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Camphor Revisited: Involvement of a Unique Monooxygenase in Metabolism of 2-Oxo-Δ^3-4,5,5-Trimethylcyclopentenylacetic Acid by *Pseudomonas putida*

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Previously, *Pseudomonas putida* was shown to degrade (+)-camphor, and cleavage of the first ring of the bicyclic structure involved two monooxygenases (a hydroxylase and a ring oxygen-inserting enzyme), a dehydrogenase, and spontaneous cleavage of an unstable oxygenation product (lactone). Cleavage of the second ring was not demonstrated but was assumed also to occur by ring oxygen insertion, since the predicted oxygenation product was extracted from whole-cell incubation systems. Our investigation established that metabolism of the first ring cleavage intermediate, 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid, occurred through the sequential action of two inducible enzymes, a coenzyme A ester synthetase and an oxygenase. The oxygenase was purified to homogeneity and had a molecular weight of 106,000. This enzyme carried a single molecule of flavin adenine dinucleotide and consisted of two identical subunits. Iron was not present at a significant level. The oxygenase was specific for NADPH as the electron donor and absolutely specific for the coenzyme A ester of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid as the substrate. The reaction stoichiometry was compatible with this enzyme being a monooxygenase, and a mass spectral analysis of the methyl ester of the product confirmed the insertion of a single oxygen atom. The enzyme appeared to be analogous to, although distinct from, 2,5-diketocamphane 1,2-monooxygenase in catalyzing a "biological Baeyer-Villiger" reaction with the formation of a lactone. Structural analogy suggested that this lactone, like the first, was also unstable and susceptible to spontaneous ring opening, although this was not experimentally established.

Reactions for the partial oxidation of (+)-camphor by *Pseudomonas putida* C1 have been described previously (2, 5) and have been shown to proceed by an initial hydroxylation at carbon 5, followed by dehydrogenation to form 2,5-diketocamphane and ring oxygenation by a 1,2-monooxygenase, resulting in the formation of a lactone. This lactone may be unstable, as a result of acidic properties conferred upon the methyl group at carbon 6 by the keto group at carbon 5, and may undergo either spontaneous or enzyme-catalyzed nonhydrolytic cleavage to form 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid (Fig. 1). Cleavage of the second ring of the bicyclic structure was presumed to occur after an analogous ring oxygen insertion (18) since the predicted reaction product, the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid, was reported to accumulate when (+)-camphor-grown cells were incubated with 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid in the presence of 2,2'-dipyridyl (2). However, the ring oxygenase catalyzing oxygen insertion into 2,5-diketocamphane was inactive toward 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid (15), and, although (+)-camphor-grown *P. putida* effectively oxidized this compound, the implicated oxygenase was never demonstrated. No subcellular system capable of catalyzing significant metabolic transformation of the compound was found. Therefore, it follows that the demonstrated degradative enzymes involved in (+)-camphor oxidation by *P. putida* are confined to cleavage of only one ring of the bicyclic structure. Further metabolic steps and the enzymes which catalyze them, which are presumably encoded on the *P. putida* CAM plasmid, are currently unknown. In this paper we describe two additional reactions involved in the oxidation of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid and the purification and characterization of the enzyme catalyzing one of them, an unusual monooxygenase.

MATERIALS AND METHODS

Growth of the organism. *P. putida* C1 (ATCC 17453) was obtained from the American Type Culture Collection and was maintained on nutrient agar slants. For

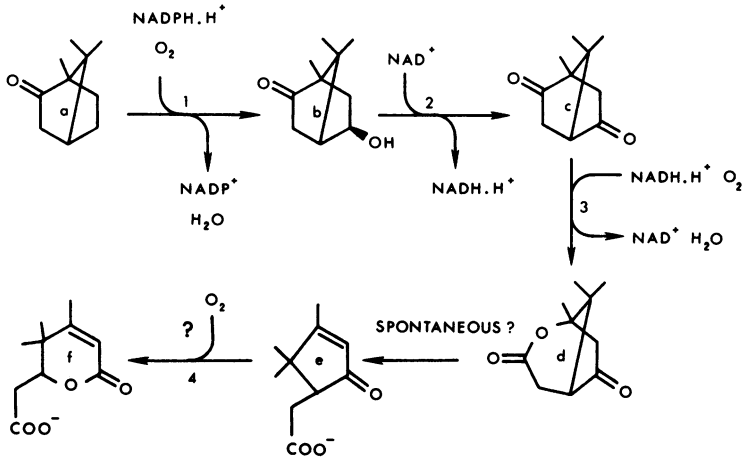

FIG. 1. Partial pathway for (+)-camphor oxidation by *P. putida* proposed by Gunsalus and co-workers (2,5). The following compounds are indicated: (+)-camphor (a); 5-*exo*-hydroxycamphor (b); 2,5-diketocamphane (c); 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid (e); δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid (f). The following enzymes are involved in this pathway: (+)-camphor 5-hydroxylase (1); 5-hydroxycamphor dehydrogenase (2); 2,5-diketocamphane 1,2-monooxygenase (3). It has been suggested that reaction 4 is catalyzed by a ring oxygen-inserting monooxygenase, but this has not been established experimentally.

growth with (+)-camphor as the sole source of carbon, the medium contained 2 g of KH_2PO_4 per liter, 4 g of Na_2HPO_4 per liter, 1 g of $(NH_4)_2SO_4$ per liter, and 4 ml of a trace element solution (29). Initially, a 20-ml starter culture in a 100-ml Erlenmeyer flask containing 4 g of sodium succinate per liter was inoculated from an agar slant and grown in a gyratory shaker at 150 rpm and 30°C for 24 h. Subsequently, 10% (vol/vol) inocula were transferred to 20-, 50-, and 500-ml volumes of medium containing (+)-camphor at a concentration of 2.5 g/liter (before autoclaving) at 12-h intervals. Two 500-ml cultures were then used to inoculate 9 liters of medium in a New Brunswick Microferm laboratory fermentor, which was stirred at 250 rpm and aerated by the passage of sterile air at a rate of 4 liters/min. This culture was then used to inoculate 30 liters of medium in a 50-liter plastic vessel adapted to fit the fermentor, aerated by passage of sterile air at a rate of 8 liters/min, and stirred at 800 rpm. Additions of (+)-camphor (25 ml of a 3.8 M solution in dichloromethane; rather similar to the procedure previously described [16]) were made when the values of absorbance at 580 nm (1-cm light path) were 1, 2, 3, 3.5, and 4 U, and the culture was harvested when the absorbance at 580 nm (1-cm light path) was 4.5 to 5 U after 6 h of growth; the yield was about 7 g of cell paste per liter. The cell paste was suspended in 1.5 volumes of 42 mM phosphate buffer (pH 7.1) and stored frozen at −20°C until it was used.

