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Carbon Dioxide Fixation in the Metabolism of Propylene and Propylene Oxide by *Xanthobacter* Strain Py2

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Evidence for a requirement for CO_2 in the productive metabolism of aliphatic alkenes and epoxides by the propylene-oxidizing bacterium *Xanthobacter* strain Py2 is presented. In the absence of CO_2, whole-cell suspensions of propylene-grown cells catalyzed the isomerization of propylene oxide (epoxypropane) to acetone. In the presence of CO_2, no acetone was produced. Acetone was not metabolized by suspensions of propylene-grown cells, in either the absence or presence of CO_2. The degradation of propylene and propylene oxide by propylene-grown cells supported the fixation of $^{14}CO_2$ into cell material, and the time course of ^{14}C fixation correlated with the time course of propylene and propylene oxide degradation. The degradation of glucose and propionaldehyde by propylene-grown or glucose-grown cells did not support significant $^{14}CO_2$ fixation. With propylene oxide as the substrate, the concentration dependence of $^{14}CO_2$ fixation exhibited saturation kinetics, and at saturation, 0.9 mol of CO_2 was fixed per mol of propylene oxide consumed. Cultures grown with propylene in a nitrogen-deficient medium supplemented with $NaH^{13}CO_3$ specifically incorporated ^{13}C label into the C-1 (major labeled position) and C-3 (minor labeled position) carbon atoms of the endogenous storage compound poly-β-hydroxybutyrate. No specific label incorporation was observed when cells were cultured with glucose or *n*-propanol as a carbon source. The depletion of CO_2 from cultures grown with propylene, but not glucose or *n*-propanol, inhibited bacterial growth. We propose that propylene oxide metabolism in *Xanthobacter* strain Py2 proceeds by terminal carboxylation of an isomerization intermediate, which, in the absence of CO_2, is released as acetone.

In recent years bacteria which are capable of aerobic growth with aliphatic alkenes and epoxides as carbon and energy sources have been isolated (6). One such bacterium is *Xanthobacter* strain Py2, which was isolated with propylene as a source of carbon and energy (19). In this bacterium and other alkene-oxidizing bacteria, the metabolism of alkenes is initiated by monooxygenases which catalyze the O_2- and reductant-dependent oxidation of the alkenes to the corresponding epoxides, as illustrated for the substrate propylene and product propylene oxide in the following equation: propylene + O_2 + NADH + H^+→propylene oxide + H_2O + NAD^+.

The epoxides thus formed are further metabolized enzymatically, although the mechanisms and pathways involved remain largely uncharacterized. There is considerable interest in biological mechanisms for the disposal of aliphatic epoxides because of the toxicity, mutagenicity, and potential carcinogenicity of these compounds (3, 20). Epoxides are highly electrophilic molecules, and their detrimental properties arise from their ability to covalently modify cellular macromolecules, including proteins and DNA (3, 20). Epoxides are formed through the cytochrome P450-catalyzed epoxidation of alkenes (11) and are also synthesized in large quantities by the petrochemical industry for a wide range of applications. Epoxide hydrolases found in the endoplasmic reticulum and nuclear envelope of mammalian cells play an important role in the detoxification of epoxides by catalyzing their hydration to the corresponding *trans*-dihydrodiols, as shown in Fig. 1A (18).

Several bacteria, including a *Nocardia* strain capable of growing with propylene oxide as a carbon source (2) and a *Pseudomonas* strain capable of growing with epichlorohydrin (3-chloropropylene oxide) as a carbon source (8), have been shown to metabolize epoxides by hydrolytic reactions similar to those catalyzed by mammalian epoxide hydrolases. Another route for epoxide degradation has been identified in styrene-utilizing bacteria, which degrade styrene oxide by an isomerization reaction producing phenylacetaldehyde as the product, as shown in Fig. 1B (7, 13). Recent studies of epoxide metabolism in *Xanthobacter* strain Py2 by using resting-state whole-cell suspensions (15) and cell extracts (21) have led to the identification of ketones as products of epoxide degradation, suggesting that isomerization to ketones may be involved in epoxide metabolism in this organism (Fig. 1C). In whole-cell suspensions with propylene oxide (epoxypropane) or butylene-1,2-oxide (1,2-epoxybutane) as a substrate, acetone or methyl ethyl ketone, respectively, accumulated as a nonstoichiometric product (10 to 20% yield) of epoxide degradation (15). In cell extracts, a number of aliphatic epoxides were stoichiometrically converted to the corresponding ketones in a reaction requiring NAD^+ and a dithiol (21), leading to the conclusion that ketones such as acetone may be intermediates in the metabolism of aliphatic epoxides such as propylene oxide (16). However, cells grown with propylene as the carbon source do not readily metabolize acetone (15), suggesting that acetone may not be a discrete, physiologically relevant metabolite of propylene oxide degradation.

