Characterization of Five Catalytic Activities Associated with the NADPH:2-Ketopropyl-coenzyme M [2-(2-Ketopropylthio)ethanesulfonate] Oxidoreductase/Carboxylase of the *Xanthobacter* Strain Py2 Epoxide Carboxylase System†

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ABSTRACT: The bacterial metabolism of propylene proceeds by epoxidation to epoxypropane followed by carboxylation to acetoacetate. Epoxypropane carboxylation is a minimetabolic pathway that requires four enzymes, NADPH, NAD^+, and coenzyme M (CoM; 2-mercaptoethanesulfonate) and occurs with the overall reaction stoichiometry: epoxypropane + CO_2 + NADPH + NAD^+ + CoM → acetoacetate + H^+ + $NADP^+$ + NADH + CoM. The terminal enzyme of the pathway is NADPH:2-ketopropyl-CoM [2-(2-ketopropylthio)ethanesulfonate] oxidoreductase/carboxylase (2-KPCC), an FAD-containing enzyme that is a member of the NADPH:disulfide oxidoreductase family of enzymes and that catalyzes the reductive cleavage and carboxylation of 2-ketopropyl-CoM to form acetoacetate and CoM according to the reaction: 2-ketopropyl-CoM + NADPH + CO_2 → acetoacetate + $NADP^+$ + CoM. In the present work, 2-KPCC has been characterized with respect to the above reaction and four newly discovered partial reactions of relevance to the catalytic mechanism, and each of which requires the formation of a stabilized enolacetone intermediate. These four reactions are (1) NADPH-dependent cleavage and protonation of 2-ketopropyl-CoM to form $NADP^+$, CoM, and acetone, a reaction analogous to the physiological reaction but in which H^+ is the electrophile; (2) $NADP^+$-dependent synthesis of 2-ketopropyl-CoM from CoM and acetoacetate, the reverse of the physiologically important forward reaction; (3) acetoacetate decarboxylation to form acetone and CO_2; and (4) acetoacetate/$^{14}CO_2$ exchange to form $^{14}C_1$-acetoacetate and CO_2. Acetoacetate decarboxylation and $^{14}CO_2$ exchange occurred independent of NADP(H) and CoM, demonstrating that these substrates are not central to the mechanism of enolate generation and stabilization. 2-KPCC did not uncouple NADPH oxidation or $NADP^+$ reduction from the reactions involving cleavage or formation of 2-ketopropyl-CoM. *N*-Ethylmaleimide inactivated the reactions forming/using 2-ketopropyl-CoM but did not inactivate acetoacetate decarboxylation or $^{14}CO_2$ exchange reactions. The biochemical characterization of 2-KPCC and the associated five catalytic activities has allowed the formulation of an unprecedented mechanism of substrate activation and carboxylation that involves NADPH oxidation, a redox active disulfide, thiol-mediated reductive cleavage of a C−S thioether bond, the formation of a CoM:cysteine mixed disulfide, and enolacetone stabilization.

Several bacteria are capable of aerobic growth with short-chain aliphatic alkenes such as ethylene, propylene, or 1-butylene as a source of carbon and energy (*1*−*3*). Of these bacteria, *Xanthobacter* strain Py2 and *Rhodococcus rhodochrous* strain B276 have been extensively studied with regard to the metabolism of propylene (*4*−*6*). In both of these organisms, propylene metabolism is initiated by highly stereoselective NADH-dependent monooxygenases producing an enantiomeric excess of (*R*)-epoxypropane (*7*−*9*). In the presence of CO_2, the enantiomers of epoxypropane are further metabolized to acetoacetate by four enzymes that have been called the "epoxide carboxylase system" (*10*, *11*). Recently, coenzyme M (CoM),[1] a cofactor once thought to be restricted to methanogenic Archaea, was discovered as the central C3 carrier in the *Xanthobacter* Py2 epoxide carboxylase system (*12*). This discovery has unveiled the epoxide carboxylase system as not a true multiprotein enzyme complex but actually a three-step metabolic pathway with distinct intermediates (Figure 1). As a result, the way has been opened for each enzyme of the *Xanthobacter* Py2 epoxide carboxylase system/pathway to be characterized individually.

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[1] Abbreviations: 2-KPCC, NADPH:2-ketopropyl-CoM oxidoreductase/carboxylase; CoM, coenzyme M (2-mercaptoethanesulfonate); 2-ketopropyl-CoM, 2-(2-ketopropylthio)ethanesulfonate; 2-ketopropyl-mercaptopropanesulfonate, 3-(2-ketopropylthio)propanesulfonate; 2-hydroxypropyl-CoM, 2-(2-hydroxypropylthio)ethanesulfonate; Tris, tris-(hydroxymethyl)aminomethane; DTT, dithiothreitol; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy; HPLC, high-pressure liquid chromatography; NEM, *N*-ethylmaleimide.

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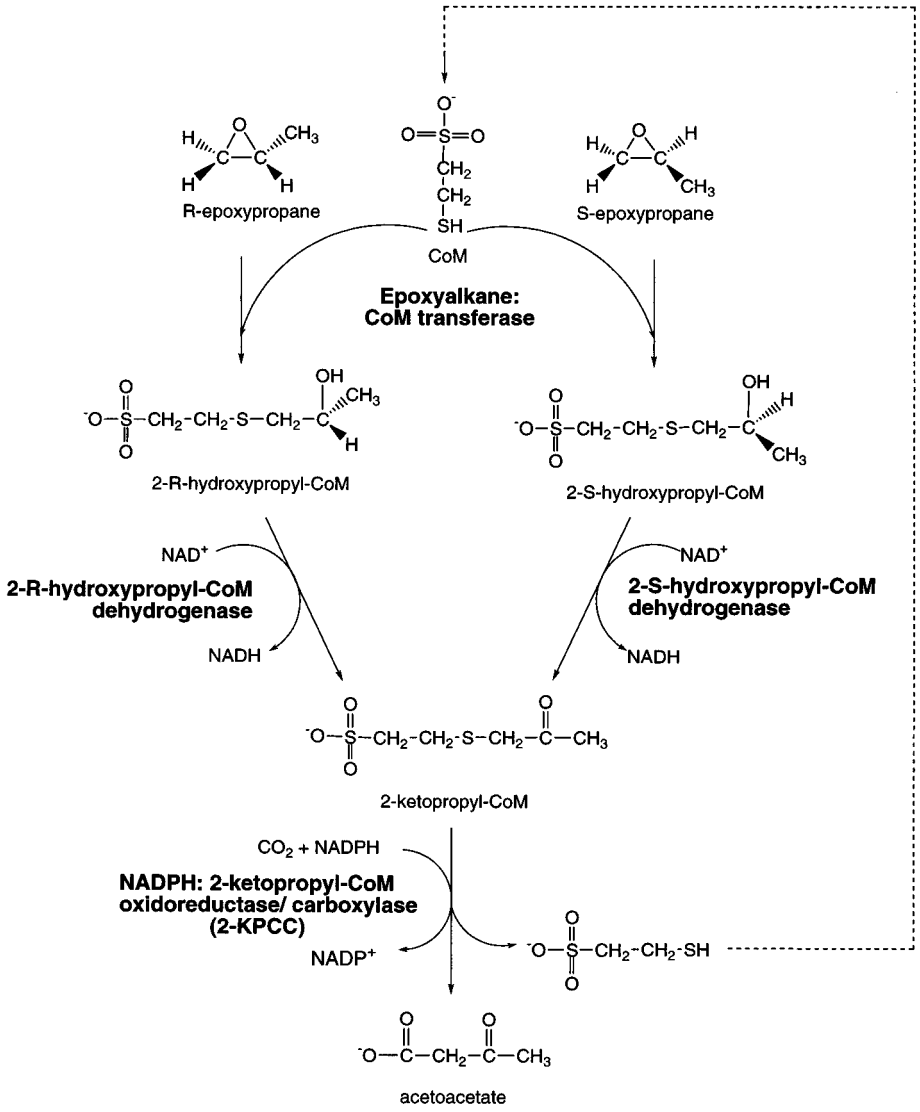


FIGURE 1: Pathway of epoxypropane carboxylation in *Xanthobacter* strain Py2 and role of NADPH:2-ketopropyl-CoM oxidoreductase/carboxylase (2-KPCC) in the regeneration of CoM. The four enzymes depicted constitute the *Xanthobacter* Py2 epoxide carboxylation system.