Batch cultures (500 ml) of succinate- and octanoate-grown *P. putida* were obtained by using the regime described above with succinate (2 g/liter) or octanoate (1 g/liter) incorporated into the basal salts medium. The pH of each medium was adjusted to 7.1 with 1 M NaOH.

Cell-free extracts. Cell suspensions were thawed at room temperature and were broken by a single passage through a French pressure cell, as previously described (9). The crushed material was incubated with 1 mg of crystalline DNase for 15 min at 2°C and then centrifuged at 48,000 × *g* and 4°C for 45 min to remove the cell debris.

Protein estimation. The protein contents of crude extracts were routinely measured by the biuret method (11), using crystalline bovine serum albumin as the standard; typically the protein concentration was 25 to 30 mg/ml. Corroborative measurements of the protein contents of purified enzyme preparations were made by using bromophenol blue (10) and tannin-gum arabic (21) procedures.

Washed cell suspensions. Cells from logarithmic-phase 500-ml shake cultures were harvested by centrifugation at 23,000 × *g* and 4°C for 20 min, washed once by suspension in 42 mM phosphate buffer (pH 7.1), centrifuged again, and finally suspended in phosphate buffer at a concentration of 5 to 10 mg (dry weight) per ml.

Mating experiment. The recipient organism, strain PaW340, was a streptomycin-resistant, CAM^-, tryptophan auxotroph derivative of *P. putida* (*arvilla*) mt-2 and was provided by P. A. Williams, University College of North Wales, Bangor, Wales. Donor and recipient bacteria were grown overnight without shaking in 5 ml of nutrient broth at 30°C. About 3 h before mating, 0.5 ml of each culture was transferred to 5 ml of nutrient broth and incubated at 30°C without shaking. To initiate conjugation, 0.5-ml portions of the two cultures were mixed; after 1.5 h at 30°C, 0.1-ml portions of the mixture and of 10^{-1}, 10^{-2}, and 10^{-3} dilutions in sterile 50 mM Na^+,K^+-phosphate buffer (pH 7.0) were spread onto basal medium plates containing 1 mg of streptomycin sulfate per ml and 50 μg of tryptophan per ml. The carbon source, (+)-camphor, was supplied in the vapor phase, and colonies were counted after 5 days of incubation at 30°C. The numbers of recipients and donors in the mating mixture were determined by plate counts of serial dilutions on nutrient agar. No colonies were observed

after 7 days of incubation at 30°C when 0.1-ml volumes of the separate donor and recipient cultures were spread onto selection plates.

Measurement of oxygen consumption. Oxygen consumption by washed cell suspensions or cell-free extracts were measured either by conventional Warburg manometry or polarographically at 30°C by using an oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) fitted with a Clark type of electrode.

Measurement of changes in proton concentration. The generation or consumption of protons in enzyme-catalyzed reactions was followed in essentially buffer-free stirred aqueous reaction systems at 20°C, which were maintained at the desired pH by controlled addition of 0.5 mM HCl or 0.5 mM NaOH, as appropriate, from a pH-stat (Pye Unicam Ltd., Cambridge, England).

Enzyme assays. 2,5-Diketocamphane 1,2-monooxygenase was assayed either by measuring NADH oxidation spectrophotometrically at 340 nm (using camphor as the substrate) or by measuring oxygen consumption polarographically. A typical reaction system contained the following (in a total volume of 3 ml): 0.4 μmol of NADH, 120 μmol of phosphate buffer (pH 7.1), and 0.025 to 0.25 U of enzyme. After the endogenous rate was measured, the reaction was started by adding 1 μmol of 2,5-diketocamphane or 1 μmol of (+)-camphor.

2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl coenzyme A (CoA) synthetase was assayed by an adaptation of the procedure described previously for *Escherichia coli* fatty acyl-CoA synthetase (26). A typical reaction system contained (in a total volume of 2 ml) 50 μmol of Tris-hydrochloride (pH 8.0), 5 μmol of $MgCl_2$, 5 μmol of dithiothreitol, 10 μmol of ATP, 0.5 μmol of CoA, 1 mmol of freshly neutralized hydroxylamine, and 3 to 9 mg of cell extract protein. Reactions, including a range of controls, were started by adding 10 μmol of sodium 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate and terminated by adding 1.5 ml of a mixture containing equal volumes of 5% (wt/vol) $FeCl_3 \cdot 6H_2O$ in 0.1 M HCl, 3 M HCl, and 10% (wt/vol) trichloroacetic acid. Protein was removed by centrifugation at 27,000 × *g* and 4°C for 15 min, and the ferric hydroxamate content was measured spectrophotometrically at 540 nm.

2-Oxo-Δ^3-4,5,5-trimethylcyclopentenyl-acetyl-CoA monooxygenase was routinely assayed by measuring substrate-stimulated NADPH oxidation spectrophotometrically at 340 nm and 30°C. The reaction mixture contained (in a total volume of 0.5 ml) 50 μmol of Tris-hydrochloride (pH 9.0), 0.12 μmol of NADPH, and 0.004 to 0.04 U of activity. After the endogenous rate was measured, the reaction was started by adding 20 μl of a standard preparation of the CoA ester substrate. For anaerobic studies in Thunberg cuvettes and polarographic measurement of enzyme activity, the assay mixture was scaled up to a volume of 3 ml.

Purification of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase. All procedures were performed at 2 to 4°C in subdued light.

(i) Step 1. About 300 ml of crude cell extract was brought to 50% saturation by slowly adding an equal volume of a neutral saturated solution of ammonium sulfate, and precipitated protein was removed by centrifugation at 27,000 × *g* for 20 min. Solid ammonium sulfate was added to the clear yellow supernatant until 75% saturation was achieved. Centrifugation, performed as described above, produced a yellow protein pellet, which was dissolved in 50 ml of 21 mM phosphate buffer (pH 7.1) and dialyzed against two 5-liter portions of the same buffer.

(ii) Step 2. The dialyzed protein solution was applied to the top of a DEAE-cellulose column (2.5 by 25 cm), and a linear gradient constructed from 500 ml of 21 mM phosphate buffer and 500 ml of the same buffer containing 0.5 M KCl was passed through the column. Fractions (5 ml) were collected. Typically, fractions 43 to 53 (0.10 to 0.12 M KCl) were pooled, and the protein was precipitated by adding 3 volumes of saturated ammonium sulfate and harvested by centrifugation at 27,000 × *g* for 20 min. The bright yellow protein pellet was then treated in one of two ways.

(iii) Step 3a. The protein pellet was dissolved in 6 ml of 0.1 M Tris-hydrochloride (pH 8.0) and applied to a Sephacryl S-200 column (2.5 by 80 cm). The protein was eluted by passing the same buffer through the column, and 5-ml fractions were collected. Fractions 35 to 38 were pooled, and the protein was precipitated by adding 3 volumes of saturated ammonium sulfate and harvested by centrifugation at 27,000 × *g* for 20 min. The enzyme was dissolved in 2 ml of 42 mM phosphate buffer, dialyzed against 500 ml of the same buffer for 8 h, and stored at −20°C.