In this paper, a requirement for CO_2 for the productive metabolism of propylene oxide in *Xanthobacter* strain Py2 is described. Evidence that propylene oxide degradation proceeds by a carboxylation reaction which uses CO_2 as a cosubstrate is presented (Fig. 1D). These studies reveal a new biochemical mechanism for the conversion of a novel three-carbon substrate to a central four-carbon metabolite which bears similarities to recently described pathways for the CO_2-dependent metabolism of acetone (1, 9, 10, 14).

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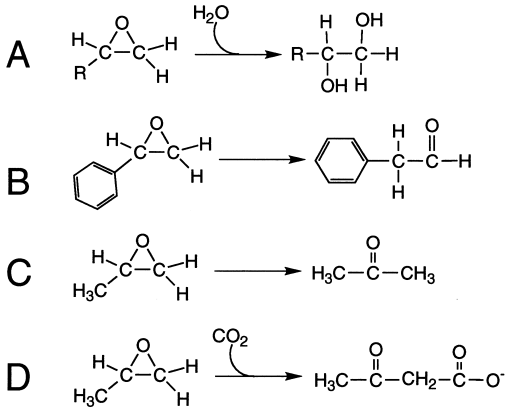

FIG. 1. Biological epoxide conversions. (A) Hydration. (B) Isomerization to an aldehyde. (C) Isomerization to a ketone. (D) Proposed carboxylation to a β-keto acid as described in the present work.

MATERIALS AND METHODS

Materials. Propylene (99.0% minimum) was obtained from Matheson Gas Products, Cucamonga, Calif. Carbon dioxide was obtained from Liquid Air, Walnut Creek, Calif. $NaH^{14}CO_3$ (specific activity, 54.4 mCi/mmol) was obtained from ICN Radiochemicals, Irvine, Calif. Organic compounds and $NaH^{13}CO_3$ were obtained from Aldrich Chemical Co., Milwaukee, Wis. All other chemicals used were of analytical grade.

Growth of bacteria and preparation of cell suspensions. *Xanthobacter* strain Py2 was cultured at 30°C in shake flasks as described previously (15). The carbon sources for growth were as follows: propylene, 10% (vol/vol) gas phase; *n*-propanol, 40 mM; acetone, 25 mM; or glucose, 10 g/liter. Cell growth (optical density) was monitored by removing a portion of cells from the cultures and measuring the A_{600}. Cells were harvested by centrifugation, washed, and resuspended in buffer (50 mM potassium phosphate, pH 7.2) as described previously (4, 15). Protein concentrations in whole-cell suspensions were determined by the biuret assay (5) after the cells were solubilized in 3 M NaOH for 30 min at 65°C. All subsequent studies with resting-state whole-cell suspensions were performed with 50 mM potassium phosphate (pH 7.2) as the buffer.

Assays of substrate depletion and product formation. Assays of organic substrate degradation and product formation by cell suspensions were performed with shaking at 30°C in sealed 9-ml serum vials which contained buffer, cells, and a substrate(s) in a total volume of 1 ml as described previously (15). Propylene oxide, propionaldehyde, and acetone were quantified by both gas phase and liquid phase analyses of the assay vials by using gas chromatography as described previously (15). For the addition of CO_2 and bicarbonate to assays, both CO_2 gas and $NaHCO_3$, from a 0.5 or 1 M stock solution of $NaHCO_3$ prepared in water, were added to the sealed assay vials in a ratio (1.2 mol of CO_2 to 1.0 mol of $NaHCO_3$) which maintained a constant pH of 7.2. CO_2 and $NaHCO_3$ were allowed to equilibrate with the buffer and cells in the assay vials for 5 min prior to initiation of the assays by the addition of other substrates. To deplete CO_2 from assay vials, sealed vials containing cells and buffer were bubbled for 10 min with CO_2-free air. CO_2-free air was prepared by sparging compressed air through an aqueous solution of 6 M KOH by using a gas dispersion tube, followed by passage through soda lime packed in a drying tube. To remove CO_2 generated by the cells during the course of assays, some assay vials contained a cut-off microcentrifuge tube in which 0.2 ml of 6 M KOH was absorbed onto a Whatman GF-A glass fiber filter disk (2.1-cm diameter). Gas-tight Hamilton microsyringes were used for all additions to assay vials and for removing samples for analysis.

