Characterization of Three Protein Components Required for Functional Reconstitution of the Epoxide Carboxylase Multienzyme Complex from *Xanthobacter* Strain Py2

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Epoxypropane from *Xanthobacter* strain Py2 catalyzes the reductant- and NAD$^+$-dependent carboxylation of aliphatic epoxides to $\beta$-keto acids. Epoxypropane fraction Py2 has been resolved from cell extracts by anion-exchange chromatography into three protein components, designated I, II, and III, that are obligately required for functional reconstitution of epoxide carboxylase activity. Component II has been purified to homogeneity on the basis of its ability to complement components I and III in restoring epoxide carboxylase activity. Purified component II had a specific activity for epoxide carboxylation of 41.8 mU·min$^{-1}$·mg$^{-1}$ when components I and III were present at saturating levels. The biochemical properties of component II reveal that it is the flavin-containing NADPH:disulfide oxidoreductase that was recently shown by other means to be associated with epoxide degradation activity in *Xanthobacter* strain Py2 (J. Swaving, J. A. M. de Bont, A. Westphal, and A. Dekok, *J. Bacteriol.* 178:6644–6646, 1996). The rate of epoxide carboxylation was dependent on the relative concentrations of the three carboxylase components. At fixed concentrations of two of the components, epoxide carboxylation rates were saturated in a hyperbolic fashion by increasing the concentration of the third variable component. Methylepoxypropane has been characterized as a time-dependent, irreversible inactivator of epoxide carboxylase activity that is proposed to be a mechanism-based inactivator of the enzyme. The addition of component I, but not that of component II or III, to methylepoxypropane-inactivated cell extracts restored epoxide carboxylase activity, suggesting that component I contains the epoxide binding and activation sites.

There is considerable interest in biological mechanisms for the degradation of aliphatic epoxides due to their toxic, mutagenic, and potential carcinogenic properties (4, 13). A novel strategy for epoxide metabolism has recently been demonstrated for *Xanthobacter* strain Py2, a gram-negative bacterium which is able to grow at the expense of aliphatic alkenes and epoxides such as propylene and epoxypropane (propylene oxide) (6). The metabolism of epoxypropane was shown to proceed by a CO$_2$-dependent carboxylation reaction that resulted in the formation of acetocacetate as the product (10). In the absence of CO$_2$, the epoxide-converting enzyme catalyzed the isomerization of terminal and internal epoxides to the corresponding ketones (e.g., epoxypropane to acetone and 2,3-epoxybutane to methylethyl ketone), although these reactions are apparently not of physiological significance (9). The epoxide-converting enzyme is thus both an epoxide isomerase and carboxylase, the nature of the reaction depending on the availability of the cosubstrate CO$_2$.

Initial studies of *Xanthobacter* epoxide carboxylase/isomerase suggest a novel and complex catalytic mechanism. In cell extracts, both the isomerase and carboxylase activities are dependent on the addition of NAD$^+$ and a reductant (e.g., di-thiothreitol [DTT]), which are reduced and oxidized, respectively, as shown by the equation epoxypropane + DTT$_{\text{red}}$ + CO$_2$ + NAD$^+$ $\rightarrow$ acetocacetate + DTT$_{\text{ox}}$ + NADH + H$^+$ (1, 14). These requirements are intriguing, since there is no net oxidation-reduction in either isomerizing or carboxylating aliphatic epoxides. Based on studies of epoxide isomerase in cell extracts, Weijers and coworkers proposed a mechanism for epoxide isomerization that involves (i) nucleophilic attack of a sulfhydryl on the C-1 carbon atom of a terminal epoxide, leading to ring opening and the formation of a covalently bound $\beta$-hydroxythioether intermediate; (ii) abstraction of the C-2 hydrogen atom as a hydride and its transfer to NAD$^+$ in concert with oxidation of the $\beta$-hydroxythioether to a $\beta$-keto-thioether; (iii) heterolytic cleavage of the C=S bond promoted by formation of a disulfide bond between the thioether sulfur and a second sulfhydryl group, followed by protonation of the ketene carbanion and product release; and (iv) reduction of the disulfide by the added reductant (e.g., DTT) (14). Epoxy carboxylation could occur by a similar mechanism in which CO$_2$, rather than a proton, is attacked by the carbanion in step 3.