(iv) Step 3b. In later preparations the protein obtained from DEAE-cellulose chromatography was dissolved in 5 to 10 ml of 10 mM Tris-hydrochloride (pH 7.5) and dialyzed for 20 h against 500 ml of the same buffer. This protein was then applied to the top of an Affi-Gel Blue column (1.5 by 9 cm), and the column was washed by passage of 35 ml of the same buffer. The enzyme was eluted by adding 2 mM NADPH to another sample of buffer passed through the column, precipitated, dissolved in 42 mM phosphate buffer, and dialyzed as described above (step 3a).

Chromatographic procedures. Ascending paper chromatography of flavins was performed on Whatman no. 1 chromatography paper developed with solvent A (*n*-butanol, acetic acid, and water, 4:3:3) or solvent B (*tert*-amyl alcohol, formic acid, and water, 6:2:2). High-pressure liquid chromatography of flavins was performed on an octadecanyl sulfate Hypersil column (particle size, 5 μm) developed with methanol–10 mM Na^+,K^+-phosphate buffer (pH 6.0) (1:4), and the flavins were detected at 450 nm.

Mass spectra. Mass spectra of methylated compounds were determined at an ionization potential of 24 eV with an A.E.I. model MS 30 instrument coupled to a Pye Unicam series 104 gas-liquid chromatograph fitted with a 3% (wt/wt) OV1 column.

Nuclear magnetic resonance spectra. Proton magnetic resonance spectra were recorded at 90 MHz. Samples were dissolved in $CdCl_3$, and chemical shifts are reported in τ units; tetramethylsilane was used as an external standard in a coaxial capilliary tube.

Polyacrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis was carried out on 4.5, 6.4, and 10.0% polyacrylamide gels in the apparatus described by Rogers (28), using the procedure of Davies (8). Sodium dodecyl sulfate (SDS) disc gel electrophoresis was carried out by the procedure of Weber and Osborn (34). An enzyme preparation (500 μg) in 10 mM sodium phosphate buffer containing 1% SDS and

1% β-mercaptoethanol was boiled for 2 min and then cooled on ice. Standard marker proteins were similarly treated. Samples of the treated enzyme and marker proteins containing 20 to 40 μg of each component were subjected to electrophoresis on 5% SDS–polyacrylamide gels together with tracking dye in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS. Electrophoresis was conducted at 7 mA/gel until the tracking dye was 0.5 cm from the bottom of the tube. Gels were stained with Coomassie brilliant blue G-250 and destained with methanol-acetic acid-water (2:3:35).

Gas-liquid chromatography. Gas-liquid chromatography was performed by using a series 104 chromatograph (Pye Unicam) and glass columns (4 mm by 1.5 m) packed with 1.25% (wt/wt) diglycerol or 3% (wt/wt) OV1 on acid-washed Chromosorb W (60 to 80 mesh). A judicious use of these two column systems in combination with isothermal and temperature-programmed operating conditions allowed the range of camphor metabolites encountered to be separated from each other.

Preparation of compounds. 1,2-Campholide (the lactone of 4-hydroxy-4,5,5-trimethylcyclopentane-1-acetic acid) was prepared by the method of Sauers (30), using peracetic acid prepared as described by Greenspan (12) and recrystallized from petroleum spirits (bp, 40 to 60°C). The purity of the compound was assessed by gas-liquid chromatography, and the lactone was assayed by the procedure of Cain (3). 2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid was extracted from *P. putida* liquid growth medium (1) and recrystallized four times from boiling diethyl ether. The physical properties, melting point (102 to 104°C), λ_{max} (226.5 nm), ε (14,000), and mass spectral analysis of the methyl ester (M^+, 196), were all compatible with the assigned structure (1).

2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl CoA was prepared from its *N*-hydroxysuccinimide ester (20) by the procedure of Al-Arif and Blecher (1). For routine assays of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase activity in cell extracts and column fractions, a crude preparation was used directly. For more precise experimental work, the compound was purified by chromatography on a column (1.5 by 22 cm) of Sephadex G-15 (17) before use. CoA ester preparations and column fractions were monitored by treatment with the nitroprusside reagent of Toennis and Kolb (32), followed by hydrolysis with alkali.

Several procedures were used for quantitative assay of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA preparations, including the following: (i) controlled hydrolysis of the compound in 0.5 M NaOH coupled with spectrophotometric analysis at 232 nm and an assumed ε of about 4.6×10^6 mol^{-1} cm^{-1} (31); (ii) formation of the hydroxamate ester at pH 9, followed by conversion to the ferric hydroxamate and comparison with a calibration curve prepared by using the hydroxamate formed from the recrystallized *N*-hydroxysuccinimide ester; and (iii) enzymatic assay in which total substrate-stimulated O_2 consumption or NADPH oxidation was measured by conventional procedures, using the assay systems described above for purified oxygenase. Although none of these procedures was entirely satisfactory, a rough correlation was usually obtained, and the most specific procedure (enzymatic analysis) typically gave values of 4 to 6 μmol/ml in crude substrate preparations (a yield of about 5%). Attempts to improve upon this yield were not successful.

All of our attempts to isolate the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid by incubating resting cells of (+)-camphor-grown *P. putida* with 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid in the presence of 2,2'-dipyridyl, as previously described (2), were unsuccessful.

J. Shapiro (University of Chicago, Chicago, Ill.) generously supplied us with a range of *P. putida* strains bearing cam^- mutations on the CAM-OCT plasmid. These strains were also screened for growth with 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid, and $acid^-$ strains were checked for the ability to accumulate the δ-lactone by growth on succinate in the presence of (+)-camphor, followed by gas-liquid chromatography of the accumulated acidic metabolites.

The majority of the cam^- $acid^-$ mutants accumulated 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid. However, one strain, a cam^- trp^- $acid^-$ mutant (strain PpS1790) carrying Tn*5*, also accumulated small amounts of a second acidic metabolite, which had a polarity compatible with its being the required lactone. Accordingly, this strain was subjected to a more detailed examination.

Mutant PpS1790 was grown in shake cultures (six 500-ml cultures) on medium containing 2 g of succinate per liter, 50 mg of L-tryptophan per liter, and 2.5 g of (+)-camphor per liter. After growth on the succinate-containing medium to the stationary phase, cultures were incubated for an additional 3 days, whole cells were removed by centrifugation, neutral metabolites and camphor were removed by batch extraction with diethyl ether, and the aqueous phase was then adjusted to pH 1 with 10 M HCl. Acidic metabolites were continuously extracted with diethyl ether for 12 h. The ether phase was dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to yield a pale yellow syrup, from which 140 mg of a white compound was crystallized in diethyl ether-petroleum ether (bp, 40 to 60°) (1:1, vol/vol). The identity of this compound as the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid was established from the following data: melting point, 106 to 107°C; λ_{max} in aqueous solution, 220 nm (abolished by hydrolysis of the lactone in 1 M NaOH at 100°C for 20 min); nuclear magnetic resonance peaks at 8.91, 8.83, 8.03 (doublet), 7.27 (doublet), 5.29 (triplet), and 4.19 (quadruplet); double resonance irradiation at 5.29 and 7.27 caused the respective triplet and doublet to collapse to singlets; gas-liquid chromatography and mass spectral analysis of the methyl ester of the compound gave an M^+ of 212. All of these data are compatible with the structure originally assigned to the accumulated δ-lactone (2).