Incorporation of ^{14}C from $^{14}CO_2$ into cell suspensions. Assay vials containing buffer and cells were prepared as described above. CO_2 gas and $NaH^{14}CO_3$ from a 500 mM stock solution were transferred to the sealed serum vials at the ratio described above to give final concentrations of CO_2 plus $NaHCO_3$ ranging from 1 to 100 mM. The specific activity of ^{14}C in the assays was 59 μCi/mmol of CO_2-$NaHCO_3$. Assay vials containing nonradiolabeled CO_2 and $NaHCO_3$ were prepared identically to those used to monitor incorporation of ^{14}C and were used to monitor the degradation of organic substrates during the assay. After a 2-min preincubation, assays were initiated by the addition of organic substrates to the vials. Samples of the gas and liquid phases of the nonradioactive assay vials were removed periodically, and the amounts of substrates remaining were quantified by gas chromatography. Twenty-five-microliter samples of the liquid phase were periodically removed from the assay vials containing $^{14}CO_2$ plus $NaH^{14}CO_3$ and were applied to a Millipore vacuum filtration apparatus which contained a 2.5-cm Whatman GF-A fiber filter placed on top of a 2.5-cm Millipore 0.45-μm-pore-size type HA filter. The cells on the filter were washed under vacuum by four successive additions (2.5 ml each) of 50 mM $NaHCO_3$-K_2HPO_4, pH 8.0. The filters were then removed and placed in scintillation vials containing 10 ml of scintillation fluid (Ecolume; ICN Radiochemicals). The radioactivity of the samples was measured with a Beckman LS 600 scintillation counter. Portions of the stock solution of 500 mM $NaH^{14}CO_3$ were added to filters wetted with 50 mM $NaHCO_3$-K_2HPO_4, analyzed for radioactivity by scintillation counting, and used to construct standard curves for converting disintegrations per minute to nanomoles of ^{14}C in the samples.

Production, isolation, and NMR analysis of poly-β-hydroxybutyrate. Cultures grown to stationary phase with propylene, glucose, or *n*-propanol as the growth substrate were used to inoculate flasks containing mineral salts medium lacking a source of fixed nitrogen. The volume of inoculant added was 4% of the volume of the nitrogen-free cultures. For the production of poly-β-hydroxybutyrate from propylene in the presence of nonenriched CO_2 plus $NaHCO_3$, cells were cultured in a sealed, 4-liter shake flask containing 0.8 liter of medium, 300 ml of propylene, 74 ml of CO_2, and 5.4 ml of 500 mM $NaHCO_3$. This combination of CO_2 and $NaHCO_3$ is the same ratio used in the assays described above and gave a combined concentration of CO_2 plus $NaHCO_3$ of 7.5 mM. For the production of poly-β-hydroxybutyrate from propylene in the presence of enriched (^{13}C-labeled) CO_2 plus $NaHCO_3$, cells were cultured in 0.1 liter of medium in a sealed, 0.5-liter shake flask which had been modified by the fusion of a center well (2 by 10 cm) to the bottom of the flask. After inoculation, 2 ml of H_2SO_4 was added to the center well of the culture flask. The flask was then sealed, 0.675 ml of 500 mM $NaH^{13}CO_3$ was added directly to the medium, and 0.825 ml of the same solution was added to the H_2SO_4 in the center well, resulting in the liberation of $^{13}CO_2$ gas to give the same ratio of $NaH^{13}CO_3$ to $^{13}CO_2$ as described above. Propylene (40 ml) was then added to the flask at overpressure. At 24-h intervals, the air, propylene, and nonenriched or ^{13}C-enriched CO_2 and $NaHCO_3$ in the cultures were replenished. Cultures were grown with *n*-propanol or glucose in place of propylene under conditions identical to those described above. After 3 days, the cultures were harvested, washed with water, sedimented, and suspended in 1/10 of the original culture volume of a commercial bleach solution. Poly-β-hydroxybutyrate was then isolated and quantified by the procedure of Law and Slepecky (12). Chloroform-extracted poly-β-hydroxybutyrate was dried in a vacuum oven, resuspended in deuterated chloroform, and transferred to a nuclear magnetic resonance (NMR) tube. Proton-decoupled ^{13}C NMR spectra were recorded with a Bruker ARX-400 NMR spectrometer with a frequency of 100.625 MHz and a sweep width of 333 ppm. Spectra were processed with a 5-Hz line broadening.

RESULTS

Effect of CO_2 on the conversion of propylene oxide to acetone by *Xanthobacter* strain Py2. Recent studies have identified ketones as products of aliphatic epoxide degradation in whole-

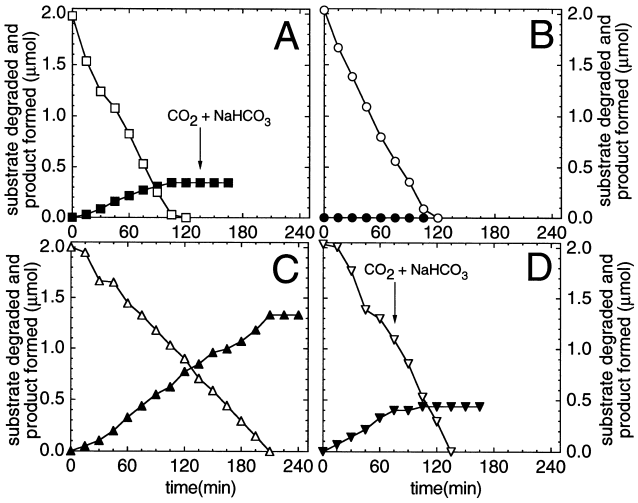

FIG. 2. Effect of CO_2 on the conversion of propylene oxide to acetone by propylene-grown *Xanthobacter* strain Py2. Assays were performed with whole-cell suspensions (0.22 mg of protein). Open symbols, propylene oxide remaining in the assay vial; closed symbols, acetone produced. (A) Normal assay conditions. CO_2 and $NaHCO_3$ were added to the assay vial to give a combined concentration of 10 mM at 135 min (arrow). (B) CO_2 and $NaHCO_3$ at a combined concentration of 10 mM were added 5 min prior to initiation of the assay with propylene oxide. (C) The assay vial contained CO_2-free air and a KOH trap prepared as described in Materials and Methods. (D) The assay vial contained CO_2-free air but no KOH trap. CO_2 and $NaHCO_3$ were added to the assay vial to give a combined concentration of 10 mM at 75 min (arrow).

