamically unfavorable reaction to yield acetoacetate. This step is catalyzed by the enzyme acetone carboxylase and is coupled with the unprecedented, concomitant hydrolysis of two phosphoanhydride bonds of ATP. This enzyme also requires two tightly bound Mn$^{2+}$ ions, among other co-factors, for catalytic activity.

In a similar, albeit distinct manner, acetophenone carboxylase catalyzes the carboxylation of acetophenone to benzoylacetate. Reminiscent of acetone carboxylase, carboxylation activity in this enzyme is dependent on the hydrolysis of ATP to ADP and inorganic phosphate. Catalytic activity is also dependent on Zn$^{2+}$ and either of Mn$^{2+}$ or Mg$^{2+}$ as co-factors. Additionally, similar to acetone carboxylase, acetophenone carboxylase shows uncoupled ATPase activity with either bicarbonate or acetophenone in the absence of a second substrate. This indicates that both substrates may be phosphorylated.
The studies on acetone and acetophenone carboxylase have expanded our knowledge on the novel mechanisms and cofactors involved in the metabolism of toxic, reactive, xenobiotic molecules such as acetone and acetophenone. Further, as in the case of acetone carboxylase, homologs of these enzymes are found in higher organisms such as mammals. The biochemical and mechanistic properties of these enzymes may be relevant to the modes of action of these homologs. In this report, using the work currently accomplished on these enzymes, and by discussing their salient features, an effort has been made to provide a detailed insight into these enzymes.
INTRODUCTION

Background and Significance:

Acetone and acetophenone are toxic molecules. They have been observed as carbon sources and intermediates mainly in bacterial but also in mammalian metabolism or as parts of bacterial xenobiotic detoxification strategies. These compounds are frequently released into the environment either through natural sources or from spills related to human handling. Apart from this acetone and acetophenone are also continuously produced by biological processes in microorganisms and higher organisms, such as plants and animals.

The inherent stability of these xenobiotics and the wide range of health concerns associated with them have made it imperative to clean these compounds from our environment. The economic and environmental impact of discovering novel means of disposing of these molecules will only gain in importance in the current world, which is increasingly dependent upon industrial chemical processes. Consequently, it is important to gain a deeper understanding of the microbial metabolism of acetone and acetophenone, in particular of the novel metabolic pathways and enzymes which catalyze the conversion of these toxic molecules to inert metabolites. Understanding the physiology and biochemistry of bacterial acetone and acetophenone metabolism will also provide new insights into how these molecules may be metabolized in mammalian systems, for which
the mechanism of metabolism is relatively unknown. The focus of the current study was to put together the work thus far accomplished in this field and present an insight into the unique mechanisms by which novel bacterial enzymes convert these toxic molecules into relatively non-reactive metabolites.

**Sources of acetone:** Anthropogenically acetone is produced directly or indirectly from propylene. Most commonly, in the cumene process, benzene is alkylated with propene and the resulting cumene (isopropylbenzene) is oxidized to give phenol and acetone.

In biological systems acetone is produced as an intermediate or end product of several processes: bacterial fermentation, bacterial propane metabolism and bacterial alkene and epoxide metabolism. It is also formed and accumulated in high levels in mammals under conditions of starvation.

**Bacterial:** The classic bacterial fermentative pathway producing acetone is found in *Clostridium acetobutylicum*, where acetone is produced as a major end product from the decarboxylation of acetoacetate, in reactions catalyzed by acetoacetate decarboxylases (27, 94) (Scheme 1). It is also an intermediate of bacterial propane metabolism wherein it is activated by an initial hydroxylation reaction producing isopropanol. This is further oxidized with the aid of a secondary alcohol dehydrogenase to acetone (Scheme 2).
Scheme 1

\[
\text{OOC} + \text{H}^+ \rightarrow \text{O} \quad \text{CO}_2
\]

Scheme 2

Mammalian: In mammals, under conditions of starvation, acetyl CoA is formed in high quantities from the \( \beta \) -oxidation of fatty acids. Two molecules of these are condensed to form acetoacetate in the liver. Acetone is produced from the spontaneous and enzymatic decarboxylation of some of this acetoacetate (2, 51, 68). The remaining undergoes reduction to \( \beta \)-hydroxybutyrate. In healthy mammals less than 2% acetone is found in blood, however, under starvation conditions or in diabetics abnormally high concentrations of ketone bodies are found. Blood acetone levels as high as 1.6 and 8.9 mM in fasting individuals and diabetics respectively have been reported (2, 62).
Ultimately the acetone formed by this process is used in the hepatic and peripheral tissues for conversion into gluconeogenic precursors (2, 36, 44).

**Reactivity of acetone:** Despite being an electrophillic molecule, a result of its carbonyl moiety, acetone is widely used as a solvent due to its relative inertness and low cost compared to other industrial solvents. The widespread use of acetone has made it necessary to study the effects of our exposure to it in our workplace and environment (both endogenous and exogenous). Acetone has also been known to cause bronchial irritation and general intoxication resulting in a change in heart rate and a fall in body temperature (43, 80). It has also been reported to have neurotoxic effects (60), although this is subject to debate (33).