Determination of protein molecular weight. The molecular weight of the native 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase was determined by the method of Yphantis (36). The rotor speed used was 20,410 rpm, and the initial protein concentration was 0.5 mg/ml. A double sector cell was used with the enzyme solution in one compartment, and 0.1 M Na^+, K^+-phosphate buffer (pH 7.0; diffusate) was used in the other. The enzyme was stable for the duration of the experiment (24 h).

Iron content of 2-oxo-Δ^3-4,5,5-trimethylcyclopenten-

ylacetyl-CoA oxygenase. The iron contents of solutions of the enzyme (4 to 5 mg of protein per ml) in 42 mM Na^+,K^+-phosphate buffer (pH 7.1) were measured by using a Pye Unicam model SP9 atomic absorption spectrophotometer. This instrument was calibrated with solutions of $FeCl_3$ containing 1 to 5 μg of Fe per ml, and phosphate buffer, against which the enzyme had been dialyzed for 21 h, was used for measuring the background level of iron.

Chemicals. Affi-Gel Blue was supplied by Bio-Rad Laboratories Ltd.; NAD, NADP, NADPH, and CoA were obtained from the Boehringer Corp.; dicyclohexylcarbodiimide was obtained from BDH Ltd.; Chromosorb W and diglycerol were obtained from the Pierce Chemical Co.; and *N*-hydroxysuccinimide was supplied by Wilkens Instruments. 2,5-Diketocamphane was a generous gift from I. C. Gunsalus. The organic solvents used were the purest commercially available and were dried and redistilled when they were used in organic preparations.

RESULTS

Growth experiments. *P. putida* ATCC 17453 grew rapidly on (+)-camphor as the sole source of carbon, with a doubling time of about 100 min. Growth with 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate as the sole source of carbon was slower, with a doubling time of 180 min, and the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid served as a very poor growth substitute, with a doubling time in excess of 40 h.

Oxidation of substrates by whole cells. Freshly harvested (+)-camphor-grown cells of *P. putida* were capable of rapid and immediate oxidation of the growth substrate (8,5 mol of O_2 consumed per mol of substrate), 2,5-diketocamphane, and 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate (6.5 mol of O_2 consumed per mol of substrate). The δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid was oxidized at a rate that was less than 10% of the rate observed with (+)-camphor. No significant differences in rate or extent of oxygen consumption were observed with 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate-grown cells, as previously reported (14).

These results support the suggested involvement of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid as an intermediate directly involved in (+)-camphor oxidation by *P. putida* (2, 4–6).

Metabolism of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate by extracts of *P. putida*. In common with previous workers (6), we were unable to detect any NADPH-linked dehydrogenase activity toward 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate, nor was any oxygenase activity demonstrable with or without the addition of reduced pyridine nucleotides. Attempts to observe enzyme-catalyzed hydration of the double bond of the cyclopentene ring by following changes in absorbance at 227 nm were unsuccessful. In addition, a partially purified sample of 2,5-diketocamphane 1,2-monooxygenase from *P. putida*, which was prepared as previously described (33), was inactive toward 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate.

An alternative approach to the problem was clearly required, and our observation that cyclohexaneacetic acid, another compound with an acetate side chain, was metabolized by initial conversion into its CoA ester (24, 25) provided the required impetus.

CoA ester synthetase activities in extracts of *P. Putida*. When an extract of (+)-camphor-grown *P. putida* was incubated with 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate in the presence of CoA, ATP, and additional components of a CoA ester synthetase assay system (26), the synthesis of a CoA ester was detected (Fig. 2). The rate of production of the CoA ester was approximately linear with respect to assay time for at least 30 min and was directly proportional to the amount of protein included (2 to 10 mg). A pH optimum of 8.0 was determined experimentally.

The specific activities of CoA ester synthetases assayed by established procedures are typically low and probably do not reflect the in vivo situation (19, 23, 26). Assays of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA synthetase appear to be no exception.

To assess the importance of the CoA ester synthetase in relation to (+)-camphor oxidation, we prepared extracts from succinate- and octanoate-grown cells of *P. putida*. Measurement of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA synthetase activity in extracts of succinate-grown cells established the inducible nature of the enzyme, and an assay of octanoyl-CoA

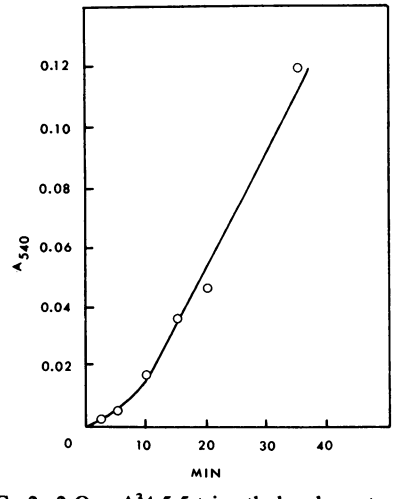

FIG. 2. 2-Oxo-$\Delta^3$4,5,5-trimethylcyclopentenylacetyl-CoA synthetase in extracts of (+)-camphor-grown *P. putida*. The enzyme was assayed as described in the text. Each assay flask contained 4.9 mg of crude cell extract protein. A_{540}, Absorbance at 540 nm.

synthetase activity in extracts of octanoate-grown cells showed that this enzyme, which is presumably mandatory for metabolism of octanoate, was induced at a level comparable to the level of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA synthetase (Fig. 3A).

CoA ester dehydrogenase activities of (+)-camphor-, octanoate-, and succinate-grown cells. The involvement of CoA ester synthetase in the first step of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxidation by (+)-camphor-grown *P. putida* stimulated speculation on the nature of the subsequent metabolic steps.

In the case of cyclohexaneacetic acid metabolism by *Arthrobacter* CA1, formation of the CoA ester is followed by a dehydrogenation step in preparation for side chain cleavage by a lyase (25).

Extracts of octanoate-grown *P. putida* contained an inducible CoA ester dehydrogenase compatible with a conventional β-oxidative attack upon octanoate. However, extracts of (+)-camphor-grown cells showed only a basal level of dehydrogenase activity toward octanoyl-CoA and no detectable activity toward 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA (Fig. 3B). Therefore, it follows that the formation of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA is not directed toward side chain elimination, and an alternative sequence of reactions must be involved.