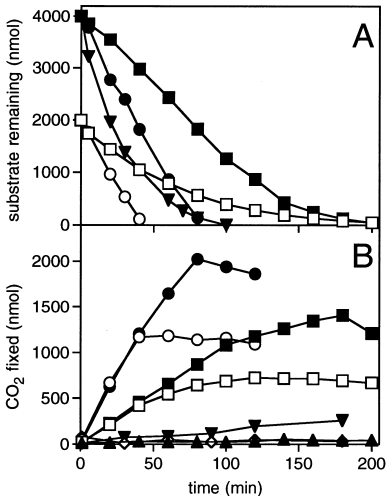

FIG. 3. Propylene- and propylene oxide-dependent fixation of $^{14}CO_2$ by propylene-grown *Xanthobacter* strain Py2. Assays were performed with propylene-grown cells (0.545 mg of protein), 10 mM CO_2-$NaHCO_3$, and 2,000 or 4,000 nmol of organic substrate. (A) Time course for the depletion of substrates. (B) Time course for the fixation of ^{14}C. Symbols for substrates added: □ and ■, 2,000 and 4,000 nmol of propylene, respectively; ○ and ●, 2,000 and 4,000 nmol of propylene oxide, respectively; ▼, 4,000 nmol of propionaldehyde; ▲, 4,000 nmol of glucose; ◇, 4,000 nmol of acetone.

cell suspensions (15) and cell extracts (21) of *Xanthobacter* strain Py2. Acetone is the ketone isomer of the growth substrate propylene oxide and is itself capable of serving as a growth-supporting substrate for *Xanthobacter* strain Py2 (19). However, suspensions of cells grown with propylene as the carbon source do not degrade acetone, and the acetone product which accumulates from propylene oxide degradation in cell extracts and whole-cell suspensions does not undergo further degradation, suggesting that acetone may not be a discrete intermediate of propylene oxide metabolism. Possibly, a physiologically important component of propylene oxide degradation is limiting in these assays, leading to the formation of acetone as a product.

Recent studies have revealed that in certain aerobic and anaerobic bacteria, acetone metabolism proceeds by a carboxylation reaction producing acetoacetate or an acetoacetyl derivative (e.g., acetoacetyl coenzyme A [acetoacetyl-CoA]) as an intermediate product (1, 9, 10, 14). Since propylene oxide is an isomer of acetone and since CO_2 could be a limiting factor in the assays described above, we examined a possible role for CO_2 in the degradation of propylene oxide and formation of acetone in suspensions of *Xanthobacter* cells grown with propylene as the carbon source. As shown in Fig. 2, the presence or absence of CO_2, added as a combination of $NaHCO_3$ and CO_2 gas to maintain a constant pH, affected both the rate of propylene oxide degradation and the amount of acetone which accumulated as a product. Under normal assay conditions (washed whole-cell suspensions in phosphate buffer), acetone accumulated as a nonstoichiometric product of propylene oxide degradation to an amount that was approximately 15% of the original amount of propylene oxide added to the assays (Fig. 2A). Acetone was not degraded by the cell suspensions upon further incubation, and the addition of bicarbonate and CO_2 did not stimulate further acetone degradation. When bicarbonate and CO_2 were included in the assays from the outset of the reaction, no acetone product was observed (Fig. 2B). In assay vials depleted of CO_2 by sparging with CO_2-free air and including a KOH trap in the vial, the rate of propylene oxide degradation was decreased about twofold and acetone accumulated to a level significantly higher (67% yield) than that observed under normal assay conditions (Fig. 2C). Another assay vial was prepared which had been sparged with CO_2-free air but which lacked a KOH trap to remove additional CO_2. CO_2 and bicarbonate were then added to the assay vial at the indicated time during the course of the assay (Fig. 2D). Further acetone accumulation ceased immediately upon the addition of CO_2 to the assay vial, while the acetone that had been formed prior to the addition of CO_2 remained in the vial.