**Biological acetone metabolism:**

A variety of pathways have been proposed for both mammals and bacteria (Fig 1). The first studies of bacterial strategies for acetone metabolism were conducted with the anaerobic phototroph *Rhodopsuedomonas gelatinosa* (82). Since then a number of bacteria, both aerobic and anaerobic, have been shown to be capable of utilizing acetone as a growth supporting substrate and sole source of carbon (30). In aerobic bacteria, it has long been proposed that the initial step in acetone metabolism occurs via an acetone monooxygenase (30, 55, 88, 91). A recent study, contributing much to the greater understanding of bacterial propane metabolism, has provided first evidence for a Baeyer-
Villiger monooxygenase (BVMO) dependent acetone metabolism in *Gordonia* sp strain *Ty-5* (49). Acetone is oxidized to methyl acetate by a novel Baeyer-Villiger monooxygenase and the methyl acetate produced is hydrolyzed to acetate and methanol by an esterase (Fig 1) (49). In facultative or strictly anaerobic bacteria the initial step in acetone metabolism is proposed to occur via a carboxylation reaction (7, 38, 70, 71, 82). This includes sulfate reducers, denitrifiers and fermentative enrichments (8, 38, 70-72). Most evidence supporting CO$_2$ dependent anaerobic acetone metabolism has been indirect, based mostly upon whole cell studies and all attempts to reconstitute AcCx activity in vitro have been unsuccessful. AcCx activity has also been reported in cell-free extracts of another photosynthetic bacterium *Rhodomicrobium vannielli* (7). Acetone is carboxylated to acetoacetate which could then be converted to acetoacetyl CoA by β-ketoacyl CoA transferase. Acetoacetyl CoA is presumed to subsequently undergo thiolysis with another molecule of CoA resulting in two molecules of acetyl CoA that can enter the central metabolic pathways. Recently, a gram negative proteobacterium, *X. autotrophicus* strain Py2, was the first aerobic bacterium proven not to utilize a monooxygenase enzyme for oxidation of acetone. The enzymes required for acetone metabolism in *X. autotrophicus* were shown to be inducible and the degradation of acetone in both whole cells and cell lysates required carbon dioxide (85). Acetone was shown to be carboxylated, forming acetoacetate in a CO$_2$ and ATP dependent manner. Carboxylation was also demonstrated to be the initial step of acetone metabolism in three other subsequently studied bacteria, in the gram negative purple non sulfur photosynthetic soil bacterium *Rhodobacter capsulatus* strain B10, the gram positive obligate anaerobe *Rhodococcus rhodochrous* strain B276 (18, 83, 84) and the aerobic
soil-dwelling organism *Azotobacter vinelandii* (Scheme 3). This suggests that carboxylation may be a ubiquitous strategy for bacterial acetone metabolism. The enzyme catalyzing this carboxylation, coined acetone carboxylase, was isolated to homogeneity from *X. autotrophicus* and *R. capsulatus* and partially purified from *R. rhodochrous* (18, 83, 84).

\[
\text{O} + \text{ATP} + \text{O} = \text{C} = \text{O} \rightarrow \text{OOC} + \text{AMP} + 2\text{Pi} + \text{H}^+ 
\]

*Scheme 3*

**Sources of acetophenone:** Industrial acetophenone is produced mainly as a by-product of the oxidation of ethylbenzene, which gives ethylbenzene hydroperoxide for use in the production of propylene oxide (45). Acetophenone and other aryl ketones can also be produced directly from the atmospheric breakdown of ethylbenzene (a major petroleum component) or following the abiotic conversion of ethylbenzene to ethylphenol and its subsequent biological conversion to acetophenone (45).
Fig 1: Possible pathways of bacterial acetone metabolism. Figure adapted from (30)
Biological acetophenone metabolism:

**Aerobic:** The aromatic nature of acetophenone confers it with high chemical stability. As a result biological mechanisms have evolved to make it more susceptible to oxidation. Acetophenone possesses several positions at which it is susceptible to oxidation and these sites vary with the source and specificity of the enzyme system involved. A variety of pathways have been proposed for acetophenone metabolism and, similar to aerobic acetone metabolism, the initial oxidation reactions are catalyzed mostly by mono- or dioxygenases (22-24). Molecular oxygen, acting as a strong oxidizing agent, is incorporated into acetophenone with the help of these oxygenases (56).

An *Arthrobacter* sp. and a *Nocardia* sp. have been reported to oxidize acetophenone to catechol via a Baeyer Villiger mechanism (23, 24). Side chain elimination is achieved by the action of the first enzyme of the catabolic sequence, acetophenone oxygenase, a classical mixed function oxidase (23, 56). The product of this reaction, phenyl acetate, is converted to catechol, which is finally degraded by the β-oxoadipate pathway (66). Acetophenone has also been shown to be oxidized to 2-hydroxyacetophenone by a naphthalene dioxygenase from *Psuedomonas* sp.strain *NCIB 9816-4* (52). Acetophenone is also known to be a common intermediate in the bacterial metabolism of monoaromatic hydrocarbons such as ethylbenzene and toluene (22, 90). The oxygenated intermediates are then further oxidized via conventional metabolic pathways.
Anaerobic: Oxygenase catalyzed reactions are used to activate aromatic hydrocarbons, such as acetophenone, under aerobic conditions. However, such activation mechanisms are not possible under anaerobic conditions (3). Thus, the degradation of aromatic compounds under anaerobic conditions was not thought to occur until it was first postulated in 1984 (3). However, in the last 15 years, the field has seen significant advances and several pure cultures under denitrifying, sulfate-reducing and iron-reducing conditions have been isolated (3).

As in the case of anaerobic acetone metabolism, the initial step in anaerobic acetophenone metabolism is also proposed to occur via a carboxylation reaction. The anaerobic mineralization of ethylbenzene, previously reported in denitrifying strains such as *Aromatoleum aromaticum* species strain EB1 and strain EbN1, proceeds via S-(−)-1-phenylethanol and acetophenone as intermediates in the pathway (Fig 2). The degradation is initiated by conversion of ethylbenzene to (S)-(−)-1-phenylethanol, by the molybdenum containing enzyme ethylbenzene dehydrogenase (Fig 2) (41, 46, 75). An alcohol dehydrogenase catalyzes the conversion of (S)-(−)-1-phenylethanol to acetophenone (37, 47). Further conversion to benzoyleacetaetate is proposed to involve a carboxylation reaction, followed by the thiolytic removal of an acetyl CoA moiety. Benzoyl-CoA is then further oxidized via a reductive ring cleavage to carbon dioxide (Fig 2) (40, 41, 50, 76). Though this pathway has been demonstrated to be carbon dioxide dependent, the evidence supporting utilization of CO2 as a co-substrate in the carboxylation of acetophenone is mostly indirect and based on CO2 dependent growth of bacteria in particular (3, 15, 50).