The first enzyme of the epoxide carboxylase pathway is an epoxyalkane:CoM transferase (*12*). This enzyme is a homohexameric zinc metalloenzyme (42 kDa subunits) that catalyzes the conjugation of CoM and (*R*)- or (*S*)-epoxypropane to form the corresponding (*R*)- or (*S*)-enantiomer of 2-hydroxypropyl-CoM (Figure 1). In the second step of the epoxide carboxylase pathway, these enantiomers are oxidized by stereospecific (*R*)- or (*S*)-2-hydroxypropyl-CoM dehydrogenases to the common intermediate 2-ketopropyl-CoM (Figure 1) (*12*). The terminal enzyme of the *Xanthobacter* Py2 epoxide carboxylase pathway is the NADPH:2-ketopropyl-CoM oxidoreductase/carboxylase (2-KPCC) (*12*). This enzyme catalyzes the reductive cleavage and carboxylation of 2-ketopropyl-CoM to acetoacetate, with the concomitant regeneration of CoM for use by the epoxyalkane:CoM transferase (Figure 1).

2-KPCC is a homodimeric FAD-containing protein (57 kDa subunits) that belongs to the FAD-dependent NAD(P)H-(disulfide) oxidoreductase family of enzymes (*13*). Of the prominent members of this family (glutathione reductase, trypanothione reductase, dihydrolipoamide dehydrogenase, thioredoxin reductase, mercuric ion reductase, and NADH peroxidase), glutathione reductase has widely been used as a structural and catalytic reference for all other FAD-dependent NAD(P)H-(disulfide) oxidoreductases (*14*, *15*). Examination of the well-characterized mechanism of glutathione reductase has allowed the basic mechanism of 2-KPCC to be suggested. 2-Ketopropyl-CoM carboxylation is believed to occur via the redox-active dithiol-mediated reductive cleavage of 2-ketopropyl-CoM with simultaneous formation of (1) a mixed disulfide between CoM and 2-KPCC and (2) the enolate of acetone. As final steps of this reaction, the enolate is thought to alternatively undergo protonation to acetone or carboxylation to acetoacetate, while the CoM/2-KPCC mixed disulfide is reduced using NADPH. This mechanism is without precedent for any other known carboxylase and also represents a novel reaction within the FAD-dependent NAD(P)H-(disulfide) oxidoreductase family of enzymes.

It was only recently that the physiological substrate for 2-KPCC was determined (*12*). To date, the only characterization of purified 2-KPCC has relied upon the nonphysiological reduction of $NADP^+$ with 1,3-propanedithiol (*16*). Although this study lacked the knowledge of the true

substrate, it nevertheless provided evidence for the existence of several intermediates in the 2-KPCC catalytic cycle, most notably the formation of a substrate/enzyme mixed disulfide (*16*). To advance the knowledge of this enzyme and shed further light on its mechanism, the interaction of physiologically relevant substrates and products with 2-KPCC has been investigated in the present work. These studies have provided insight into basic kinetic parameters, the reversible nature of the 2-ketopropyl-CoM carboxylation reaction, 2-KPCC substrate specificity, the importance of the redox-active disulfide in the catalytic mechanism, and enolate formation and stabilization during catalysis.

This work is of interest because it permits a greater understanding of the important terminal carboxylase and CoM regenerating enzyme of the *Xanthobacter* Py2 epoxide carboxylase pathway. While of broader biochemical significance, the present work is of interest because it demonstrates the novelty and mechanistic versatility of the FAD-dependent NAD(P)H-(disulfide) oxidoreductase family of enzymes.

EXPERIMENTAL PROCEDURES

Materials. $NaH^{14}CO_3$ (specific activity 54.4 mCi of ^{14}C $mmol^{-1}$) was purchased from ICN Radiochemicals, Irvine, CA. All other chemicals used were of analytical grade.

Chemical Syntheses and Analyses. The enzymatic synthesis of racemic 2-hydroxypropyl-CoM and the chemical synthesis of 2-ketopropyl-CoM were performed as described previously (*12*). 2-Ketopropylmercaptopropanesulfonate was synthesized in a manner identical to that of 2-ketopropyl-CoM, with the substitution of 3-mercaptopropanesulfonate for CoM (2-mercaptoethanesulfonate). The purities of chemically synthesized compounds (2-ketopropyl-CoM, 95%; 2-ketopropylmercaptopropanesulfonate, 90%) were estimated by HPLC analysis at 210 nm. HPLC was performed using a Shimadzu SCL-10Avp system controller equipped with dual Shimadzu LC10-AT pumps, a Shimadzu SPD-10A UV−vis detector, and a Shimadzu CR501 integrator. Chromatographic separations/analyses were achieved using a Supelcosil LC-18 column (25 cm × 4.6 mm) under conditions described previously (*12*). Chemical structures of all compounds were confirmed using ^1H NMR. The spectra of 2-hydroxypropyl-CoM and 2-ketopropyl-CoM demonstrated resonances with splitting and chemical shifts identical to those previously reported for these compounds (*12*). The spectrum of 2-ketopropylmercaptopropanesulfonate demonstrated five signals: a single resonance at 3.57 ppm integrated to two protons which corresponded to the protons of the methylene flanked by the thioether sulfur and the carbonyl; triplets at 3.00 and 2.66 ppm that each integrated to two protons and corresponded to the C1 and C3 methylene protons, respectively; at 2.33 ppm, a singlet that integrated to three protons and corresponded to the methyl group protons; a quintet at 2.01 ppm that integrated to two protons and correlated with the C2 methylene protons. All samples analyzed by ^1H NMR were dissolved in D_2O, and spectra were recorded in 5-mm NMR tubes using a Bruker ARX-400 NMR spectrometer at 25 °C.

Purification of 2-KPCC. Purified 2-KPCC was prepared as described previously (*17*). 2-KPCC concentrations were determined by using the previously determined extinction coefficient (ϵ_{450} of 11 828 $M^{-1}\cdot cm^{-1}$) (*17*).

2-Ketopropyl-CoM Carboxylation Assays. Although higher rates of 2-ketopropyl-CoM carboxylation were observed at pH 8.2, pH 7.4 was chosen to reflect a physiologically relevant pH and was used for all activity assays throughout the present work. Tris was chosen as the buffer for all assays to avoid potential complicating factors of Goode buffers containing an alkylsulfonate side chain resembling CoM (e.g., MOPS, HEPES). 2-Ketopropyl-CoM carboxylation assays were performed in sealed 12-mL serum vials with shaking (200 cycles min^{-1}) in a 30 °C water bath. The standard assay mixture contained 2-KPCC (10 μg) in 100 mM Tris-HCl, pH 7.4, containing 2-ketopropyl-CoM (concentration varied), NADPH (5 mM), and 60 mM carbonate species (added as 33.5 mM CO_2 gas plus 26.5 mM $NaHCO_3$ containing 118 μCi of ^{14}C $mmol^{-1}$) in a total volume of 1 mL. Assays were started by the addition of NADPH, and acetoacetate was quantified by the amount of ^{14}C-radiolabel incorporated from $^{14}CO_2/NaH^{14}CO_3$ as described previously (*12*). All compounds evaluated as alternative substrates for carboxylation were tested at concentrations of 10 mM.

2-Ketopropyl-CoM Protonation Assays. 2-Ketopropyl-CoM protonation assays were performed in sealed 9-mL serum vials with shaking (200 cycles min^{-1}) in a 30 °C water bath. The standard assay mixture consisted of 2-KPCC in 100 mM Tris-HCl, pH 7.4, containing 2-ketopropyl-CoM (2.5 mM) and NADPH (5 mM) in a total volume of 1 mL. Assays containing added CO_2 and $NaHCO_3$ (at a combined concentration of 60 mM) contained 33.5 mM CO_2 gas and 26.5 mM $NaHCO_3$. Acetone was quantified by gas chromatography as described previously (*18*). Assays that evaluated 2-ketopropylmercaptopropanesulfonate as an alternate substrate used a concentration of 10 mM.

Assay for the Presence of a Schiff Base Intermediate. Samples of 2-KPCC (3.1 mg) were incubated for 5 min at 25 °C in 100 mM Tris-HCl, pH 7.4, containing NADPH (10 mM) and either with or without 2-ketopropyl-CoM (10 mM). Next, 100 μL of 1 M $NaBH_4$ was added to each sample, followed by an additional 20-min incubation at 25 °C. Following this incubation, 800 μL of each sample was applied to a 1.5 × 5 cm column of 25 M Sephadex G (equilibrated in 100 mM Tris-HCl, pH 7.4), and 2-KPCC was eluted into 3 mL. Samples of 2-KPCC (0.64 mg) were assayed for 2-ketopropyl-CoM protonation activity as described above. All assays were performed in triplicate.