Propylene- and propylene oxide-dependent fixation of $^{14}CO_2$. In order to determine whether the phenomena described above result from CO_2 serving as a cosubstrate in the degradation of propylene oxide, the abilities of propylene and propylene oxide to stimulate the incorporation of ^{14}C from $^{14}CO_2$ into biomass in cell suspensions were examined. In whole-cell suspensions, propylene is oxidized to propylene oxide by the enzyme alkene monooxygenase, and the propylene oxide product of this reaction is immediately degraded such that no propylene oxide intermediate can be detected. The oxidation of propylene is therefore diagnostic of the degradation of propylene oxide as well. As shown in Fig. 3, there was a direct correlation between the time courses for the degradation of propylene and propylene oxide and the time courses for the fixation of $^{14}CO_2$ into biomass. With both propylene and propylene oxide as substrates, ^{14}C fixation continued until the substrates had been completely degraded, and at that time, no further fixation of $^{14}CO_2$ occurred. Doubling the concentrations of propylene and propylene oxide added to the assays resulted in approximately twofold-higher maximal amounts of ^{14}C fixation. No significant fixation of $^{14}CO_2$ occurred in the

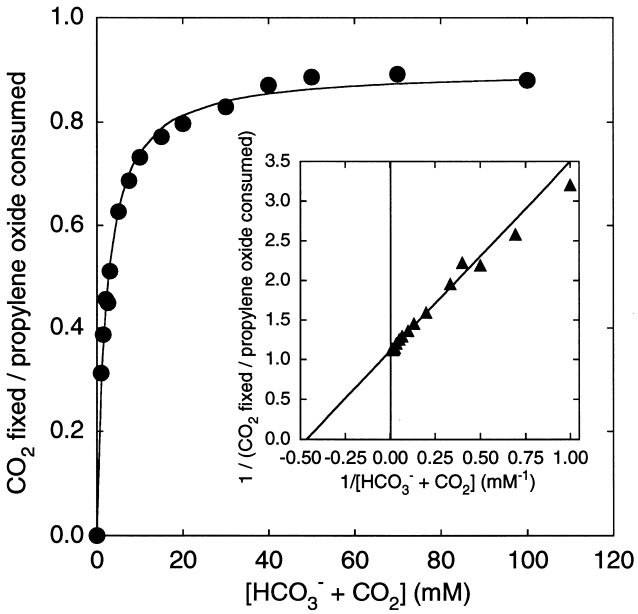

FIG. 4. Effect of CO_2-$NaHCO_3$ concentration on the ratio of CO_2 fixed to propylene oxide consumed. Assay vials contained propylene-grown cells (0.545 mg of protein) and various concentrations of $^{14}CO_2$ plus $NaH^{14}CO_3$. Assays were initiated by the addition of 2,000 nmol of propylene oxide. After complete degradation of propylene oxide (40 min), the amount of ^{14}C incorporated into the cells was determined. The ratio of CO_2 fixed to propylene oxide consumed is plotted versus the combined concentration of CO_2 and $NaHCO_3$ present in the assays. The solid line was generated by a nonlinear least-square fit to the equation for a rectangular hyperbola by using Sigma Plot (Jandel Scientific). (Inset) Double-reciprocal plot of the same data. The straight line was generated from the nonlinear least-squares fit of the data.