The enzyme required for acetophenone metabolism and carboxylation in *Aromatoleum aromaticum* st. EB1 and st. EbN1 was shown to be inducible and the degradation of
acetophenone in both whole cells and lysates required carbon dioxide. The same enzyme is also involved in acetophenone metabolism in the absence of ethylbenzene (40, 41, 50). The enzyme catalyzing this carboxylation, coined acetophenone carboxylase, has been recently isolated to homogeneity, by Jobst et al., from *Aromatoleum aromaticum* EbN1 (39).
Fig 2: Proposed catabolic pathway of initial reactions in the anaerobic degradation of ethylbenzene and acetophenone in Aromatoleum aromaticum sp.strain EB1 and strain EbN1. Figure adapted from (3, 41, 50, 75)
Review of carboxylation strategies

Carboxylation is primarily a thermodynamic problem. Enzymes solve this by associating the carboxylation reaction with some exergonic process. As mentioned further in the text, this may be via nucleotide hydrolysis (ATP, as in the case of pyruvate carboxylase), hydrolysis of phosphoenolpyruvate (phosphoenolpyruvate carboxylase), or cleavage of a carbon-carbon bond (ribulose-bisphosphate carboxylase).

There are two other major challenges faced by carboxylase enzymes, 1) carbanion generation and stabilization and 2) which species of CO$_2$ to utilize. It can use CO$_2$ which is a good electrophile albeit present in low physiological concentrations or HCO$_3^-$, a poorer electrophile albeit present in plenty under physiological pH conditions. Enzymes use either one of the two; the only enzyme capable of using both is carbonic anhydrase (64). In general most carboxylase enzymes, such as (the sluggish and therefore abundant enzyme) ribulose-1,5 bisphosphate carboxylase, vitamin K-dependent carboxylases, phosphoenolpyruvate carboxykinase and carboxytransphosphorylase, use CO$_2$. The exceptions are most of the biotin dependent enzymes, phosphoenol pyruvate carboxylase and carbamoyl phosphate synthetase which activate HCO$_3^-$ for nucleophilic attack in an ATP consuming reaction (48, 64, 81).

A number of the carboxylase enzymes are biotin dependent enzymes and utilize HCO$_3^-$, which enables them to function more efficiently with a one carbon substrate (48). They use the energy derived from ATP to activate the bicarbonate to species that can subsequently react with biotin to form N-carboxybiotin (48). As shown in Fig 3, the
gamma-beta phosphodiester bond of ATP is used to activate the bicarbonate, forming carboxyphosphate. The formation of carboxyphosphate has been observed in a few enzymes; however, this species is highly unstable, hydrolyzing in aqueous solution within a few seconds (64, 96). It spontaneously breaks down into CO₂ and PO₄²⁻ (64, 96). The newly formed CO₂ is then attacked by biotin, which acting as a carbon carrier provides a substrate for the carboxylation reaction. Well known examples of biotin containing enzymes include pyruvate carboxylase (92), acetyl-CoA carboxylase, propionyl CoA carboxylase, geranyl CoA carboxylase, urea carboxylase and transcarboxylase (93). Notably, no biotin is associated with acetone carboxylase, thus indicating that if ATP hydrolysis is used to activate bicarbonate to form CO₂, the substrate is not subsequently carried by biotin for carboxylation.

Fig 3: Hypothetical mechanism for the activation of a bicarbonate ion. Phosphate group is transferred from ATP, forming carboxyphosphate and ADP. Carboxyphosphate spontaneously breaks down into carbon dioxide and inorganic phosphate. Figure adapted from O’ Leary (64)
The only known biological role of vitamin K, in higher organisms, is as a cofactor for vitamin K dependent carboxylase (VKD). The only non-mammalian species in which VKD is found to be functional is the marine snail Conus (4, 26). VKD is an integral membrane protein and uses the energy of vitamin K hydroquinone (KH$_2$) oxygenation to catalyze the carboxylation of the amino acid, glutamic acid (Glu), resulting in its conversion to gamma-carboxyglutamic acid (Gla) (Fig 4) (32). During carboxylation KH$_2$ and O$_2$ react to form a strong vitamin K base intermediate that collapses to form a vitamin K epoxide product upon protonation (Fig 4) (6, 28). It has been proposed that a cysteine (Cys) acts as the carboxylic catalytic base that deprotonates the KH$_2$, however this is under dispute as recent studies indicate the base to be an activated amine. Each round of catalysis results in the epoxidation of the VDK protein and the oxidation of the KH$_2$ to vitamin K epoxide (6, 9, 28, 73).

The direct carboxylation of pyruvate to form oxaloacetate is thermodynamically unfavourable (64). The enzymes phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase solve this problem by starting with the high energy compound phosphoenolpyruvate (PEP) (64).

Phosphoenolpyruvate carboxylase catalyzes a non reversible reaction and creates oxaloacetate from PEP (16, 31, 64). The substrate for the carboxylation is HCO$_3^-$ rather than CO$_2$ (65). The phosphate group from PEP is transferred to the bicarbonate ion creating carboxyphosphate and the enolate of pyruvate (Fig 5). The carboxyphosphate decomposes spontaneously in the active site of the enzyme, where it is protected from...
Fig 4: The VKD protein carboxylase reaction which uses the energy of vitamin K hydroquinone oxygenation to convert Glu to Gla. Figure adapted from Berkner et al (6)

hydrolysis, to form CO₂ and Pi (Fig 5). The CO₂ then reacts with the enolate to form oxaloacetate and Pi (31, 64). PEP carboxylase requires either Mg or Mn ions for catalysis and it is believed that these ions facilitate stabilization by co-ordination as shown in (Fig 6) (16, 64).
Fig 5: Reaction catalyzed by phosphoenolpyruvate carboxylase wherein phosphoenolpyruvate is converted to oxaloacetate. Figure adapted from O’Leary (64).

Fig 6: Mn ions facilitate stabilization by co-ordination. Figure adapted from O’Leary (64).
In contrast to PEP carboxylase, PEP carboxykinase catalyzes a readily reversible reaction forming oxaloacetate from PEP (63, 64). Additionally the one carbon substrate is CO$_2$ rather than HCO$_3^-$ (42). However, like PEP carboxylase, PEP carboxykinase forms an enolate by phosphate transfer from PEP, thus eliminating the thermodynamically unfavorable proton removal step. The enzyme begins by transferring a phosphate to a nucleotide diphosphate (NDP), forming a nucleotide triphosphate (NTP) (Scheme 4). This enolate then reacts with CO$_2$. This is also one of the few enzymes where there is evidence of an enzyme-metal-CO$_2$ complex (34, 35, 63, 64).