Reversibility of 2-KPCC: Identification of Enzymatically Synthesized 2-Ketopropyl-CoM. 2-Ketopropyl-CoM synthesis was performed using 0.50 mg of 2-KPCC in 100 mM Tris, pH 7.4, containing $NADP^+$ (2 mM), CoM (5 mM), and acetoacetate (100 mM). After a 5-min incubation at 30 °C, the mixture was centrifuged (5000*g* for 25 min) in a Centricon-30 concentrator (30 kDa molecular mass cutoff; Amicon, Inc.). Eight separate 20-μL samples of the flow-through were subjected to HPLC (under conditions referenced in Chemical Syntheses and Analyses), and in each case the peak corresponding to 2-ketopropyl-CoM (as evident by comparison to standards) was collected and pooled. The sample pool was lyophilized in a speed-vac concentrator overnight, then dissolved in 350 μL of D_2O, and subjected to ^1H NMR. An identical assay lacking 2-KPCC was performed as a control.

Continuous Spectrophotometric Assay for 2-Ketopropyl-CoM Formation. 2-Ketopropyl-CoM formation was moni-

tored by measuring the increase in absorbance at 340 nm as a result of $NADP^+$ reduction (ϵ_{340} of 6.22 $mM^{-1}\cdot cm^{-1}$ for NADPH) according to the reaction $NADP^+$ + CoM + acetoacetate → NADPH + CO_2 + 2-ketopropyl-CoM. Assays were performed in stoppered quartz cuvettes (1-cm path length) in a Shimadzu Model UV160U spectrophotometer containing a thermostated cell holder maintained at 30 °C. The standard assay mixture contained 2-KPCC in 100 mM Tris-HCl, pH 7.4, containing $NADP^+$ (5 mM), CoM (5 mM), and acetoacetate (concentration varied) in a total volume of 1 mL. Assays were initiated by the addition of acetoacetate or alternate organic compound.

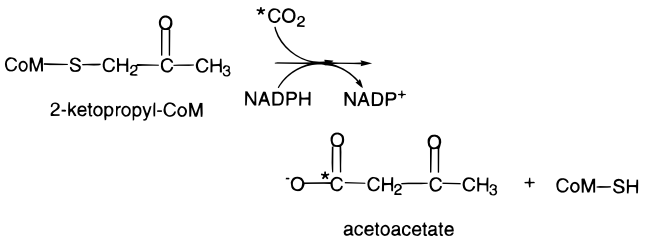
Acetoacetate Decarboxylation Assays. Assays for acetoacetate decarboxylase activity were performed in sealed 9-mL serum vials with shaking (200 cycles min^{-1}) in a 30 °C water bath. Vials were depleted of carbonate species by including a KOH-containing trap as described previously (*18*). The standard assay mixture (without CoM) consisted of 2-KPCC (1.0 mg) in 100 mM Tris-HCl, pH 7.4, containing acetoacetate (250 mM) in a total volume of 1 mL. The standard assay mixture (with CoM) consisted of 2-KPCC (0.25 mg) in 100 mM Tris-HCl, pH 7.4, containing acetoacetate (100 mM) and CoM (5 mM) in a total volume of 1 mL. Assays were initiated by the addition of acetoacetate, and acetone was quantified by gas chromatography as described previously (*18*).

Assay of $^{14}CO_2$/Acetoacetate Exchange. Assays were performed in sealed 12-mL serum vials with shaking (200 cycles min^{-1}) in a 30 °C water bath. The standard assay mixture consisted of 2-KPCC (assays without CoM contained 1.0 mg; assays with CoM contained 0.25 mg) in 100 mM Tris-HCl, pH 7.4, containing acetoacetate (concentration varied) and 60 mM carbonate species (added as 33.5 mM CO_2 gas plus 26.5 mM $NaH^{14}CO_3$ containing 118 μCi of ^{14}C $mmol^{-1}$) in a total volume of 1 mL. Assays were started by the addition of $NaH^{14}CO_3$, and radiolabeled acetoacetate was quantified as described previously (*12*). All assays examining the ability of other compounds to substitute for CoM used 100 mM acetoacetate.

NEM Inhibition of 2-Ketopropyl-CoM Carboxylase. 2-KPCC was subjected to six preassay treatments prior to performing activity assays. Preassay treatments were carried out for 30 min at 25 °C in 50 mM Tris-HCl, pH 8.2, containing 0.76 mg of 2-KPCC in total volumes of 40 μL. After treatment, 2-KPCC was immediately diluted for use in activity assays. For 2-ketopropyl-CoM carboxylation assays, 260 μL of 50 mM Tris, pH 8.2 (20% glycerol), was added to each preassay treatment, and 4 μL of this dilution (10 μg of 2-KPCC) was used in assays containing 2-ketopropyl-CoM (5 mM); this resulted in a 2-KPCC dilution of 1:1875. Acetoacetate decarboxylation and $^{14}CO_2$/acetoacetate exchange assays lacking CoM utilized 35 μL of each preassay treatment (0.66 mg of 2-KPCC); this resulted in a 2-KPCC dilution of 1:29. All other activity assays used 10 μL of each preassay treatment (0.19 mg of 2-KPCC), which resulted in 2-KPCC dilutions of 1:100. Except for the changes indicated here, all activity assays were performed as described under the appropriate heading in Experimental Procedures.

Data Analysis. Kinetic constants (K_m and V_{max}) were calculated by fitting initial rate data to the Michaelis−Menten equation using the software SIGMAPLOT.

Scheme 1



RESULTS

2-Ketopropyl-CoM Carboxylation. As shown in Scheme 1, 2-KPCC catalyzes a reaction of unprecedented chemistry: the reductive cleavage and carboxylation of a β-ketothioether to form a β-keto acid and CoM.

This role for 2-KPCC (formerly epoxide carboxylase component II) in the pathway of aliphatic epoxide carboxylation in *Xanthobacter* Py2 was unknown until recently, and accordingly no data exist on the basic kinetic properties of this enzyme. Furthermore, the specificity of 2-KPCC and its sensitivity to inhibition by other intermediates in the pathway of epoxide carboxylation are unknown. As initial steps in the investigation of the mechanism of 2-KPCC, these properties were investigated further.

Carboxylation assays were performed in which NADPH and CO_2/$NaHCO_3$ were held at fixed saturating concentrations, while the 2-ketopropyl-CoM concentration was varied in individual assays. 2-KPCC was found to be saturatable with respect to 2-ketopropyl-CoM. The experimental data were used to calculate apparent K_m, V_{max}, and k_{cat}, values of 633 ± 40 μM 2-ketopropyl-CoM, 412 ± 9.7 nmol of acetoacetate formed·min^{-1}·mg^{-1}, and 23.7 min^{-1}, respectively. Of several compounds evaluated as alternative substrates for carboxylation, no detectable activity was observed with 2-ketopropylmercaptopropanesulfonate, racemic 2-hydroxypropyl-CoM, chloroacetone, acetone, acetone oxime, or phosphoenolpyruvate. Inhibition of 2-KPCC was investigated using racemic 2-hydroxypropyl-CoM, enantiomers of which are the direct precursors of 2-ketopropyl-CoM in the pathway of epoxide carboxylation (Figure 1). Using saturating concentrations of 2-ketopropyl-CoM (5 mM), it was found that 1 mM racemic 2-hydroxypropyl-CoM inhibited 2-ketopropyl-CoM carboxylation by 14%. Of the two physiological products of 2-ketopropyl-CoM cleavage and carboxylation, CoM was investigated as an inhibitor due to its structural homology to 2-ketopropyl-CoM. In assays containing saturating concentrations of 2-ketopropyl-CoM (5 mM), it was found that 1 mM CoM inhibited 2-ketopropyl-CoM carboxylation by 28%.