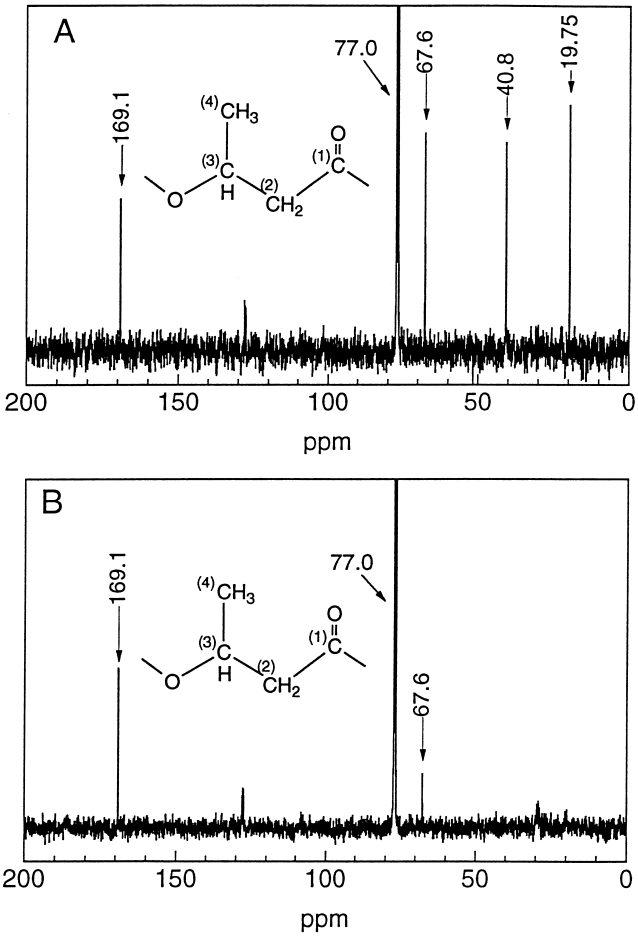

FIG. 5. Proton-decoupled ^{13}C NMR spectra of poly-β-hydroxybutyrate isolated from propylene-grown *Xanthobacter* cultures. The structural formula for the repeating unit of poly-β-hydroxybutyrate is shown in each panel. Resonances for individual carbon atoms: C-1, 169.1 ppm; C-2, 40.8 ppm; C-3, 67.6 ppm; and C-4, 19.75 ppm. The resonance at 77.0 ppm is due to the deuterated chloroform solvent. The magnitudes of the *y*-axis scales are different in the two panels. (A) Poly-β-hydroxybutyrate isolated from cells grown in the presence of nonenriched (natural-abundance) CO_2 and $NaHCO_3$. The concentration of poly-β-hydroxybutyrate in the sample was approximately 1 M. The spectrum represents the average from 3,260 scans. (B) Poly-β-hydroxybutyrate isolated from cells grown in the presence of ^{13}C-enriched CO_2 and $NaHCO_3$. The concentration of poly-β-hydroxybutyrate in the sample was approximately 0.06 M. The spectrum represents the average from 1,055 scans.

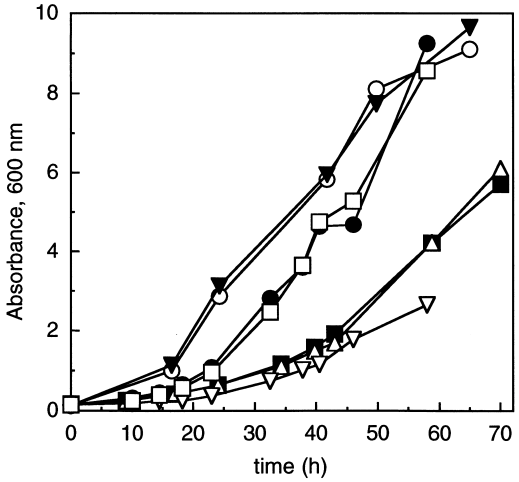

FIG. 6. Effect of CO_2 depletion on the growth of *Xanthobacter* strain Py2 with propylene, glucose, and *n*-propanol as carbon sources. Cultures were grown in 25 ml of medium in sealed shake flasks (250 ml) which had been modified by the fusion of a center well (1.5 by 8 cm) to the bottom of the flasks. To determine the CO_2 requirement for growth, a 9-cm-diameter disk of filter paper (Whatman no. 5) and 3 ml of 6 M KOH were added to the center well. Cultures were inoculated with 1 ml of stationary-phase cells grown with various carbon sources. Samples of the cells were removed periodically and analyzed for growth by measuring the A_{600} of the cells. At approximately 24-h intervals, the flasks were opened, sparged with 400 ml of sterile air, and resealed, and fresh propylene, CO_2-$NaHCO_3$, and *n*-propanol were added to the appropriate cultures. Symbols: □, propylene-grown cells; ●, propylene-grown cells plus 25 mM CO_2-$NaHCO_3$; ▽, propylene-grown cells plus KOH trap; ▼, *n*-propanol-grown cells; ○, *n*-propanol-grown cells plus KOH trap; △, glucose-grown cells; ■, glucose-grown cells plus KOH trap.

absence of an added carbon source. The addition of acetone, which was not degraded by the suspensions, did not support CO_2 fixation (Fig. 3B). Two additional substrates, propionaldehyde and glucose, were examined to determine whether they would support $^{14}CO_2$ fixation. As shown in Fig. 3A, propionaldehyde was degraded at a rate slightly higher than that for propylene oxide. However, only low levels of $^{14}CO_2$ were incorporated into biomass in the presence of propionaldehyde, and this incorporation continued at a constant, low level after complete consumption of propionaldehyde (Fig. 3B). Glucose, which is oxidized by propylene-grown cell suspensions at a rate that is about one-third of the rate of propylene oxide and propionaldehyde oxidation as determined from O_2 consumption measurements (4), did not support detectable $^{14}CO_2$ fixation (Fig. 3B). As a further control, suspensions of glucose-grown cells, which do not express the alkene- and epoxide-degrading enzymes, were incubated with $^{14}CO_2$ and propionaldehyde or glucose as described above. The amounts of ^{14}C incorporated into biomass with the glucose-grown cells were comparable to the low levels observed with propionaldehyde and glucose as substrates with propylene-grown cells (data not shown). No significant ^{14}C incorporation occurred when glucose-grown cells were incubated with either propylene or propylene oxide.

Stoichiometry of propylene oxide-dependent $^{14}CO_2$ fixation. The studies whose results are presented in Fig. 2 and 3 were conducted at concentrations of CO_2 and bicarbonate equal to 10 mM total CO_2 and carbonate species. Although this concentration of CO_2 was sufficient to completely prevent formation of acetone as a product in the assays whose results are shown in Fig. 2, it did not support stoichiometric $^{14}CO_2$ fixation as a function of propylene and propylene oxide degradation in the assays whose results are presented in Fig. 3. It seems reasonable to assume that the whole-cell suspensions used in these assays have endogenous storage reserves which, under aerobic conditions, can be oxidized to CO_2, thereby serving as a source of CO_2 in addition to the exogenous CO_2 supplied to the assays. Previous studies have demonstrated that whole-cell suspensions consume O_2 in the absence of added substrates (4), supporting this assumption. Since the cells are actively respiring, metabolites produced upon propylene oxide degradation will be further oxidized, resulting in the release of a portion of the fixed CO_2. For Fig. 2, the addition of KOH to trap CO_2 increased the amount of acetone produced from propylene oxide degradation, but the stoichiometry did not attain a 1:1 ratio. This result is consistent with endogenous CO_2 serving as a cosubstrate for the conversion of a portion of the propylene oxide to a product other than acetone.