2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase. Experiments aimed at elucidating enzyme-catalyzed reactions subsequent to CoA ester formation revealed the presence of an NADPH-linked activity which was initially assumed to be a dehydrogenase associated with attack on the double bond of the cyclopentene ring of the CoA ester. Further investigation proved that this was incorrect since substrate-stimulated NADPH oxidation was dependent upon the presence of oxygen (anaerobic Thunberg cuvettes) and accompanied by oxygen consumption. These characteristics are typical of a mixed-function (mono)oxygenase.

Since the direct insertion of an oxygen atom into 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate had previously been proposed on the basis of the accumulation of the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid by whole cells (2), it seemed possible that this oxygen-consuming reaction, demonstrated for the first time in cell extracts, may be the lactone-forming step and thus worthy of further investigation.

2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase induction and gene transference. The obligatory involvement of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase in (+)-camphor degradation was indicated by the observation that, like the CoA ester

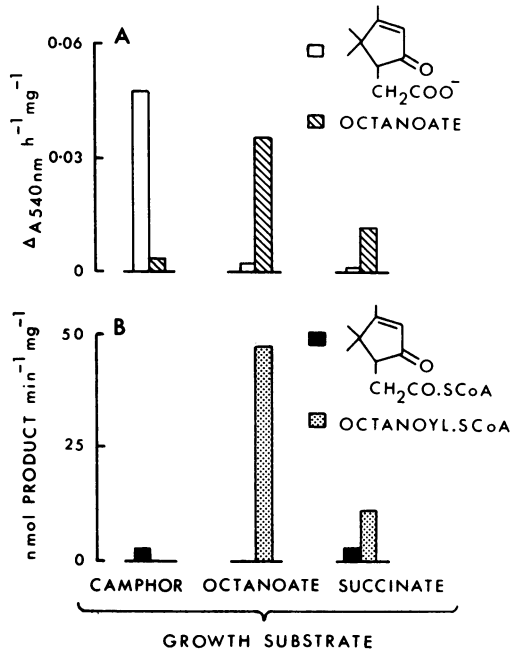

FIG. 3. Enzymes of β-oxidation in extracts of (+)-camphor-, octanoate-, and succinate-grown *P. putida*. (A) CoA ester synthetase activities. Δ_{A540nm}, Change in absorbance at 540 nm. (B) CoA ester dehydrogenase activities. The substrates utilized in the assays are indicated.

synthetase, this enzyme was induced by growth with (+)-camphor or 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid (Table 1), but was undetectable in extracts of octanoate- and succinate-grown cells.

The ability to grow on (+)-camphor was transferred to a recipient strain, *P. putida* PaW340, which is a streptomycin-resistant tryptophan auxotroph that was derived from *P. putida* (arvilla) mt-2. Streptomycin-resistant, (+)-camphor-positive transconjugants were isolated at a frequency of 10^{-5}. Concomitant transfer of the CAM plasmid and the inducible CoA ester synthetase and oxygenase demonstrated that the genes coding for the two additional enzymes are

TABLE 1. Induction of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase in *P. putida*

Growth substrate	Oxygenase activity[a]
(+)-Camphor	0.027
2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate	0.031
Succinate	<0.001
Octanoate	<0.001

[a] Results are expressed as micromoles converted per milligram of protein per minute.

TABLE 2. Enzymes of (+)-camphor oxidation in *P. putida* ATCC 17453, *P. putida* PaW340, and transconjugants

Strain	Enzyme activities			
	2,5-Diketocamphane 1,2-monooxygenase[a]	5-Hydroxycamphor dehydrogenase[a]	2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA synthetase[b]	2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase[a]
P. putida ATCC 17453	0.20	1.22	0.23	0.03
P. putida PaW340	<0.01	<0.01	<0.03	<0.004
Transconjugant 1	0.21	1.55	0.20	0.03
Transconjugant 2	0.23	0.70	0.22	0.03
Transconjugant 3	0.31	1.00	0.18	0.06

[a] Results are expressed as micromoles converted per milligram of protein per minute.
[b] Results are expressed as units of absorbance at 540 nm per 8 mg of extract protein per hour, corrected for endogenous activity.

located on the plasmid. Agarose gel electrophoresis (35) showed that transconjugants capable of growth with (+)-camphor contained plasmids of varying sizes. Three different transconjugants were selected on the basis of plasmid size and grown in medium containing (+)-camphor and 50 μg of tryptophan per ml, and enzyme activities were determined. Transconjugant 1 contained a plasmid similar in size to the plasmid in the donor strain (molecular weight, 300×10^6), whereas transconjugants 2 and 3 contained smaller plasmids. In a control experiment, *P. putida* PaW340 grown on succinate (2 g/liter) in the presence of (+)-camphor had no detectable CoA synthetase or CoA ester oxygenase activity (Table 2).

Purification of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase. 2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase was initially purified by a sequence of conventional procedures that included ammonium sulfate fractionation, DEAE-cellulose chromatography, and passage through Sephacryl S-200. Polyacrylamide gel electrophoresis of this purified enzyme yielded a yellow band that was visible to the naked eye; this band corresponded to the major protein band on stained gels and could be eluted from unstained gels and shown to possess oxygenase activity. Enzyme prepared by this method still contained approximately 25% impurities, as judged by scanning stained gels at 600 nm with a Gilford model 2400-S spectrophotometer fitted with a gel-scanning attachment. Although this enzyme was not pure enough for physical studies, it was adequate for metabolic investigations.

In subsequent preparations the procedure was improved by substitution of a terminal affinity chromatography step (Table 3), which yielded pure protein on the basis of polyacrylamide gel electrophoresis. A single protein band, which was detectable either by staining with Coomassie brilliant blue or by scanning gels at 280 nm, was obtained at all gel concentrations tested, and this band corresponded to an absorbance peak at 445 nm. The results obtained with a 6.4% gel are shown in Fig. 4.

Molecular weight and subunit structure. The molecular weight of pure 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase was determined by the Yphantis (36) method to be 106,000. The subunit composition of pure enzyme was investigated by gel electrophoresis of the reduced enzyme in the presence of 0.1%

TABLE 3. Purification of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase

Step	Treatment	Vol (ml)	Protein (mg)	Enzyme units[a]	Sp act[b]	Purification (-fold)	Recovery (%)
Crude cell extract		264	10,290	327	0.032		100
1	Ammonium sulfate (50–80%)	81	2,100	142	0.068	2.2	43
2	DEAE-cellulose	43	90	63	0.70	22	19
3	Affi-Gel Blue	2.5	13.8	37	2.68	84	11

[a] One unit was the amount of enzyme required to oxidize 1 μmol of NADPH per min under standard assay conditions.
[b] Units per milligram of protein.