Scheme 4

Any discussion upon carboxylases and their mechanisms would be incomplete without a mention of ribulose 1, 5 diphosphate carboxylase oxygenase, considered the most abundant protein on Earth (19, 20). It catalyzes the primary reaction by which inorganic
carbon enters the biosphere. The enzyme utilizes a metal ion cofactor Mg$^{2+}$, in conjunction with proton abstraction by a lysyl carbamate general base, to facilitate endiolization for attack on CO$_2$ (19, 64).

**Concluding remarks:** This study is devoted to summarizing, and presenting an insight into the work currently accomplished in studying the unique mechanisms by which novel bacterial carboxylases convert acetone and acetophenone into relatively non reactive metabolites. The introduction went into great detail on the various pathways of acetone and acetophenone metabolism as well as the catalytic mechanisms of other similar carboxylase enzymes. It is hoped that this will help the reader orient themselves to better follow the discussion herein.
ACETONE CARBOXYLASE

Molecular properties and the genetic footprint of AcCx:

Acetone carboxylase (AcCx) catalyzes the carboxylation of acetone, a toxic organic molecule, to acetoacetate, a central metabolite, with the concomitant hydrolysis of ATP to AMP and two inorganic phosphates (11, 12, 83). The production of AMP and inorganic phosphate (Scheme 3) is unprecedented among ATP dependent carboxylases.

AcCx is a multimeric acetone utilizing enzyme that was first purified and characterized from Xanthobacter autotrophicus strain Py2 (83). It has been isolated from different bacteria; it has been purified to homogeneity from R. capsulatus strain B10 and partially purified from R. rhodochrous strain B276 (18, 83, 84) and A. vinelandii (unpublished data). AcCx is an inducible enzyme, expressed in high levels (~20% of total cell protein) in acetone grown cells. Interestingly neither of the two well characterized AcCx systems, from X. autotrophicus or R. capsulatus, is subject to catabolite repression, as the presence of alternative carbon substrates, such as glucose or malate, does not affect AcCx expression. AcCx is a heterohexameric protein comprised of three polypeptides having molecular weights of 86, 78 and 19 Da (83). The polypeptides are arranged as a trimer of dimers, $(\alpha_2 \beta_2 \gamma_2)$ and it is interesting to note that such diverse bacteria contain an enzyme with a conserved subunit structure (18, 83, 84). Additionally the genes encoding the AcCx subunits are arranged in operons consisting of the genes acxABC, which encode
the beta, alpha and gamma subunits of the enzyme respectively (11, 84) (unpublished data). This characteristic operon, consisting of 3 ORF’s, was identified using a highly efficient transposon mutagenesis system (84). A BLAST analysis of these genes further highlighted the uniqueness of this operon, and, of the genomes sequenced to date, a similar organization of genes was observed only in the case of the three *Helicobacter pylorii* strains (1, 14, 84, 89). The putative products of these genes share homology with the subunits of AcCx (encoded by *acxABC*) from *Xanthobacter autotrophicus* strain Py2 and *Rhodobacter capsulatus* strain B10. Indeed recent studies by Hoover et al., with *H. pylori* SS1, provide evidence that a functional *acxABC* operon operates in *H. pylori* and appears to help enhance colonization by the bacteria in mice. It is further hypothesized that products of the *H. pylori acxABC* operon may function primarily in acetone utilization or may catalyze a related reaction that is important for survival or growth of *H.pylori* in the host.

This is intriguing since *H.pylori* is in the epsilon subclass of proteobacteria with a fairly different physiology and morphology. *Helicobacter pylorii* are pathogenic bacteria, colonizing the human stomach and are an etiological agent of peptic ulcer disease. Further they have no known non-human reservoir (14, 21, 78, 84).

The amino acid sequences of the three subunits share high identity with each other (70-84%). However, they share no sequence homology with the biotin-dependent carboxylases, rubisco, or any enzyme catalyzing a reaction similar to acetone carboxylation such as PEP carboxylase and PEP carboxykinase (11, 84). The only enzymes with which AcCx shares any significant identity are proteins in the hydantoinase
subfamily of the cycloamindohydrolases, which show 15-30% identity with the alpha and beta subunits (11, 84). Importantly the gamma subunit does not share significant identity with any known enzyme (11, 84). Hydantoinases use zinc to facilitate substrate hydrolysis and N-methylhydantoinase requires ATP for substrate hydrolysis (57, 87).

**Regulation of acetone degradation:** Another interesting observation, with regards to the genetic footprint of AcCx, all of the acetone carboxylase operons identified to date have an associated $\sigma^{54}$ or $\sigma^{70}$ dependent transcriptional activator (84). The only exceptions are the *Helicobacter* strains. The $\sigma^{54}$ dependent activator, termed AcxR, has features unique to $\sigma^{54}$ dependent transcriptional activators, including a distinctive switch I motif (consensus SELFGXXXGAFTGA) (84, 97). It also has the signature -24/-12 promoter element, required for $\sigma^{54}$ holoenzyme transcription initiation (5, 84). In *R. capsulatus*, the AcxR has features more reminiscent of enhancer binding proteins that function with the housekeeping $\sigma^{70}$-RNA polymerase (67, 84). There is a defined precedent in *R. capsulatus* for using the $\sigma^{70}$ in conjunction with cis-acting elements to regulate gene expression (10, 67, 84, 97). Indeed interaction of NtrC with $\sigma^{70}$ has been found to regulate nitrogen metabolism in *R. capsulatus* (10, 84, 97).
Mechanistic Studies of AcCx

Nucleotide hydrolysis, products of hydrolysis and isotope exchange studies: AcCx from *X. autotrophicus* was found to exhibit an obligate requirement for ATP as a cofactor (83). The other three acetone carboxylases obtained to date also require nucleotide hydrolysis for substrate carboxylation (18, 83, 84). The enzyme partially purified from *R. rhodochrous* strain B276 prefers to use GTP as the nucleotide of choice, whereas the others show a preference for ATP (18). AcCx from *R. rhodochrous* is also capable of using ITP, CTP, UTP and XTP to drive carboxylation, albeit at lower rates (~50% lower) as compared to GTP (18, 84).