2-Ketopropyl-CoM Protonation. In previous studies, aliphatic epoxides were found to undergo isomerization to the corresponding ketones in cell suspensions and extracts when CO_2 was excluded from the assays (*5*, *19*). The subsequent discovery that 2-ketopropyl-CoM is reductively cleaved and carboxylated to acetoacetate has helped to explain these results. Epoxide isomerization can now be explained by assuming that the enolate of acetone is formed upon the reductive cleavage of 2-ketopropyl-CoM. An enolate formed in this manner can be carboxylated to acetoacetate, or in the absence of CO_2 a proton can serve as an alternative electrophile for acetone production. This proposal is con-

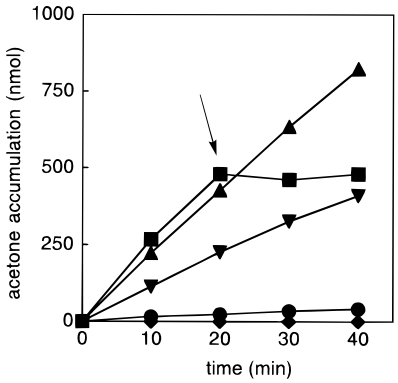
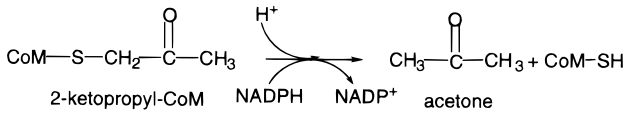
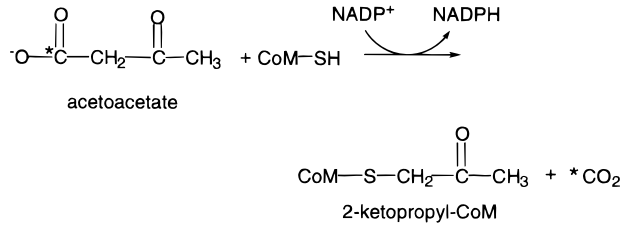


FIGURE 2: Effect of CO_2 on the reductive cleavage and protonation of 2-ketopropyl-CoM to acetone. Assays were performed in duplicate and contained the standard assay mixture with 0.25 mg of 2-KPCC unless otherwise noted: (♦) assays lacking 2-KPCC or NADPH, (▲) assays containing no added CO_2 or $NaHCO_3$, (▼) assays containing no added CO_2 or $NaHCO_3$ utilizing 0.125 mg of 2-KPCC, (●) assays containing CO_2 and $NaHCO_3$ (combined concentration 60 mM), and (■) assays containing no added CO_2 or $NaHCO_3$; at the time indicated by the arrow, CO_2 and $NaHCO_3$ were added to a combined concentration of 60 mM.

Scheme 2



Scheme 3



sistent with the reaction shown in Scheme 2. As shown in Figure 2, 2-ketopropyl-CoM was indeed cleaved with formation of acetone when CO_2 and $NaHCO_3$ were not included in assays. The rate of 2-ketopropyl-CoM protonation doubled when the enzyme concentration was doubled, and this activity was NADPH-dependent (Figure 2). The specific activity of 2-ketopropyl-CoM protonation was determined to be 81.4 nmol of acetone formed·min^{-1}·mg^{-1}, or approximately 4-fold less than the specific activity observed for 2-ketopropyl-CoM carboxylation at an equivalent concentration of 2-ketopropyl-CoM. When CO_2 and $NaHCO_3$ were included from the onset of assays, 2-ketopropyl-CoM protonation was almost completely prevented, and when CO_2 and $NaHCO_3$ were added to an assay mixture that was actively protonating 2-ketopropyl-CoM, subsequent formation of acetone was abruptly halted (Figure 2). As observed for the carboxylation reaction, the natural substrate analogue with the CoM portion of the molecule lengthened by one carbon (2-ketopropylmercaptopropanesulfonate) was not utilized.

Reversibility of 2-KPCC. The reversibility of the 2-ketopropyl-CoM carboxylase reaction was investigated with the presumption that 2-ketopropyl-CoM formation would occur according to the reaction shown in Scheme 3. In confirmation of this, when assays were performed that contained 2-KPCC, acetoacetate, CoM, and $NADP^+$, two products were resolved

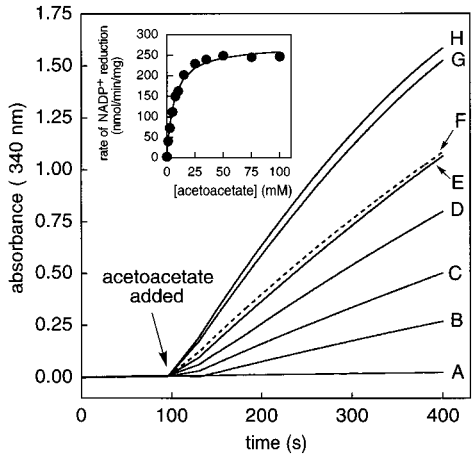


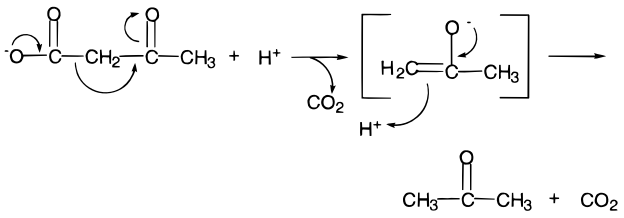
FIGURE 3: Spectrophotometric assays of the 2-ketopropyl-CoM carboxylation reverse reaction (2-ketopropyl-CoM formation): the effect of acetoacetate concentration on the acetoacetate-dependent reduction of $NADP^+$. Assays were performed in duplicate and contained the standard assay mixture with 0.25 mg of 2-KPCC. At the indicated time, acetoacetate was added to the following concentrations: (A) no acetoacetate, (B) 1.0 mM, (C) 2.5 mM, (D) 5.0 mM, (E) 7.5 mM, (F) 100 mM (F also contained 1 mM 2-ketopropyl-CoM at the assay onset), (G) 25 mM, and (H) 100 mM. Inset: Initial rates of $NADP^+$ reduction were derived and plotted against acetoacetate concentration; additional concentrations are shown that were omitted from the A_{340} vs time plot for clarity.

by HPLC that were absent from assays lacking 2-KPCC. Comparison to standards suggested these peaks to be 2-ketopropyl-CoM and NADPH. Samples of the peak believed to be 2-ketopropyl-CoM were subjected to ^1H NMR, and the resultant spectrum demonstrated a pattern of resonances with splitting and chemical shifts identical to those of 2-ketopropyl-CoM.

A real-time assay for 2-ketopropyl-CoM formation was developed that relies on the reduction of $NADP^+$ to NADPH accompanying the process shown in Scheme 3. 2-Ketopropyl-CoM formation assays were performed in which $NADP^+$ and CoM were held at fixed saturating concentrations, while the acetoacetate concentration was varied in individual assays. As shown in Figure 3, prior to the addition of acetoacetate, low background rates of $NADP^+$ reduction were observed. The addition of acetoacetate resulted in an increase in the rate of $NADP^+$ reduction, and this increase was proportional to the concentration of acetoacetate added (Figure 3). The rates of $NADP^+$ reduction reached steady-state levels within about 30 s of acetoacetate addition, although a lag in reaching the maximal rates was observed (Figure 3). The magnitude of the lag was more pronounced for assays containing low acetoacetate concentrations. At present the origin of this lag is not understood. Acetoacetate-dependent $NADP^+$ reduction decreased markedly when 2-ketopropyl-CoM was included in assays (Figure 3). The inset of Figure 3 demonstrates the relationship between the rates of $NADP^+$ reduction (taken from the linear portions of the progress curves) and acetoacetate concentration. These data was used to calculate apparent K_m,V_{max}, and k_{cat}, values of 6.3 ± 0.5 mM acetoacetate, 274 ± 5.5 nmol of NADPH formed·min^{-1}·mg^{-1}, and 15.8 min^{-1}, respectively.

2-KPCC Reverse Reaction: Evaluation of Alternative Substrates. A variety of organic compounds were tested (at 100 mM) for their ability to substitute for acetoacetate in 2-ketopropyl-CoM formation assays. No detectable increase

Scheme 4



in the rate of $NADP^+$ reduction was observed with pyruvate, oxaloacetate, acetopyruvate (2,4-dioxopentanoate), α-ketoglutarate, DL-β-hydroxybutyrate, phosphoenolpyruvate, 1,3-acetone dicarboxylate, or acetone. The lack of activity with acetone is particularly noteworthy, as this reaction would represent the reversal of the reaction shown in Scheme 2.

Several reducing agents and CoM analogues (β-mercaptoethanol, DTT, 3-mercaptopropanesulfonate, and 3-mercaptopropionate) were tested (at 5 mM) for their ability to substitute for CoM in 2-ketopropyl-CoM formation assays. Of these, β-mercaptoethanol did not support $NADP^+$ reduction with or without acetoacetate present. DTT supported very low rates of $NADP^+$ reduction (approximately 15% of the background rate observed in Figure 3), but this activity was not stimulated by acetoacetate. 3-Mercaptopropanesulfonate and 3-mercaptopropionate substituted for CoM in acetoacetate-dependent $NADP^+$ reduction, with rates that were 1% and 11% of the corresponding CoM-dependent rate, respectively.