In order to determine whether a stoichiometric relationship between CO_2 fixation and propylene oxide degradation exists,

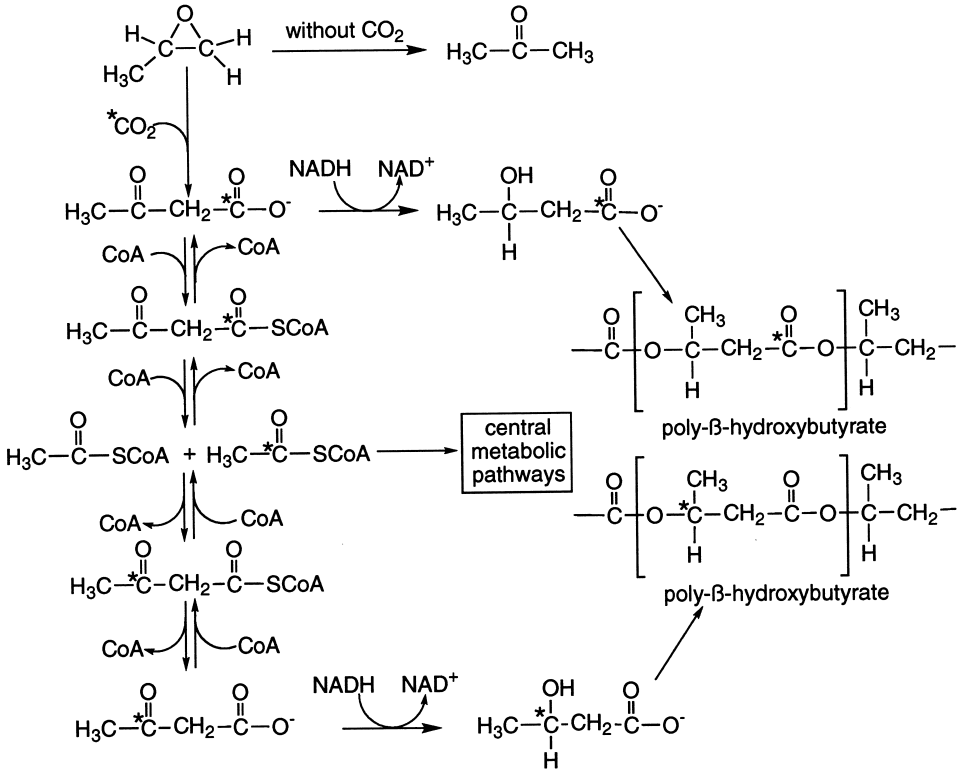

FIG. 7. Proposed pathways for propylene oxide metabolism in *Xanthobacter* strain Py2.

the amounts of ^{14}C fixed into biomass were quantified for various concentrations of $^{14}CO_2$ upon complete degradation of a fixed concentration of propylene oxide. As shown in Fig. 4, the relationship between $^{14}CO_2$ concentration and ^{14}C fixation followed saturation kinetics. A kinetic analysis of the data presented in Fig. 4 demonstrates that, at saturation, 0.90 ± 0.0088 mol of CO_2 was assimilated into biomass for each mol of propylene oxide degraded.

Evidence for four-carbon and two-carbon intermediates of propylene oxide carboxylation. As mentioned above, aerobic and anaerobic bacteria have been isolated which metabolize acetone via carboxylation reactions which have been proposed to produce an acetoacetyl derivative (free acetoacetate or acetoacetyl-CoA) as the immediate product (1, 9, 10, 14). Since propylene oxide and acetone are isomers, the carboxylation of propylene oxide, or a reactive intermediate derived from propylene oxide isomerization, might be expected to give this same four-carbon product. The acetoacetyl derivative could then be further metabolized by thiolytic cleavage to produce 2 mol of acetyl-CoA. Alternatively, acetoacetate could undergo reduction to β-hydroxybutyrate for incorporation into the endogenous storage compound poly-β-hydroxybutyrate.