Acetone carboxylation is a thermodynamically unfavorable process (ΔG° for acetone carboxylation with bicarbonate is ~17.1 kJ/mol) and it is not surprising that it is coupled to an exergonic reaction, nucleotide hydrolysis, to drive carboxylation. As mentioned earlier, the carboxylation of acetone to acetoacetate was shown to be coupled to the formation of AMP as a stoichiometric product (1:1 mol ratio) (83). Additionally no pyrophosphate formation was observed, indicating that acetone carboxylation requires the hydrolysis of both the γ-β and β-α phosphodiester bond of a single molecule of ATP. The purified enzyme has no visible spectra indicating that biotin is associated with AcCx and is a required co-factor for the enzyme (83-85).

As the initial step in catalysis, all organic substrate carboxylations require the formation of a carbanion for attack on electrophilic CO₂ or an activated CO₂ species (such as carboxyphosphate) (29, 48). Carboxylase substrates such as acetyl-CoA, pyruvate and
propionyl-CoA contain an electrophillic carbonyl \( \alpha \) to the C-H bond to be cleaved, allowing stabilization of the carbanion via keto-enolate tautomerization (64). Acetone is a symmetrical molecule and does not have any electron withdrawing groups which would increase the acidity of the protons \( \alpha \) to the carbonyl group. This results in a very high pKa (~20) of the methyl protons which need to be abstracted to form enolacetone (69). Thus it is believed that the ATP/nucleotide hydrolysis required for carboxylation may have two functions: 1) to allow the formation/stabilization of the enolacetone and 2) to activate bicarbonate as an electrophile.

Elegant experiments, by Boyd et al., using isotopic exchange studies to investigate the kinetic mechanism of acetone carboxylase established that, indeed, the enolate of acetone is a distinct intermediate in catalysis (12). A unique GC/MS based assay was developed that allowed for the monitoring of isotope exchange within the acetone molecule. For a concerted reaction, \( \alpha - \text{H}^+ \) abstraction would occur only in the presence of \( \text{CO}_2 \) and isotopic exchange between \( \alpha - \text{CH} \) and solvent would not occur, or occur only at low rates. However it was found that in the absence of \( \text{CO}_2/\text{HCO}_3^- \), the enzyme is capable of removing the protons from acetone and replacing them with deuterons from solution (\( \text{H}_2\text{O} \)). Further isotope exchange was found to occur with the production of ADP rather than AMP, in stark contrast to the stoichiometry. Isotope studies also showed that deuteron/proton abstraction from acetone is not rate-limiting, however the removal of a proton/deuteron from water is. These data indicated that ATP hydrolysis and /or the release of the ATP hydrolysis product plays a significant role in the rate limiting step of the exchange reaction.
**Catalytic mechanism:** Based on the studies mentioned earlier, Boyd et al. proposed a working model for the initial step in acetone carboxylation (Fig 7). According to this model, \( \alpha -H^+ \) abstraction from acetone occurs in concert with \( \gamma \)-phosphoryl group transfer from ATP to acetone, thereby stabilizing enol acetone as phosphoenol acetone and forming enzyme bound ADP. In the absence of the electrophile CO\(_2\), phosphoenol acetone can either hydrolyze and dissociate from the active site or reabstract a proton from solution in concert with the re-formation of ATP (precedents for which exist) (12, 58). Acetone carboxylation also requires the additional hydrolysis of the phosphoryl bond from ADP. It has been speculated by Boyd et al. that perhaps a carboxyphosphate is formed from this phosphoryl transfer from the bound ADP. This carboxyphosphate could then provide the necessary activated CO\(_2\) species for C-C bond formation and the reaction would be completed by the hydrolysis of phosphoenol acetone. This scenario is supported in part by experimental data showing that ADP rather than AMP is the predominant product of CO\(_2\) independent proton deuteron exchange in acetone, while no ADP is seen for the complete carboxylation reaction (12).
Fig 7: Initial step in the reaction catalyzed by acetone carboxylase, formation of phosphoenol acetone – In the absence of CO₂, phosphoenol acetone is reprotonated to reform acetone. A concomitant isotopic exchange occurs when the reaction uses D₆-acetone as the substrate in buffer containing H₂O. Figure adapted from Boyd et al. (12).
Fig 8: A combination of the two reactions of PEP synthase and PEP carboxylase reactions compared with the reaction course of acetone carboxylase. Recent studies have provided evidence for the formation of the phosphoenol acetone intermediate (12). Preliminary studies also point towards HCO₃⁻ being the substrate (12). Figure adapted from (17).

The proposed phosphoenol acetone structure is similar to the structure of PEP and interestingly acetone carboxylation resembles the combination of two reactions that when added together achieve pyruvate carboxylation: PEP synthase plus PEP-C (Fig 8) (61).

However, despite this interesting fact, there is no significant homology between either of PEP-C or PEP synthase and AcCx. While PEP is a relatively stable molecule in aqueous solution, the proposed phosphoenol acetone is unstable and has not been directly trapped or visualized. In PEP-C, the phosphoryl group of PEP is transferred to bicarbonate from carboxyphosphate and enol pyruvate (13,29 from enol paper). Enol pyruvate then serves as the electrophile to attack CO₂ formed carboxyphosphate decomposition (16, 64). However, it has been difficult to answer whether phosphoenol acetone (if indeed formed)
reacts with CO₂ or HCO₃⁻. The difficulty lies in the slow turnover (29 min⁻¹) of AcCx coupled with the rapid equilibrium of CO₂ and bicarbonate in aqueous solution. Preliminary studies using the novel coupled assay developed by Boyd et al. indicate that HCO₃⁻ is indeed the enzyme substrate; however this conclusion awaits more detailed studies (12).