Partial Reactions of 2-KPCC: Acetoacetate Decarboxylation. The results presented above are consistent with the formation of the enolate of acetone as an intermediate in the forward and reverse reactions of 2-KPCC. The enolization of acetone is an extremely difficult and endergonic process due to the high pK_a (19.3) of its α-hydrogens and instability of the resulting enolate. 2-KPCC is apparently unreactive toward acetone, as evidenced by its inability to reverse 2-ketopropyl-CoM protonation according to the equation acetone + CoM + $NADP^+$ → 2-ketopropyl-CoM + NADPH. To date, the only enzymes that have been shown to catalyze acetone carboxylation are bacterial acetone carboxylases, enzymes that couple acetone enolization and carboxylation to the hydrolysis of ATP (or GTP) via a phosphoenolacetone (or other activated acetone intermediate) as illustrated for the reaction catalyzed by the *Xanthobacter* strain Py2 enzyme: acetone + CO_2 + ATP → acetoacetate + AMP + 2 P_i (*18*, *20*). In this regard, it should be noted that nucleotides (i.e., ATP, ADP, AMP, GTP) and pyrophosphate have no stimulatory or inhibitory effects on 2-KPCC activities.

To provide further evidence for the formation of enolacetone as an obligate intermediate of 2-KPCC-catalyzed reactions, partial reactions of 2-KPCC in which enolacetone would be formed have been investigated. The first of these is acetoacetate decarboxylation, a thermodynamically favorable reaction that occurs spontaneously in aqueous solutions according to Scheme 4. Figure 4 shows the time course of 2-KPCC-catalyzed acetoacetate decarboxylation under several different conditions. 2-KPCC alone catalyzed acetoacetate decarboxylation at a very low rate (4.1 nmol of acetone formed·min^{-1}·mg^{-1} above the abiotic rate of acetoacetate decarboxylation). However, when CoM was included in the assay, the rate of 2-KPCC-catalyzed acetoacetate decarboxylation increased 77-fold to 317 nmol of acetone formed·min^{-1}·mg^{-1}. NADPH also had a stimulatory effect on acetoacetate decarboxylation, although the stimulation (11-fold) was significantly less than that observed with CoM (Figure 4). The simultaneous addition of CoM and NADPH stimulated acetoacetate decarboxylation to the same level as observed for CoM alone, while the addition of $NADP^+$ or 2-ketopropyl-CoM inhibited CoM-dependent acetoacetate decarboxylation (Figure 4).

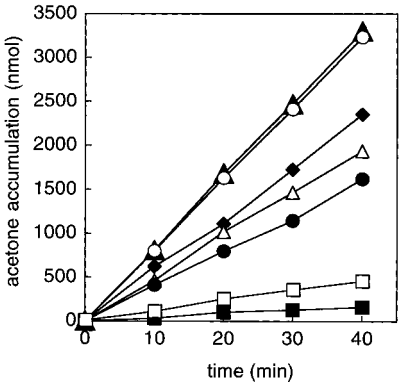
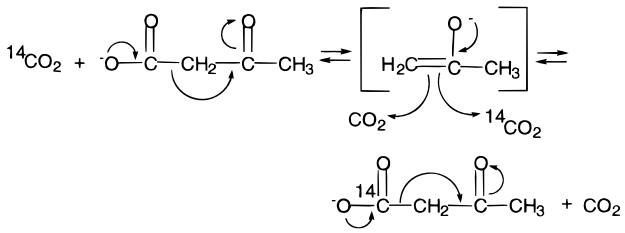


FIGURE 4: Time course of acetone accumulation from the decarboxylation of acetoacetate. The standard assay mixture for each assay was dictated by the presence of CoM and is described in Experimental Procedures. Assays contained 0.25 mg of 2-KPCC unless otherwise noted: (■) assays containing 1.0 mg of 2-KPCC, (□) assays containing 5 mM NADPH, (●) assays containing 0.125 mg of 2-KPCC and 5 mM CoM, (▲) assays containing 5 mM CoM, (○) assays containing 5 mM CoM and 5 mM NADPH, (△) assays containing 5 mM CoM and 5 mM $NADP^+$, and (♦) assays containing 5 mM CoM and 1 mM 2-ketopropyl-CoM. All assays were performed in duplicate, and background rates of acetoacetate decarboxylation were subtracted from all assays.

Scheme 5



Partial Reactions of 2-KPCC: Acetoacetate/$^{14}CO_2$ Exchange. 2-KPCC was found to catalyze the exchange of $^{14}CO_2$ into the C1 position of acetoacetate, a reaction that must also proceed through enolacetone as an intermediate as shown in Scheme 5. This assay was found to be superior to acetoacetate decarboxylation for performing quantitative kinetic measurements in that no detectable abiotic acetoacetate/$^{14}CO_2$ exchange occurred in assays lacking 2-KPCC. Figure 5 demonstrates the relationship between the initial rates of $^{14}CO_2$/acetoacetate exchange and acetoacetate concentration in the presence or absence of CoM. As observed for acetoacetate decarboxylation, the presence of CoM resulted in significantly higher exchange rates. The presence of CoM resulted in an increase in V_{max} for the exchange reaction from 7.4 (±0.2) to 285 (±2.3) nmol of $^{14}CO_2$ exchanged·min^{-1}·mg^{-1}, while the K_m for acetoacetate was decreased from 45.3 (±5.1) to 7.6 (±0.2) mM.

The stimulatory effect of CoM on exchange activity was further investigated by comparing the kinetics of this

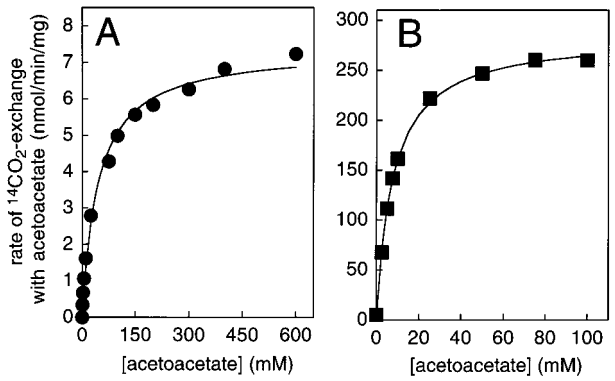


FIGURE 5: Effect of CoM and acetoacetate concentration on the rate of $^{14}CO_2$ exchange with acetoacetate. All assays were performed in duplicate. Panels: (A) assays containing 1.0 mg of 2-KPCC; (B) assays containing 0.25 mg of 2-KPCC and saturating CoM (500 μM).

"activation" to the kinetics of CoM utilization as a substrate in the reverse physiological reaction (Scheme 3). The apparent K_m and V_{max} values, determined as a function of CoM concentration, were statistically identical for the two reactions. The K_m for CoM utilization as a substrate in the reverse reaction was calculated to be 18.7 $\pm$ 1.5 μM while the "$K_{1/2}$" value for stimulation of the exchange reaction was 19.7 $\pm$ 2.3 μM CoM. By comparison, the K_m for 2-ketopropyl-CoM for the forward reaction was determined to be 633 μM. These results demonstrate that CoM has a sigificantly higher affinity for 2-KPCC than the ketopropyl derivative of CoM used as the substrate for the physiologically important reaction.

$^{14}CO_2$/Acetoacetate Exchange: Evaluation of Alternate Substrates and Activators. A variety of organic acids were tested (at 100 mM) for their ability to substitute for acetoacetate in the $^{14}CO_2$/acetoacetate exchange assay (with 500 μM CoM present). Pyruvate, oxaloacetate, acetopyruvate (2,4-dioxopentanoate), α-ketoglutarate, DL-β-hydroxybutyrate, and phosphoenolpyruvate were each unable to exchange their carboxylate functions with $^{14}CO_2$. Only 1,3-acetone dicarboxylate demonstrated exchange with $^{14}CO_2$, with a rate 15% of the acetoacetate-dependent rate.

To examine the specificity of 2-KPCC for CoM and elucidate the structural and functional group properties of CoM that allow it to stimulate 2-KPCC catalyzed $^{14}CO_2$/acetoacetate exchange, three categories of compounds (based on whether they had a thiol, charged moiety, or both) were screened. All of these compounds were tested at concentrations of 5 mM in the presence or absence of NADPH (5 mM) to assess any synergistic or antagonistic effects resulting from the redox state of 2-KPCC.