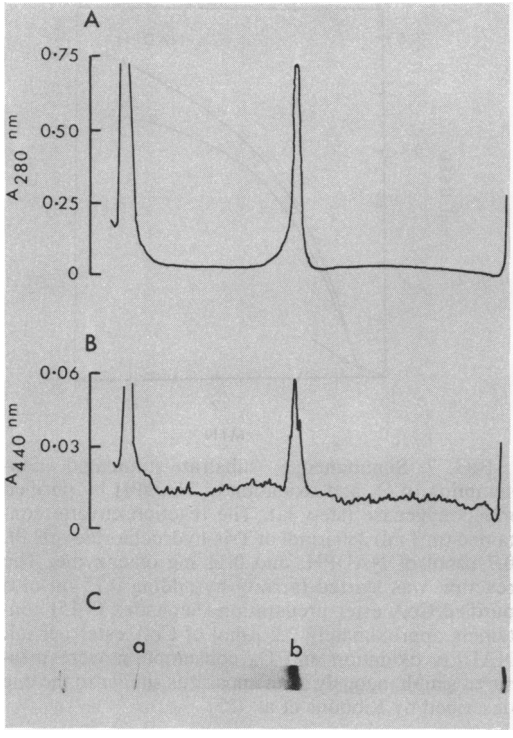

FIG. 4. Polyacrylamide gel electrophoresis of pure monooxygenase. Enzyme (110 μg, step 3b) was subjected to electrophoresis on a 6.4% gel. After electrophoresis the gel was quickly scanned with a Gilford model 2400-S spectrophotometer fitted with a gel-scanning attachment. (A) Absorbance at 280 nm (A_{280} nm). (B) Absorbance at 440 nm (A_{440} nm). (C) Gel stained with Coomassie brilliant blue G-250 (a) Tracking dye marker; (b) stained protein band.

SDS and 0.1% mercaptoethanol, which yielded a single detectable band of protein. A comparison of the distance migrated by the subunit with the migration distances of selected polypeptide chains of known molecular weights resulted in a calculated subunit molecular weight of 56,000 (Fig. 5). Thus, the native oxygenase is a dimer constructed from two identical subunits.

Absorption spectrum and prosthetic group. The pure enzyme was bright yellow, and the absorption spectrum showed maxima at 274, 378, and 438 nm and a ratio of absorbance at 274 nm to absorbance at 438 nm of 10.2:1 (Fig. 6). The pronounced shoulder at 460 nm was indicative of the hydrophobic environment occupied by the flavin in the holoenzyme.

The flavin component of the enzyme was dissociated from the protein by heating at 100°C in the dark for 1 min; centrifugation then yielded a clear yellow supernatant and a white pellet of denatured protein. A chromatographic analysis of the dissociated flavin showed that it consisted only of flavin adenine dinucleotide (FAD). Spectrophotometric measurements of the amounts of FAD released from known amounts of the holoenzyme gave values which ranged from 1.09 to 1.16 mol of FAD per 106,000 g of protein (close to unity and indicative that the two identical subunits of the holoenzyme together bind a single molecule of FAD).

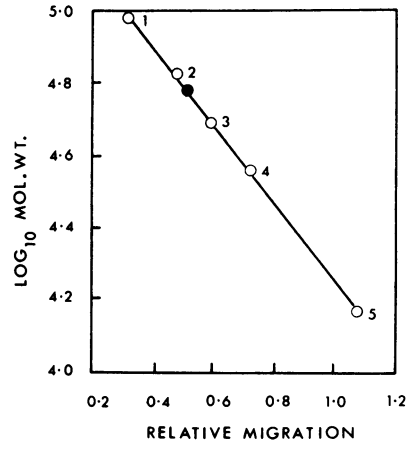

FIG. 5. SDS-polyacrylamide gel electrophoresis of the monooxygenase subunit. Enzyme and standard proteins were treated with SDS and mercaptoethanol and subjected to electrophoresis on 5% acrylamide gels containing 0.1% SDS. The following protein standards were used: 1, phosphorylase *a* (subunit molecular weight, 94,000); 2, serum albumin (molecular weight, 68,000); 3, glutamate dehydrogenase (subunit molecular weight, 53,000); 4, glyceraldehyde-3-phosphate dehydrogenase (subunit molecular weight, 36,000); 5, myoglobin (molecular weight, 17,500). The solid circle indicates the oxygenase subunit.

Iron content of the enzyme. In previous work it had been suggested that the 2,5-diketocamphane

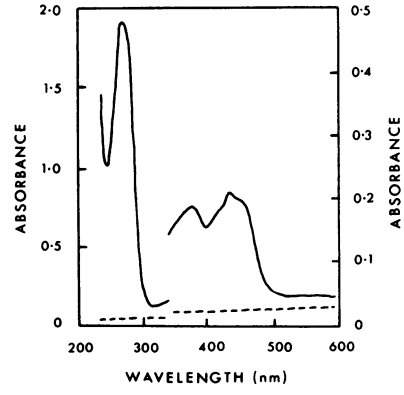

FIG. 6. Absorbance spectrum of the monooxygenase. A cuvette with a 1-cm light path contained (in 1 ml of 42 mM Na^+,K^+-phosphate buffer) 0.92 mg of enzyme purified to step 3b. Solid lines, Absorbance spectrum; dashed lines, base line.

monooxygenase of *P. putida*, which is an assembly of flavoproteins (6, 33, 37), also carries catalytically functional nonheme iron (6). In view of this assertion, a sample of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase (from step 3b) was analyzed for total iron as described above. A solution containing 4.95 mg of protein per ml was found to contain 0.22 μg of Fe per ml. This was equivalent to less than 0.1 atom of Fe per molecule of enzyme and may be attributed to nonspecific association that has no catalytic role. The lack of involvement of transition metal ions in the catalytic action of the oxygenase was further supported by the observed failure of 2,2'-dipyridyl (5 mM), EDTA (10 mM), and Tiron (4,5-dihydroxy-*m*-benzenedisulfonic acid; 1 mM) to cause significant inhibition of activity. 2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase is a simple flavoprotein.

Substrate specificity. The pH optimum for enzymatic activity was 9.0, and all assays were routinely performed in 0.1 M Tris-hydrochloride at this pH.

The enzyme was absolutely specific for the CoA ester of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid. Neither the sodium salt of the acid nor CoA was utilized, and alkali hydrolysis of the substrate, followed by careful neutralization, rendered it ineffective. NADH was not utilized as an electron donor, and the enzyme was inactive toward 2,5-diketocamphane (Table 4).

In marked contrast, a partially purified preparation of 2,5-diketocamphane 1,2-monooxygenase (33) (DEAE-cellulose column fractions eluted by 0.14 to 0.16 M KCl) was inactive toward 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA, thus confirming the separate identities of the two oxygenases.

TABLE 4. Substrate specificity of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenase[a]

Substrate	Electron donor	Relative activity[b]
2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA	NADPH	100
2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA	NADH	<5
2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA (hydrolyzed)[c]	NADPH	<5
2-Oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate	NADPH	<5
CoA	NADPH	<5
2,5-Diketocamphane	NADPH	<5

[a] Purified through step 3a (see text).