**Metal co-factor(s):** Among the unique characteristics of acetone carboxylase are its tightly bound metal ions. Enzymes isolated from *X. autotrophicus* st. Py2 and *R. capsulatus* st. B10 contained significant amounts of iron, zinc and manganese. Among these manganese (Mn) was present in the highest amounts and in stoichiometric quantities (1.3-1.5 mol Mn/mol α₂ β₂ γ₂ protomer) (83, 84). The high quantities of manganese found associated with AcCx were intriguing, since in many enzyme systems enolate intermediates are stabilized by metal ion complexation, especially in the absence of a cationic imine (93). Recent studies by Boyd et al. into the kinetic mechanism of AcCx have established that, indeed, the enolate of acetone is a distinct intermediate in catalysis (12). Complexation of the oxygen of the keto and enol forms can increase the acidity of an adjacent carbon-hydrogen bond by four-six orders of magnitude (64). For example complexation with Mg²⁺ lowers the pKa at C-3 of oxaloacetate (OAA) from 13 to 9 (53), a similar shift is seen with pyruvate (Fig 9) (59). Enolates of α-keto acids can also be effectively stabilized by metal complexation (64).
Boyd et al. studied the metal ions present in the AcCx purified from *R. capsulatus* st. B10 with respect to their function in the catalytic mechanism of the enzyme. The ratios of the metals differ in the respective AcCx's purified, but the one from *R. capsulatus* contains two metals per holoenzyme. As mentioned earlier, manganese was found to be associated in the most significant quantities with AcCx and intriguingly it remains bound to the enzyme through purification and is not removed by metal chelators. Additionally, when the concentration of Mn$^{2+}$ in the growth medium of the bacteria was raised from 0.5 µM Mn$^{2+}$ to 50 µM Mn$^{2+}$, the enzyme purified was found to be fully loaded with Mn$^{2+}$ and had an increased specific activity (11). As expected from this, in media lacking in manganese, *R. capsulatus* demonstrated poor growth with acetone as the carbon source. The doubling times in log phase, of the manganese depleted cells were 19% of those observed for manganese supplemented cells. In contrast, there was no effect of manganese depletion in cells grown on malate as the carbon source. This underlined the requirement of manganese for optimal acetone dependent growth of *R. capsulatus*. AcCx purified from cells grown with 50 µM Mn$^{2+}$ showed a 1.6 fold higher specific activity and 1.9 fold higher manganese content than cells grown with 0.5 µM Mn$^{2+}$. Manganese is a
cofactor for a number of enzymes, but rarely remains tightly bound and associated with enzymes during purification, as observed in AcCx. This is in contrast to the situation for most Mn dependent enzymes where Mn is lost during enzyme purification and must be added back to restore activity (25). Thus Mn is best viewed as an integral cofactor of the enzyme rather than a dissociable metal ion.

In order to obtain more information on the nature of the metal center(s) in AcCx, Boyd et al. analyzed samples of cells grown with optimal manganese by EPR spectroscopy (11). White et al. have reported five distinguishing features of a mononuclear Mn$^{2+}$ binding site as determined for bacteriophage γ phosphoprotein phosphatase (95). The spectra obtained for AcCx form R. capsulatus (Fig 10) by Boyd et al. were found to exhibit all of these features, apart from a few additional ones (whose origin and relevance are uncertain) (11).

Further, AcCx is colorless and the addition of sodium dithionite to the enzyme was found to have no effect on the EPR spectrum. This indicated that the Mn is divalent and probably does not have a redox role in acetone carboxylation (11, 77). The effect of an increase in temperature on the EPR of acetone carboxylase was also studied. New features were seen in the 31K spectrum. Based on similar spectra observed by Heinrich and co-workers (54), it is believed that this signal may arise from coupled manganese(II) centers (11). The EPR properties of AcCx were also investigated in the absence and presence of substrate(s) molecules (ATP, acetone, CO$_2$), products(s) (AMP, Pi, acetoacetate) and cofactor molecules.
Fig 10: X-band EPR spectra of acetone carboxylase at 5.6 K. The concentration of acetone carboxylase was 0.14 mM. A, spectrum from 0 to 6000 G. Inset in A, spectrum of 0.1 mM MnSO₄ in buffer A. B, expanded scale of the 0–3000 G region of the spectrum. The letters a–c show the valleys used to determine the average hyperfine splitting. Experimental conditions for spectra were as follows: temperature, 5.6 K; modulation frequency, 100 kHz; modulation amplitude, 12.6 G; time constant 81.92 ms; microwave frequency 9.654 GHz; microwave power, 2.0 milliwatts. Figure from Boyd et al (11).
Dramatic perturbations in the signals were observed upon the simultaneous addition of magnesium and nucleoside phosphates. The most profound difference was seen upon addition of Mg.AMP (11). It is important to remember here that many enzymes such as pyruvate kinase, creatine kinase, and PEP carboxykinase (which are also nucleoside triphosphate dependent enzymes) which require divalent ions, such as manganese and magnesium are able to utilize them interchangeably with little or no effect on activity. AcCx, however, requires Mg$^{2+}$ (apart from Mn$^{2+}$) for activity since it is an ATP-dependent enzyme (11).

This observation, that the magnesium complexes of ATP, ADP and AMP perturb the coordination environment of manganese in acetone carboxylase, suggests that manganese plays a role in nucleotide activation rather than in some other aspect(s) of catalysis (11).

**Monovalent metal ion requirement for carboxylation activity:** Monovalent ions are known to be either required for or to stimulate the activity of ATP-dependent enzymes (12, 13, 86). The carboxylation activity in the well studied AcCx’s, isolated from *X. autotrophicus* and *R. capsulatus*, and the partially purified enzyme from *R. rhodochrous* was shown to be stimulated by potassium ions (12, 83, 84). It was also shown to be required for activity in the partially purified AcCx from *A. vinelandii* (S. Ensign, unpublished results). Although this requirement was not rigorously established, the assays were conducted in the absence of any source of a potassium ion. Interestingly enough, in contrast to manganese, magnesium and potassium ions do not form tightly bound complexes with the enzyme. It was also established by Boyd et al (12), that both
Rb$^+$ and NH$_4^+$ were effective substitutes for K$^+$. In contrast the smaller Li$^+$ and Na$^+$ ions were not effective substitutes (12). These results were in agreement with those observed for other enzymes, such as pyruvate carboxylase (12, 79, 86).