Compounds of the first category contained a charged group(s) and a thiol and included 3-mercaptopropanesulfonate, 3-mercaptopropionate, 2-amino-3-mercaptopropionate (L-cysteine), mercaptoacetate, 2-mercaptoethylamine, reduced glutathione, and reduced lipoate. Of these compounds, only 3-mercaptopropanesulfonate and 3-mercaptopropionate stimulated $^{14}CO_2$/acetoacetate exchange rates. In the absence of NADPH, 3-mercaptopropanesulfonate and 3-mercaptopropionate stimulated $^{14}CO_2$/acetoacetate exchange to rates 31% and 61%, respectively, of the calculated maximum with CoM. In contrast to the results with CoM, the presence of NADPH decreased 3-mercaptopropanesulfonate- and 3-mercaptopropionate-dependent exchange rates by approximately 5-fold (data not shown).

Compounds of the second category each contained a charged group but lacked a thiol function and included ethanesulfonate, 2-hydroxyethanesulfonate, 2-aminoethanesulfonate, propanesulfonate, propionate, acetate, and sodium bisulfite ($NaHSO_3$). None of these compounds stimulated $^{14}CO_2$/acetoacetate exchange to any measurable degree, with or without NADPH present.

Compounds of the third category were charge neutral and contained a thiol(s) and included ethanethiol, propanethiol, β-mercaptoethanol, and dithiothreitol. None of these compounds stimulated $^{14}CO_2$/acetoacetate exchange in either the absence or presence of NADPH. Together, the above results demonstrate that the combination of thiol, sulfonate, and alkyl chain length is crucial to 2-KPCC function. To determine whether these moieties could be donated by separate molecules, combinations of "category 2" and category 3" molecules were assessed for possible stimulatory effects on $^{14}CO_2$/acetoacetate exchange. The combinations of ethanethiol + $NaHSO_3$ and ethanesulfonate + ethanethiol did not stimulate exchange rates to any measurable degree, in either the absence or presence of NADPH.

NEM Inhibition of 2-Ketopropyl-CoM Carboxylase. As described in the introduction, 2-KPCC is a member of the FAD-dependent NAD(P)H-(disulfide) oxidoreductase family. All members of this family (with the exception of NADH peroxidase) contain a conserved redox-active disulfide that is integral to their catalytic mechanism (*14*). Classically, one method of probing the catalytic role of the redox-active disulfide has used sulfhydryl modifying agents such as *p*-hydroxymercuribenzoate, iodoacetate, or *N*-ethylmaleimide (NEM). Early work has demonstrated that epoxide degradation and isomerization in cell extracts of propylene-grown *Xanthobacter* Py2 are sensitive to inhibition by NEM (*21*, *22*). More recently, NEM was identified as an inactivator of purified 2-KPCC for a nonphysiological reaction: $NADP^+$ reduction using 1,3-propanedithiol as reductant (*16*). To determine the importance of the redox-active disulfide to the five catalytic activities described in this paper, the effects of NEM treatment on each activity were investigated. As shown in Table 1, incubation of 2-KPCC with NEM and NADPH led to a significant loss in activity of 2-KPCC-catalyzed reactions. The inactivating effect of NEM was dependent upon the addition of NADPH in all cases, suggesting that the redox-active thiols are fully oxidized to the disulfide state in the as-isolated enzyme and thus protected against reaction with NEM. For the acetoacetate decarboxylation and $^{14}CO_2$ exchange activities, NEM had no effect on the low activities observed in the absence of CoM (Figures 4 and 5A) but inhibited the much higher CoM-dependent rates (Figures 4 and 5B). These results are consistent with NEM modification of the specific thiol to which CoM binds. As shown in Table 1, the substrate analogue 2-ketopropylmercaptopropanesulfonate (the physiological substrate lengthened by one methylene) partially protected 2-KPCC from NEM inactivation, a result consistent with the target thiols being in proximity to the substrate binding site of the enzyme.

The Catalytic Mechanism of 2-KPCC Does Not Involve a Schiff Base. Enzymatic carboxylations often involve the

Table 1: *N*-Ethylmaleimide (NEM) as an Inhibitor of the Five Catalytic Activities Associated with 2-KPCC[a]

	relative specific activity (%)						
				acetoacetate decarboxylation		$^{14}CO_2$/acetoacetate exchange	
preassay treatment[b]	2-ketopropyl-CoM carboxylation	2-ketopropyl-CoM protonation	2-ketopropyl-CoM formation	without CoM	with CoM[c]	without CoM	with CoM[c]
none	100	100	100	100	100	100	100
NADPH	100	100	97.8 ± 1.4	100	88.7 ± 0.8	100	100
NADPH + 2-KPS	100	100	100	100	88.4 ± 1.6	100	100
NEM	100	92.6 ± 2.5	100	100	98.8 ± 5.2	95.2 ± 0.7	100
NEM + NADPH	10.0 ± 0.8	6.3 ± 0.4	4.9 ± 0.6	100	13.8 ± 1.1	98.3 ± 3.3	6.7 ± 0.5
NEM + NADPH + 2-KPS	45.5 ± 1.6	50.8 ± 0.5	68.5 ± 3.5	100	29.3 ± 0.7	100	33.3 ± 1.6

[a] All activity assays were performed in triplicate as described under NEM Inhibition of 2-KPCC in Experimental Procedures. [b] All preassay treatments were performed for 30 min in 500 μL microcentrifuge tubes at 25 °C containing 0.76 mg (0.33 mM) of 2-KPCC in 100 mM Tris, pH 7.4, and the additional components as indicated in a total volume of 40 μL: none, 2-KPCC only; NADPH, contained NADPH (1.25 mM); NADPH + 2-KPS, contained NADPH (1.25 mM) and 2-ketopropylmercaptopropanesulfonate (5 mM); NEM, contained NEM (1.25 mM); NEM + NADPH, contained NEM (1.25 mM) and NADPH (1.25 mM); NEM + NADPH + 2-KPS, contained NADPH (1.25 mM) and 2-ketopropylmercaptopropanesulfonate (5 mM), which was incubated for 1 min at 25 °C prior to addition of NEM (1.25 mM). [c] Assays contained 5 mM CoM.

reaction of CO_2 or an enzyme-bound form of CO_2 with a carbanion. Inherently unstable, a carbanion can be stabilized by electron delocalization with an adjacent electron-deficient atom such as a carbonyl or imine carbon. A number of enzymes are known to stabilize carbanions by carbonyl group conversion into a protonated imine, or Schiff-base, by nucleophilic addition of a protein- or cofactor-originating amine (*23*). The imine carbon/nitrogen double bond is readily reduced to a secondary amine by agents such as sodium borohydride ($NaBH_4$), and $NaBH_4$ inhibition of an enzyme (in the presence of its substrate) has been a classical way to detect Schiff base formation (*23*). This technique was employed here to determine if a Schiff base intermediate is formed in the catalytic mechanism of 2-KPCC.

2-KPCC was incubated under conditions favoring 2-ketopropyl-CoM protonation either with or without 2-ketopropyl-CoM, followed by addition of $NaBH_4$, as described in Experimental Procedures. In subsequent 2-ketopropyl-CoM protonation assays, it was determined that enzyme activity in both cases was identical. These results favor the absence of a Schiff base intermediate in the catalytic mechanism of the 2-KPCC.

DISCUSSION

The present work provides insights into the mechanism of bacterial epoxide carboxylation by characterizing five relevant activities associated with 2-KPCC, the terminal "carboxylating" component of this system (Figure 1). The stoichiometry and cofactor requirements of these transformations highlight the novelty of the 2-KPCC-catalyzed reaction: it is the only carboxylase known which uses a thioether as substrate and which forms a carbanionic nucleophile from reductive cleavage of a thioether C−S bond. To date, only a few other enzymes (operating in reverse) have been reported to catalyze NADPH-dependent reductive carboxylations, as illustrated by the following comparative enzymes and reactions (*24*−*27*):

(1) 2-ketopropyl-CoM carboxylase (present work)

2-ketopropyl-CoM + CO_2 + NADPH →
acetoacetate + $NADP^+$ + CoM

(2) $NADP^+$-malic enzyme

pyruvate + CO_2 + NADPH → L-malate + $NADP^+$

(3) isocitrate dehydrogenase

α-ketoglutarate + CO_2 + NADPH →
isocitrate + $NADP^+$

(4) $NADP^+$-pyruvate dehydrogenase

acetyl-CoA + CO_2 + NADPH →
pyruvate + $NADP^+$ + CoA

(5) 6-phosphogluconate dehydrogenase

ribulose 5-phosphate + CO_2 + NADPH →
6-phosphogluconate + $NADP^+$

An examination of the catalytic mechanisms of reactions 2, 3, and 5 reveals that the function of NADPH is fundamentally different from that of the 2-KPCC-catalyzed reaction in that NADPH is used solely for reduction of the keto groups. Reaction 4 is essentially a reversal of the pyruvate dehydrogenase complex-catalyzed reaction linking glycolysis to the citric acid cycle. This reaction bears some similarity to that catalyzed by 2-KPCC in that a redox-active dithiol (lipoamide) mediates NADPH oxidation and the cleavage of the C−S bond of acetyl-CoA. This reaction differs fundamentally, however, in the requirement of thiamin pyrophosphate, which mediates C−S bond cleavage and carboxylation.