[b] Expressed as percentages of the activity observed with 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA. No activity was observed under anaerobic conditions or with boiled enzyme.

[c] CoA ester hydrolyzed in 1 M NaOH at 20°C for 5 min and readjusted to pH 9 for the assay.

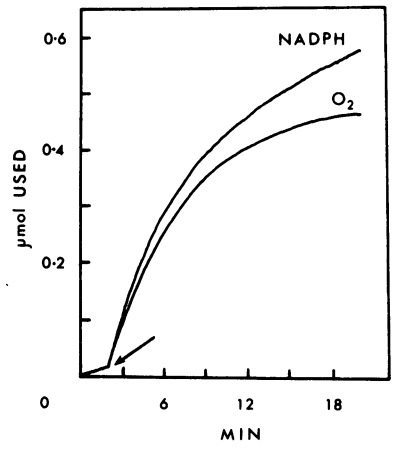

FIG. 7. Simultaneous substrate-stimulated consumption of O_2 and oxidation of NADPH by purified monooxygenase (step 3a). The reaction cuvette contained (in 3 ml) 280 μmol of Tris-hydrochloride (pH 9), 0.7 μmol of NADPH, and 0.11 mg of enzyme. The reaction was started (arrow) by adding 0.15 ml of a purified CoA ester preparation (Sephadex G-15) containing approximately 2.2 μmol of CoA ester per ml. NADPH oxidation and O_2 consumption were measured simultaneously in an apparatus similar to the one described by Ribbons et al. (27).

Reaction stoichiometry. A precise stoichiometric correlation of amount of substrate added, NADPH oxidation, and oxygen consumption was not obtained. Difficulties in precisely assaying the amount of substrate included in the assay, other than enzymatically, made independent assessment of the amount of substrate used unreliable. However, the amounts of NADPH and oxygen utilized were directly proportional to the volume of a given CoA ester preparation used and were approximately equal (Fig. 7). Precise ratio calculations were not possible because of the failure of the substrate-stimulated NADPH oxidation rate to return to the endogenous level when stimulated oxygen consumption had ceased.

Identity of the oxygenation product. Identification was difficult because poor yields of the substrate 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA obtained by chemical synthesis frustrated our attempts to obtain more than 1 to 2 mg of reaction product. In an accumulation experiment a double sidearm Warburg flask contained 200 μmol of Tris-hydrochloride (pH 9.0) and approximately 15 μmol of substrate (Sephadex G-15 preparation). One sidearm contained 20 μmol of NADPH, and the other contained 0.9 U of oxygenase purified through step 3a (see above). The CoA ester was omitted from a

control flask. The reaction was initiated by tipping the contents of the two sidearms, and when substrate-stimulated oxygen consumption had ceased (13.2 μmol), 1 ml of 5 M HCl was added to the contents of each flask, which were then subjected to a single freeze-thaw cycle; the denatured protein was removed by centrifugation at 27,000 × *g* and 4°C for 20 min. Attempts to extract material with absorption in the UV range from the aqueous phase into diethyl ether were unsuccessful. On the assumption that this was because the reaction product was also a CoA ester, 2 ml of 5 M NaOH was added to the aqueous phase, which was then incubated at 20°C for 60 min to hydrolyze CoA esters. Subsequent acidification and extraction with diethyl ether yielded a compound from the ether phase with an absorption maximum at 220 nm (aqueous). Conversion of this compound into a methyl ester followed by gas-liquid chromatography showed that it was more polar than the methyl ester of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid, and mass spectral analysis of the gas-liquid chromatography peak gave a molecular ion (M^+ 212), demonstrating the addition of a single oxygen atom to the substrate. The product was unequivocally identified as the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid by comparison of its mass spectrum with that of the authentic lactone accumulated by *P. putida* mutant PpS1790, as described above. The two mass spectra were identical in all respects.

The isolation of the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid after oxygenation of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA by the purified oxygenase was indicative of ring oxygen insertion to generate a lactone and compatible with the requirement for an electron donor. Studies of the metabolism of other alicyclic ketones have shown that typically ring oxygen insertion either is followed by lactone hydrolase-catalyzed ring cleavage (9, 22) or occurs spontaneously (7). A comparison of the structures of the lactone formed from 2,5-diketocamphane and of the proposed 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA oxygenation product showed important structural similarities (Fig. 8). The keto group at carbon 5 of 2,5-diketocamphane confers acidic properties upon carbon 6, and the resulting electron shifts initiate ring cleavage. Although it has been suggested that this may occur spontaneously (6), an enzyme-catalyzed nonhydrolytic ring cleavage reaction has not been unequivocally eliminated. The formation of the CoA ester of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetate confers keto properties upon the carboxyl carbonyl group, with the consequent potential for cleavage of the lactone formed therefrom by an identical mechanism (Fig. 8). Experimental handling of the oxygenation product was complicated by (i) the necessity to remove the CoA group before the compound could be isolated and (ii) the known tendency of hydroxy acids formed by the hydrolysis of δ-lactones to ring close in hydrophobic solvents. Therefore, we attempted to investigate ring cleavage in a pH-stat.

Experiments in the pH-stat. Studies in the pH-

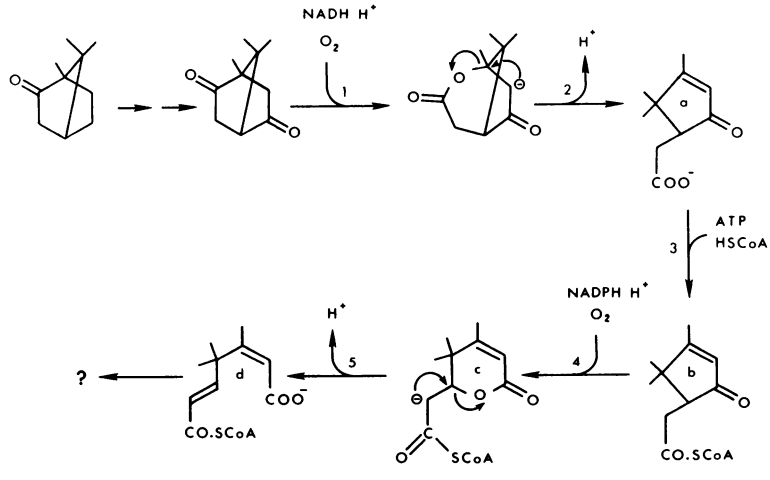

FIG. 8. Role of the CoA ester synthetase and CoA ester oxygenase in the cleavage of (+)-camphor. The following compounds are indicated: 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid (a); 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA (b); δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelyl-CoA (c); $\Delta^{2,5}$-3,4,4-trimethylpimelyl-CoA (d). The following enzymes are indicated: 2,5-diketocamphane 1,2-monooxygenase (1); 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA synthetase (3); 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA 1,2-monooxygenase (4). Reactions 2 and 5 appear to be parallel steps that may take place spontaneously.

stat, in which enzymatic hydrolysis of ε-caprolactone by a crude extract of cyclohexanol-grown *Nocardia globerula* CL1 was used as a check system (22), confirmed that an extract of (+)-camphor-grown *P. putida* was incapable of hydrolytic cleavage of chemically prepared 1,2-campholide and the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid (Fig. 1, compound f). Formation of stable 1,2-campholide from (+)-camphor by 2,5-diketocamphane 1,2-monooxygenase fractions from a DEAE-cellulose column was clearly distinguishable from the lactonization and cleavage of 2,5-diketocamphane (Fig. 9).