**Substrate range in carboxylation:** The most versatile of the acetone carboxylases obtained to date is the partially purified enzyme from *R. rhodochrous*. It was found to have broad substrate specificity, catalyzing the CO$_2$ and ATP dependent consumption/carboxylation of longer chain 2-ketones and 3-pentanone. 2-butanone (methylethyl ketone) was consumed at a rate identical to that of acetone, while 2-pentanone, 3-pentanone, and 2-hexanone were degraded at rates that were 70%, 40% and 42% of the corresponding acetone dependent rate. These results are dramatically different from those seen for the more well studied carboxylases from *X. autotrophicus* and *R. capsulatus*. They were unable to degrade 2-pentanone, 3-pentanone, or 2-hexanone. 2-butanone was degraded by the enzyme albeit at a rate 60% lower than that for acetone.
Molecular and biochemical studies:

Acetophenone carboxylase (ApcCx) catalyzes the carboxylation of acetophenone, a toxic organic molecule, to benzoylacetaet. Reminiscent of acetone carboxylation, carboxylation of acetophenone is dependent on ATP hydrolysis; however unlike acetone carboxylation, where AMP is the hydrolysis product, ADP and inorganic phosphate are produced (39).

ApcCx is a multimeric protein, first purified and characterized from *Aromatoleum aromaticum* strain EbN1. It is comprised of five polypeptides of 70, 15, 87, 75 and 34 kDa. ApcCx is a probable heterooctamer (αβγδ)_2 (39). The protein was purified as two inactive subcomplexes, the larger of which contained four subunits ApcABCD and the smaller complex contained ApcE. Since activity was reconstituted upon combining the complexes, ApcE is considered a functional subunit of the enzyme (39). Interestingly the ApcE subunit could not be purified to homogeneity from the cells of strain EbN1; hence, it was expressed and purified in *E.coli*. This recombinant protein yielded higher specific activity upon combining with the core complex than the partially purified wild type protein (39). Similar to acetone carboxylase, ApcCx has a relatively low specific activity. It is presumed that both the enzymes are highly expressed and represent a sizable fraction
of total soluble protein, probably in order to compensate for the low specific activity (39, 83).

The substrate specificity of ApcCx was also investigated. Apart from its natural substrates (acetophenone and HCO\textsubscript{3}\textsuperscript{-}), only the aromatic ketones propiophenone and 4-acetyl-pyridine were converted by the enzyme. Aliphatic ketones, such as acetone (the substrate of the AcCx enzyme) exhibited no activity as substrates of ApcCx (39).

**Genetic footprint and regulation of acetophenone degradation:**

The genome of *A. aromaticum* has been sequenced recently (75). The ethylbenzene/acetophenone metabolic gene cluster of strain EbN1 consists of two major catabolic operons, the *ebd/ped* gene cluster, and the *apc/bal* gene cluster (74, 75). The *apc/bal* cluster codes for the five subunit acetophenone carboxylase, with the individual genes coding for the subunits being *apcABCDE* (74, 75). The only analogs of *apcABCDE*, apparently coding for the same enzyme, are found in *Rubrobacter xylanophilus* and *Rhodococcus jostii* (39). A PSI-BLAST analysis indicates that the only enzymes with a paralogous function are the acetone carboxylases previously mentioned in this text, however, they have fairly low sequence similarities (20-31\%) (39, 74, 75). The core ApcCx complex harbors one paralog each of the β and γ subunits of AcCx, while it has two paralogs of the α subunit (74, 75). Notably, similar to AcCx, the subunits of ApcCx also show similarity to the hydantoinase subfamily of enzymes, specifically N-methyl-hydantoinases, which catalyze the ATP dependent hydrolysis of cyclic amide bonds (74, 75).
Enzymatic and physiological studies of *A. aromaticum* suggest that the regulation of acetophenone degradation is substrate dependent (50). This was confirmed in recent studies by Kuhner et al., using a pathway-specific DNA microarray and proteomics (50). As mentioned earlier in the text, degradation activity is encoded by two major catabolic operons (74, 75). The genes coding for two separate two-component regulatory systems are located in the intervening regions between these two operons. They are supposed to regulate the two catabolic operons in a sequential manner, in response to the presence of their specific substrate, namely ethylbenzene or acetophenone (50, 74, 75). The regulation for the ApcCx genes from the *apc/bal* gene cluster is mediated by a two component regulatory system Tcsl/Tcr1 (74). It has been found to have relaxed substrate specificity and is also responsive to toluene. This regulation system is similar to that observed in *P. putida*, where the aerobic degradation of toluenes and xylenes is regulated in response to the presence of the respective substrates (74).

**Mechanistic studies:**

Reminiscent of acetone carboxylation, ApcCx was also found to exhibit an obligate requirement for ATP as a cofactor. Although different NTP’s (GTP, CTP, UTP, ITP) were tested by Jobst et al., only ATP was accepted in assays of purified ApcCx (74, 83). Acetophenone carboxylation is a thermodynamically unfavourable process. Hence, it is not surprising that in a reaction similar to acetone carboxylation it also couples the carboxylation reaction with nucleotide hydrolysis, an exergonic reaction (ΔG°" = -31 kJ/mol for the hydrolysis of ATP to ADP) (39). During the carboxylation of
acetophenone, two molecules of ATP are hydrolysed to ADP and inorganic phosphate. Jobst et al. developed a continuous spectrophotometric assay to study the ATPase activity of ApcCx. Similar to the catalytic activity, neither of the purified subcomplexes show ATP hydrolysis independently (39). In other words, all five subunits are also required for the ATP-hydrolytic activity of the enzyme. The rate of ATP hydrolysis was also found to increase substantially when either of the two substrates, HCO$_3^-$ or acetophenone, was added independent of the second substrate (i.e. an uncoupled reaction, similar to acetone carboxylation) (39).

As (has been) mentioned earlier in the text, for the initial step in catalysis, all organic substrate carboxylations require the formation of a carbanion for attack on electrophillic CO$_2$ or an activated CO$_2$ species (such as carboxyphosphate) (29, 48). For the reaction catalyzed by ApcCx, Jobst et al. have hypothesized the formation of an activated carboxyphosphate intermediate (39). Thus it was notable when carbomylphosphate, a structural analogue of carboxyphosphate was identified as an inhibitor of ApcCx. The fact that carbomylphosphate was found to inhibit both the ATPase and carboxylating activity of the enzyme indicated that the carboxyphosphate intermediate formed in the reaction needs to be cleaved prior to forming a new C-C bond (39).