The combination of insights provided from the characterization of the five catalytic activities associated with 2-KPCC in the present work and the previous spectroscopic characterization of reaction intermediates generated from 1,3-propanedithiol oxidation by 2-KPCC (*16*) has allowed the formulation of a plausible catalytic mechanism for 2-KPCC-catalyzed reactions (Figure 6). While this mechanism is believed to be fundamentally correct, the kinetic mechanism using the physiological substrate has not been determined. As such, the order of substrate binding and product release in Figure 6 is shown to follow the sequence proposed by Westphal et al. (*16*) for illustrative and descriptive purposes.

The proposed catalytic mechanism is as follows: Step 1: NADPH binds to oxidized 2-KPCC to produce an intermedi-

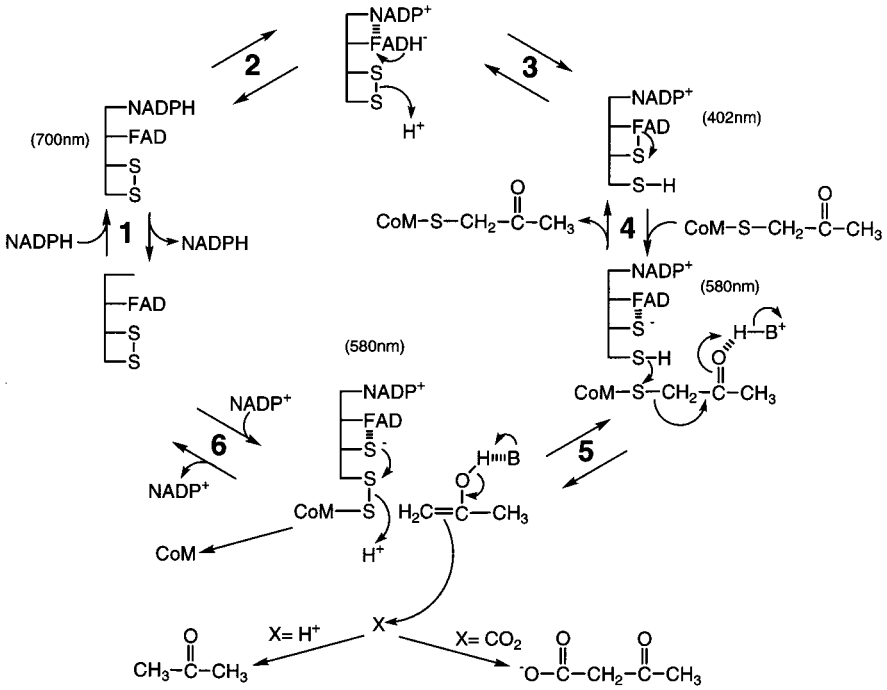


FIGURE 6: Proposed mechanism of 2-ketopropyl-CoM carboxylation based on the present work and the reaction mechanism proposed by Westphal et al. (*16*) for the reduction of $NADP^+$ with 1,3-propanedithiol.

ate observable at 700 nm. Steps 2 and 3: NADPH reduces FAD, which subsequently reduces the redox-active disulfide, and this results in the formation of a covalent bond between the FAD C(4a) and the proximal thiol, while the interchange thiol is protonated. Step 4: the transient FAD C(4a)-thiol adduct (402 nm) collapses to the thiolate−FAD charge-transfer complex (580 nm), and 2-ketopropyl-CoM binds to the enzyme where its carbonyl oxygen conceivably interacts with an active site catalytic acid through hydrogen bonding. Step 5: the 2-ketopropyl-CoM thioether sulfur undergoes nucleophilic attack by the interchange thiol, resulting in 2-ketopropyl-CoM cleavage and formation of a CoM/interchange thiol mixed disulfide and the enolate of acetone. The enolate thus formed is thought to be stabilized by proton donation from a catalytic acid. Step 6: enol deprotonation by base catalysis is concerted with electrophilic addition of either a proton or CO_2 to generate acetone or acetoacetate, respectively. Concomitant with this process, the redox-active dithiol is reoxidized, the CoM/interchange mixed disulfide is reduced (releasing CoM), and $NADP^+$ leaves, freeing the enzyme for another round of catalysis.

Comparison of the proposed mechanism for 2-ketopropyl-CoM carboxylation/protonation with the proposed mechanisms of other FAD-dependent NAD(P)H-(disulfide) oxidoreductases reveals many similarities and a few obvious differences (*14*, *15*). It is interesting that, with the exception of mercuric ion reductase and NADH peroxidase, the prominent members of this enzyme family all utilize disulfide substrates. These enzymes facilitate disulfide reduction by thiol-mediated nucleophilic attack on the disulfide (R−S−S−R) with formation of an enzyme/substrate mixed disulfide. Apparently, 2-ketopropyl-CoM carboxylase uses a similar strategy, in this case for nucleophilic attack of a thiol (i.e., cysteine) on a thioether sulfur (R−S−R). Although attack on the sulfur is consistent with the strategy of other disulfide oxidoreductases, it is conceivable that attack occurs instead on the 2-ketopropyl-CoM C3 carbon with release of CoM-SH, forming a covalent intermediate between the 2-ketopropyl moiety and the interchange thiol. If such an intermediate is indeed formed, reduced 2-KPCC might be expected to catalyze reactions with substrate analogues where CoM-SH is replaced by another leaving group. In investigating this idea, it was found that chloroacetone was not used as a substrate by 2-KPCC, a result that argues against the formation of a 2-ketopropyl-bound intermediate.

Evidence for the role of the redox-active dithiol in 2-KPCC has been deduced from the experiments of the present work. As shown in Table 1, 2-ketopropyl-CoM carboxylation, protonation, or formation activities are reduced to ≤10% by a preassay treatment of NEM in the presence of NADPH. However, in the absence of NADPH (a condition favoring the disulfide or the NEM-unreactive form of the redox-active dithiol), NEM pretreatment has little or no effect on these activities (Table 1). Furthermore, a 2-ketopropyl-CoM analogue (2-ketopropylmercaptopropanesulfonate) protected against NEM inactivation (Table 1). Accordingly, these data demonstrate the essential nature of the redox-active dithiol to catalysis and seem to suggest its proximity to the substrate binding site.

A notable feature of the proposed 2-KPCC mechanism is the involvement of a catalytic acid in enolate stabilization. Evidence for this process is inferred from the ability of 2-KPCC to catalyze acetoacetate decarboxylation and the exchange of $^{14}CO_2$ with acetoacetate. As shown in Table 1, both of the CoM-independent activities are retained after incubation with NEM and NADPH. These results demonstrate that the redox-active disulfide is not essential to the mechanisms of acetoacetate decarboxylation or the exchange of $^{14}CO_2$ with acetoacetate. Therefore, 2-ketopropyl-CoM carboxylase must be catalyzing these activities by other means. On the basis of established β-keto acid chemistry, both of these activities can be accelerated by carbanion and or enolate stabilization. Surveys of the common types of organic acids (β-keto acids, β-hydroxy acids, α-keto acids,