However, although experiments in which oxygenation of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA by a dialyzed crude cell extract or purified oxygenase was followed were not entirely satisfactory because of the small amounts of CoA ester available, they suggested that cleavage of the oxygenation product did not occur under the experimental conditions used (Fig. 9).

DISCUSSION

This investigation began with the realization that the information accumulated over a decade of study of microbial degradation of simple alicyclic compounds (7, 9, 13, 22) was potentially useful in the investigation of unsolved problems in (+)-camphor oxidation by *P. putida*. The enzymological and genetic aspects of the early steps of (+)-camphor metabolism are well understood but relate only to cleavage of the first ring of the bicyclic structure. Presumptive evidence was obtained for a "biological Baeyer-Villiger" attack upon the second ring (2) but was never supported by subcellular enzymology.

Our recent finding that cyclohexaneacetic acid was amenable to bacterial oxidation by the first two steps of a β-oxidation cycle in conjunction with a lyase (24, 25) which eliminated the side chain as acetyl-CoA prompted our study of the fate of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid, which also carries an acetyl side chain. Our discovery that growth of *P. putida* with (+)-camphor induced a specific 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA synthetase suggested a parallel metabolic route, which immediately became untenable in the absence of the necessary CoA ester dehydrogenase (Fig. 3). The discovery of a monooxygenase active toward this CoA ester was surprising since a catabolically involved oxygenase that utilizes this class of substrate has not been reported previously. In other respects this oxygenase proved to be unremarkable, being a simple dimer carrying FAD as the only detectable nonprotein component.

Therefore, cleavage of both rings of (+)-camphor requires three distinct monooxygenases, each of which bears little structural relationship to either of the others (6, 16, 33). The CoA ester oxygenase is probably structurally the simplest, 2,5-Diketocamphane 1,2-monooxygenase, which catalyzes an analogous reaction (Fig. 8), is also probably an assemblage of flavoproteins (37), although these proteins are clearly not identical (6, 37) and their association is relatively fragile (6).

If a study of the bacterial degradation of (+)-camphor had not preceded the consideration of simple alicyclic structures, such as cyclohexanol (9, 22) and cyclopentanol (13), then it would have been logically predicted that cleavage of the first camphor ring would proceed through an initial ring oxygen insertion, followed by hydrolysis of the lactone formed in this way. Generation of a keto group on the second ring would then have been predicted to be followed by the same sequence of steps. Therefore, it is of interest that (+)-camphor-grown *P. putida* cells do not possess lactone hydrolase activity toward either the 1,2-campholide or the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid. In this respect such cells resemble *Acinetobacter* TD 63, which is capable of growth only on alicyclic ketones that give rise to unstable lactones (7).

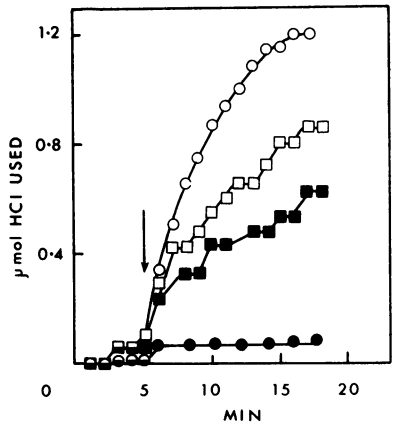

FIG. 9. Studies of oxygenation reactions in the pH-stat. The stirred reaction vessel contained (in a total volume of 3 ml) 0.4 μmol of Na^+,K^+-phosphate buffer (pH 7.1), 5 μmol of NADPH, and approximately 0.15 to 0.3 U of oxygenase activity. Substrates were added at the time indicated by the arrow, and the reaction was monitored at 20°C by controlled addition of 0.5 mM HCl. Symbols: ○ and ●, 2,5-Diketocamphane monooxygenase fractions from DEAE-cellulose with (+)-camphor and 2,5-diketocamphane (1 μmol each), respectively; □ and ■, purified (step 3a) 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA monooxygenase and dialyzed crude extract of (+)-camphor-grown *P. putida*, respectively, with 0.6 μmol of CoA ester as the substrate.

Since *P. putida* is unable to synthesize lactone hydrolases that are active toward putative intermediates of (+)-camphor oxidation, an alternative ring cleavage strategy is required. The operation of this system is clearly seen in relation to cleavage of the first ring, in which generation of the keto group at carbon 5 confers acidic properties on carbon 6 which, on losing a proton, initiates ring cleavage. Whether this occurs spontaneously or is catalyzed by the oxygenase has not been unequivocally established. The keto group at carbon 5 of the camphor remains as the keto group of the substituted cyclopentenyl ring cleavage product, which is susceptible to ring oxygen insertion and the generation of a second lactone. An understanding of the mechanism of cleavage of this second ring was frustrated by the lack of active subcellular systems and the observed stability of the putative oxygenation product in the absence of a lactone hydrolase (2).

Formation of the CoA ester of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetic acid before oxygenation seemed to acquire a logic when it was considered as a device to unmask the carbonyl properties of the carboxyl carbonyl group, with the consequent potential for ring cleavage of the lactone then to take place without recourse to a hydrolase (Fig. 8).

We encountered difficulties in establishing the precise nature of the oxygenation product. The material extracted from reaction mixtures with pure enzyme was unequivocally identified as the δ-lactone of 5-hydroxy-3,4,4-trimethyl-Δ^2-pimelic acid. However, identification was preceded by hydrolysis of the CoA ester, acidification, ether extraction, solvent evaporation, and methyl ester formation. Thus, there was the possibility that ring closure occurred during these manipulations. In an attempt to clarify this problem, CoA ester oxygenation was studied in a pH-stat with both crude extract and purified enzyme and compared with 2,5-diketocamphane 1,2-monooxygenase-mediated attack upon (+)-camphor and 2,5-diketocamphane, which form stable and unstable lactones, respectively (Fig. 9). We encountered operational difficulties because of the small amounts of 2-oxo-Δ^3-4,5,5-trimethylcyclopentenylacetyl-CoA available, but our results suggested that neither spontaneous nor hydrolytic cleavage of the oxygenation product occurred in the systems tested. This aspect of the pathway and the further metabolism of the ring cleavage metabolite remain unresolved and will be the subject of further study.

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