In order to test the hypothesis that terminal carboxylation of propylene oxide might produce an acetoacetyl derivative which can be further metabolized as discussed above, cells were grown under nitrogen-limiting conditions to stimulate the synthesis of poly-β-hydroxybutyrate. Propylene was added as the growth substrate, and the growth medium was supplemented by the addition of either natural-abundance or ^{13}C-enriched CO_2 and bicarbonate. The ^{13}C NMR spectra of poly-β-hydroxybutyrate isolated from cultures grown in the presence of natural-abundance and ^{13}C-enriched CO_2 are presented in Fig. 5A and B, respectively. Four resonances are observed in the spectrum of poly-β-hydroxybutyrate isolated from the cells grown with nonenriched CO_2 present, which correspond to the four repeating carbon atoms of the polymeric unit (Fig. 5A). In the cells grown with $^{13}CO_2$ present, the resonances corresponding to the C-1 and C-3 carbon atoms are specifically enriched, and the magnitude of the C-1 peak is greater than that of the C-3 peak (Fig. 5B). The specific labeling of the C-1 carbon atom of poly-β-hydroxybutyrate with ^{13}C is consistent with terminal carboxylation of propylene oxide to produce a four-carbon unit which is directly incorporated into poly-β-hydroxybutyrate. The lesser labeling of the C-3 carbon can be explained by assuming that the four-carbon intermediate (acetoacetate or acetoacetyl-CoA) is reversibly cleaved into two two-carbon units (acetyl-CoA) which can reassemble with scrambling of the C-1 and C-3 carbons. In contrast to the results described above, the NMR spectra of poly-β-hydroxybutyrate isolated from cells grown with *n*-propanol or glucose as a carbon source in the presence of $^{13}CO_2$ showed no enrichment of specific resonances and were identical to the spectrum shown in Fig. 5A (data not shown).

Effect of CO_2 addition and depletion on the growth of *Xanthobacter* strain Py2. The data presented above suggest that the carboxylation of propylene oxide may be an integral step in the propylene oxidation pathway. This would indicate a CO_2 requirement for growth with propylene as the carbon source. Neither CO_2 nor bicarbonate is a component of the mineral salts growth medium used to culture this bacterium (22). However, when grown with propylene, the cells are typically cultured in sealed flasks to prevent the escape of propylene from the cultures. Thus, the CO_2 generated during the course of growth is retained in the culture flasks, thereby alleviating the need for the addition of an exogenous CO_2 source.

In order to test the requirement for CO_2 for growth with

propylene, *Xanthobacter* cultures were grown in sealed shake flasks containing a center well to which KOH could be added to deplete CO_2. As shown in Fig. 6, the depletion of CO_2 by the addition of KOH inhibited the growth rates of cultures with propylene as the carbon source. In contrast, there was no effect of the presence of KOH on growth rates with either glucose or *n*-propanol as the carbon source. The addition of CO_2 and bicarbonate to cultures grown with propylene did not stimulate the growth of the bacteria, indicating that the amount of CO_2 generated through respiration, and which accumulates in the sealed flasks, is sufficiently high to allow maximal growth rates.

DISCUSSION

The present work provides evidence for a novel mechanism for epoxide degradation involving isomerization and carboxylation steps yielding a β-keto acid product as shown in Fig. 7. This proposed pathway provides a novel route for converting a nonconventional three-carbon substrate, propylene oxide, into a common four-carbon metabolite, acetoacetate, which can be converted to two molecules of the central metabolite acetyl-CoA.

The observations that propylene oxide undergoes isomerization to acetone in whole-cell suspensions in the absence of CO_2 (Fig. 2) and in cell extracts (21) are consistent with an isomerization step preceding the carboxylation step. A plausible mechanism for propylene oxide conversion catalyzed by an epoxidase enzyme might involve an initial isomerization to produce the keto or enol form of acetone, which is then carboxylated to produce acetoacetate. In the absence of CO_2, this intermediate is released from the enzyme active site as acetone. Possibly, the enolate anion of acetone is the intermediate which undergoes carboxylation, and, in the absence of CO_2, the enolate undergoes irreversible protonation to produce acetone. In any event, the acetone product of propylene oxide isomerization formed in the absence of CO_2 cannot be considered to be a catalytically competent intermediate of propylene oxide carboxylation, since it is not reactive in either the absence or presence of CO_2.

Evidence similar to that presented in this paper has been obtained for the involvement of carboxylation mechanisms in the metabolism of acetone by certain aerobic and anaerobic bacteria (1, 9, 10, 14). Other acetone-oxidizing bacteria apparently metabolize acetone via hydroxylation to acetol rather than carboxylation to acetoacetate (17). Interestingly, acetone is capable of serving as a growth-supporting substrate for *Xanthobacter* strain Py2 (19), although the metabolism of acetone apparently involves the expression of a set of enzymes different from those involved in alkene and epoxide degradation (data not shown). In preliminary studies, we have found that growth of *Xanthobacter* strain Py2 with acetone as the carbon source is CO_2 dependent and that acetone degradation supports $^{14}CO_2$ fixation in suspensions of acetone-grown cells. These data suggest that *Xanthobacter* strain Py2 can express two distinct carboxylation systems involved in either epoxide or acetone degradation, depending on the nature of the growth substrate.

In summary, a pathway for aliphatic epoxide metabolism that is distinct from the hydrolytic mechanisms described for certain other bacteria (2, 8) and mammals (18) is described in this paper. The isolation and characterization of the epoxide-converting enzyme or enzymes responsible for this carboxylation reaction are of great interest. Experiments directed towards this end are in progress.

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