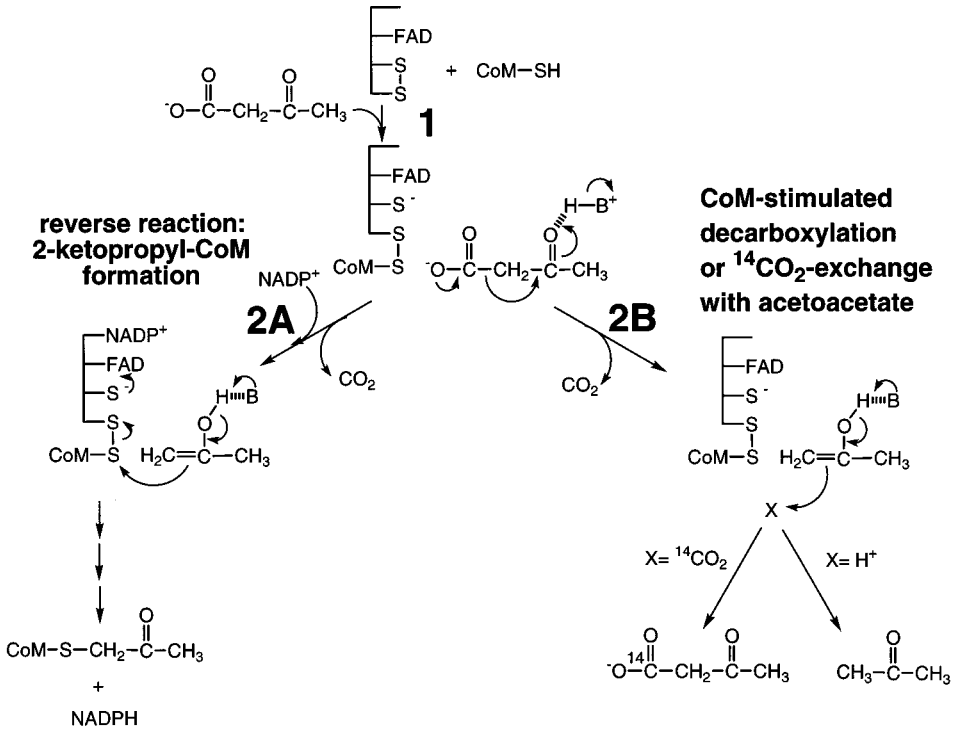


FIGURE 7: Proposed mechanisms of 2-ketopropyl-CoM formation, CoM-stimulated acetoacetate decarboxylation, and CoM-stimulated $^{14}CO_2$ exchange with acetoacetate.

and α-amino acids) that are substrates for enzymatic decarboxylation indicate several strategies used by proteins to stabilize a carbanion/enolate. Of particular relevance to the present work are the two mechanistic classes of acetoacetate decarboxylases: those that facilitate carbanion formation through carbonyl conversion to a cationic imine (Schiff base) and those that instead utilize a divalent metal ion to stabilize enolate formation (*23*). Both of these classes contrast 2-KPCC, which is shown here to not utilize a Schiff base mechanism and which has been previously shown to be devoid of metals (*17*). Similarly, 2-ketopropyl-CoM carboxylase cannot operate by the same mechanism as well-known β-hydroxy acid decarboxylases [with the exception of 6-phosphogluconate dehydrogenase (*28*)] that also utilize the divalent metal enolate stabilization strategy (*23*). An inspection of common α-keto acid decarboxylating enzymes indicates that decarboxylation is usually facilitated by the cofactor thiamin pyrophosphate (TPP). However, neither TPP nor pyridoxal phosphate, cofactors used by enzymes that catalyze α-amino acid decarboxylations, are associated with 2-KPCC. In light of these considerations, it is conceivable that 2-KPCC stabilizes carbanion/enolate formation (in concert with thioether cleavage) by (1) catalytic acid mediated proton donation to the carbonyl oxygen or (2) electrostatic stabilization using a positively charged amino acid side chain(s). Of the two possibilities, acid-catalyzed keto−enol tautomerization is well-known to organic chemists, and this strategy has been implicated in several enzyme mechanisms including triosephosphate isomerase (TIM), citrate synthase (CS), and Δ^5-3-ketosteroid isomerase (KSI) (*29*−*31*). In all three mechanisms it is proposed that a strong or low-barrier hydrogen bond (LBHB) between an active site catalytic acid and the substrate carbonyl oxygen facilitates protonation and formation of either an enediolate, enolate, or dienol for TIM, CS, and KSI, respectively (*32*). In fact, LBHB interactions are thought to provide much of the energy needed to bring about enolization of the ketone substrates for these enzymes (*32*). Although TIM and CS use a histidine catalytic acid and KSI uses a tyrosine, all of these enzymes also require the concerted action of a catalytic base (aspartate or glutamate) to initiate carbanion formation (*29*−*31*). In the case of 2-ketopropyl-CoM carboxylase, use of a catalytic base in the mechanism is not necessary because carbanion formation is initiated by the reductive cleavage of the 2-ketopropyl-CoM thioether. With respect to the above discussion, it should be noted that no direct evidence exists for the role of a catalytic acid in 2-KPCC-catalyzed reactions. However, such a role is nevertheless feasible on the basis of the activities reported here, and thus it does serve as a useful model and starting point for further experimentation.

It should be noted that the approximate specific activities (in nmol·min^{-1}·mg^{-1}) measured for 2-ketopropyl-CoM carboxylation (413), 2-ketopropyl-CoM formation (274), CoM-stimulated acetoacetate decarboxylation (317), and CoM-stimulated exchange of $^{14}CO_2$ with acetoacetate (285) are quite similar. These results may indicate a common rate-limiting step (or intermediate) for these activities, and this might explain the ability of CoM to stimulate decarboxylation (Figure 4) and exchange activities (Figure 5). While a common intermediate would be expected for the forward (2-ketopropyl-CoM carboxylation/protonation) and reverse reactions (2-ketopropyl-CoM formation), it is not immediately apparent how this could be the case for the other two activities. One possible explanation is offered in Figure 7, where 2-ketopropyl-CoM formation, CoM-stimulated acetoacetate decarboxylation, and CoM-stimulated exchange of $^{14}CO_2$ with acetoacetate are hypothesized to operate through an intermediate identical to that generated in step 5 of Figure 6 (but without $NADP^+$ bound). The hypothetical mechanism of Figure 7 is as follows: Step 1, CoM reduction of the redox-active disulfide of 2-KPCC to form a CoM/enzyme mixed disulfide, followed by acetoacetate binding.

Acetoacetate is then decarboxylated, as in step 2A or 2B to form the enol of acetone. Step 2A, collapse of the enol is coordinated with the electrophilic addition of CoM, which (in the presence of $NADP^+$) allows 2-ketopropyl-CoM to form. As an alternative to step 2A, step 2B illustrates collapse of the enol simultaneous with electrophilic addition of either a proton (decarboxylation) or $^{14}CO_2$ (exchange). Inspection of this mechanism hints that the CoM-reduced form of the enzyme (shown following step 1) might be the state responsible for enhanced decarboxylation and exchange activities. This is suggested on the basis of two observations of the decarboxylation and exchange activities: (1) CoM stimulation is much more pronounced than with NADPH, and (2) the CoM-stimulated rate is not enhanced by NADPH. Perhaps transition to the CoM reduced state occurs with a conformational change that involves catalytic acid repositioning and/or other changes. While these confomational changes for 2-ketopropyl-CoM carboxylase are purely speculative, a number of X-ray crystallography studies of prominent FAD-dependent NAD(P)H-(disulfide) oxidoreductase have suggested conformational changes (in some cases quite large) to occur upon reduction or during catalysis (*33*−*35*). Evidence for the involvement of the redox-active thiols in CoM-stimulated acetoacetate decarboxylation and exchange (as shown in Figure 7) is inferred from the data of Table 1. Here it is shown that CoM-stimulated acetoacetate decarboxylation and CoM-stimulated $^{14}CO_2$ exchange with acetoacetate activities are reduced to ≤14% by a preassay treatment of NEM in the presence of NADPH. However, in the absence of NADPH, NEM pretreatment has little or no effect on these activities (Table 1). These data, together with the NEM inhibition data for 2-ketopropyl-CoM formation, lend further support for the mechanisms of Figures 6 and 7.

To summarize, five catalytic activities associated with the 2-ketopropyl-CoM carboxylase of the *Xanthobacter* Py2 epoxide carboxylase system have been described.

The characterization of these activities has revealed insight into basic kinetic parameters, the reversible nature of the 2-ketopropyl-CoM carboxylase, 2-ketopropyl-CoM substrate specificity, the importance of the redox-active disulfide, and enolate formation and stabilization during catalysis. Collectively, this information has allowed us to propose mechanisms for 2-ketopropyl-CoM carboxylation (or protonation) and the other activities. These mechanisms, although not rigorously established, should provide a useful foundation for future structural, mechanistic, and spectroscopic studies.

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