Jobst et al. also studied the catalytic properties of ApcCx using isotopic exchange experiments, similar to those used by Boyd et al. to study acetone carboxylation. The ATPase activity of ApcCx was found to be acetophenone dependent indicating the
transfer of a second phosphoryl group to acetophenone, either directly or via a phosphoryl-enzyme intermediate (39).

**Metal co-factors (s):** As in the case of AcCx, ApcCx also requires additional monovalent and divalent metal ions for activity. Apart from Zn\(^{2+}\), Mn\(^{2+}\) or Mg\(^{2+}\) are also required as essential co-substrates by ApcCx. The purified ApcCx core complex (ApcABCD) contains zinc in stoichiometric amounts (1.1 mol Zn\(^{2+}\) per mol of αβγδ heterotetramer). No further metals were found to be associated with ApcCx. This is marked contrast to AcCx which has an absolute requirement for Mn\(^{2+}\) (tightly bound to the enzyme) (39). The addition of Mn\(^{2+}\) was also required, though it was replaceable by Mg\(^{2+}\). Although the requirement for Mn\(^{2+}\) is similar to AcCx, it is not thought to play the same role in ApcCx. This is due to the fact that 1) ApcCx is silent in EPR spectroscopy and 2) Mn\(^{2+}\) is not tightly bound to ApcCx, nor does depletion of Mn\(^{2+}\) slow the growth of strain EbN1, as compared to *X. autotrophicus*. ApcCx also requires K\(^{+}\) or NH\(_4\)\(^{+}\) at concentrations ranging from 20-40 mM (39).
Fig 11: Proposed mechanism of acetophenone carboxylase. Figure adapted from Jobst et al (39).
Proposed mechanism of ApcCx: Based on the studies mentioned above, Jobst et al. proposed a mechanism for the reaction catalyzed by ApcCx. One ATP would be used to activate HCO$_3^-$ to an activated carboxyphosphate (similar to carbamoylphosphate synthetase) (Fig 11). A second ATP would then be used for the formation of a phospho-enol-acetophenone (similar to acetone carboxylase or N-methyl hydantoinases) (Fig 11). Following the formation of this intermediate, acetophenone carboxylation probably involves the electrophillic attack of CO$_2$ liberated from the carboxyphosphate. This CO$_2$ would attack the double bond of the phospho-enol-acetophenone. The stoichiometrically bound zinc atoms may help in stabilizing the bound acetophenone in the enol state for phosphorylation and/or for orienting the activated intermediates (39).

Inhibitors: Among the monovalent and divalent ions mentioned above, Zn$^{2+}$, Ni$^{2+}$, Mn$^{2+}$ and Mg$^{2+}$ show inhibitory effects on ApcCx. Both ATPase and carboxylating activities are inhibited. Zn$^{2+}$ and Ni$^{2+}$ can inhibit at as low as 0.2 mM concentrations. This is a notable difference from AcCx, where neither of Mn$^{2+}$ or Mg$^{2+}$ shows any inhibition. As mentioned earlier, carbamoylphosphate, a structural analogue of carboxyphosphate, was also identified as an inhibitor of both activities of ApcCx. No inhibitory effects were observed for the non-hydrolysable analogues of ATP, AMP-CCP, AMP-PCP and AMP-PNP (39).
CONCLUSION

The biochemical, molecular and genetic studies on AcCx and ApcCx, the first aliphatic and aromatic ketone carboxylases to be purified, reveal that they are essentially identical yet unique from all other organic carboxylases characterized to date. They are unique in,
1. subunit composition, three for AcCx, while five for ApcCx  
2. genetic footprint, the layout of their gene operon,  
3. oligomeric structure,  
4. primary sequence,  
5. lack of biotin as a co-factor and  
6. presence of tightly bound metal co-factor, manganese in case of AcCx and zinc in case of ApcCx. In contrast to AcCx, in which the manganese is tightly bound, in ApcCx the manganese, though essential for activity, is not tightly bound, and is replaceable by magnesium.

Although these studies have greatly advanced our knowledge, some key questions remain to be answered. In the case of AcCx, its molecular structure which is currently being solved, will greatly enhance our understanding of its catalytic mechanism (61). It has been postulated that ketone bodies are an important source of carbon and energy for Helicobacter pylori (14). AcCx from H. pylori is postulated to be a key enzyme involved in the metabolism of these ketones, and structural information on this enzyme would open up the possibility of designing inhibitors and studying the molecular mechanism of any such inhibition (14). This may lead to the development of new drugs for treatment of H. pylori infections. Structural information is also expected to help clear up why acetone carboxylase exhibits such a high affinity for Mn$^{2+}$ and what is the exact nature of the protein-manganese association(s). Presently, it is also unclear as to how the phosphoenol acetone, if it is indeed formed, reacts with CO$_2$ or bicarbonate. Further if phosphoenol
acetone is indeed formed it would be interesting to test its non-hydrolysable analogs as inhibitors of AcCx, similar to the studies conducted by Jobst et al in ApcCx. To date, the only acetone carboxylases purified to homogeneity have been from aerobic bacteria. As and when more details are available, it will be intriguing to see how the acetone carboxylases from anaerobic bacteria compare in terms of the biochemical and molecular properties and the mechanistic strategies employed by them.

ApcCx has been recently purified and being a novel carboxylase further elucidation of its mechanism, would be of great interest. Similar to AcCx, the molecular structure of ApcCx, once solved, would provide a wealth of information. Till date, ApcCx has so far been proposed to exist in and has been purified only in anaerobic bacteria. However, recent studies by Ensign et al. (unpublished data) have identified, a strain of *Rhodococcus ruber*, an aerobic soil bacteria, that utilizes a CO\(_2\) dependent ethylbenzene degradation pathway proposed to proceed via an acetophenone carboxylase enzyme. A BLAST analysis also shows another aerobic, soil dwelling, bacteria *A. vinelandii* to have the putative acetophenone carboxylase genes (Ensign et al., unpublished data). If this is indeed the case then it would be intriguing to see how the acetophenone carboxylases from aerobic bacteria compare in terms of the biochemical and molecular properties and the mechanistic strategies employed by them. It would also raise the question – is there a central role for CO\(_2\) in acetophenone metabolism, and is this strategy more widespread than previously conceived. It will be intriguing to see if more bacteria are able to use to use this strategy.
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