Recent attempts to purify epoxide carboxylase have revealed that it is a multicomponent enzyme. Chion and Leak, by anion-exchange chromatography, resolved cell extracts into two fractions (designated A and B) that lacked activity individually but restored epoxide isomerase activity when recombined (2). A third fraction, which eluted prior to the first two, stimulated activity, although it was not required. Fraction A was further chromatographed over a Q-Sepharose anion-exchange column with enrichment of a 44-kDa polypeptide that may be the active component of this fraction (2). A homodimeric flavoprotein with a subunit molecular mass of 62 kDa was purified from fraction B and proposed to be the active component of this fraction. The purified protein contained NADPH-dependent lipoamide reductase activity which was inhibited by sulfhydryl-modifying reagents. However, the protein was not shown to substitute for fraction B in stimulating fraction A-dependent epoxide degradation activity (2). In a separate study, Swaving and coworkers purified the same dimeric flavoprotein on the basis of diaphorase activity and showed that the purified protein could be added to cell extracts depleted of the flavoprotein...
with 10-fold stimulation of epoxide degradation activity (11). The protein was further characterized as a pyridine nucleotide: disulfide oxidoreductase that could use NADPH as the reductant for epoxide degradation in place of DTT. The NADPH: disulfide oxidoreductase is presumably involved in reducing the dithiol required for epoxide isomerization and carboxylation as proposed by Weijers et al. (14) and discussed above. The results of the studies discussed above demonstrate that epoxide isomerization and carboxylation occur by complicated mechanisms requiring an oxidant and a reductant and involving the interplay of two or more enzymatic components. A number of important questions about this system remain unresolved. (i) How many separable proteins are required as components of the epoxide carboxylase system? (ii) What component contains the active site for the epoxide carboxylation reaction? (iii) How do the individual components associate and participate in the overall process of epoxide conversion? To contribute to the resolution of these questions, we have fractionated the components of the epoxide carboxylase system with particular emphasis on the quantitative recovery of epoxide carboxylase activity by recombining individual fractions. Based on this approach, we have identified three individual components obligately required by the system, including a component not identified in previous studies. One component, the NADPH:disulfide oxidoreductase, has been purified on the basis of its role in complementing the other two components of the epoxide carboxylase system. Finally, methylepoxypropane has been characterized as a time-dependent inactivator of epoxide carboxylase activity that is shown to have utility as an active-site probe of the enzyme system.

MATERIALS AND METHODS

Chemicals. 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS) detergent, NAD+, lactic acid dehydrogenase, and pyruvate were purchased from Sigma Chemicals. Horseradish peroxidase (HRP) color development reagent and goat anti-rabbit immunoglobulin G HRP conjugate were purchased from Bio-Rad. 3-[N-Morpholino]propanesulfonic acid (MOPS) buffer was obtained from United States Biochemicals. Epoxidepropane (99% min) was purchased from Aldrich Chemicals. Methylepoxypropane was purchased from Lancaster.

Growth of bacteria and preparation of cell extracts. Xanthobacter strain Py2 was grown in 15-liter serum bottles in continuous cultures in a Microferm fermentor (New Brunswick Scientific) as described previously (1). The carbon source for cell growth was propylene (10% [vol/vol] gas phase), acetone (25 mM), or glucose (10 mM) and was supplied as a source of enzyme (cell extracts or column fractions), NAD+ (2 mM), DTT (5 mM), epoxidepropane (1 to 2 mM), and CO2 that were saturating throughout the course of assays. Epoxypropane degradation rates were linear over the entire time course of assays, and the initial rates of epoxypropane degradation were unchanged in assays containing initial concentrations varying from 0.25 to 2 mM. Acetoacetate was quantified by removing 1-μl samples of the liquid phase from assay vials and analyzing it by gas chromatography as previously described (1). Assays of epoxidepropane degradation activity by whole-cell suspensions (0.33 mg of protein) were performed with shaking at 30°C in sealed 9-mL serum vials as described (previously 10). SDS-PAGE and immunoblotting procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% total gel; 2% cross-linker running gel) was performed in a Mini-Protean II apparatus (Bio-Rad) by following the manufacturer's instructions. Immunoblot analysis was conducted by electrotherolectrically transferring proteins from SDS-PAGE gels onto a polyvinylidene difluoride membrane. The membrane was incubated with polyclonal antiserum raised against purified component II. Cross-reacting proteins were visualized by using HRP conjugated to goat anti-rabbit immunoglobulin G.

Protein characterizations. The flavin cofactor was extracted from purified component II by acidification of a protein sample (25 μM) with trichloroacetic acid (10%, vol/vol) at 25°C. The flavin cofactor was identified with a Nova-Pak C18 reversed-phase column as previously described (8). Elution of flavins was monitored at 254 nm.

Preparation of methylepoxypropane-treated cell extracts. Frozen cell paste (10 g) was thawed and resuspended in 210 mL of potassium phosphate (50 mM, pH 7.2) buffer. Eighty-milliliter aliquots of the cell suspension were distributed into 150-mL serum bottles and crimp sealed with gray butyl stoppers. Inactivation of epoxide carboxylase activity performed by incubation with methylepoxypropane (5 mM) with shaking for 30 min at 30°C. The cell suspensions were then centrifuged for 10 min at 8,600 g at 4°C, and the supernatant was decanted, and the pellets were resuspended in 2 volumes of potassium phosphate buffer (50 mM) per g of cells and then centrifuged. This procedure was repeated twice, except that the last resuspension of pellets was in buffer A. The cell suspension was then passed three times through a French pressure cell at 110,000 kPa, and the lysate was clarified by centrifugation at 54,000 × g for 1 h at 4°C. Residual methylepoxypropane was removed by dialysis against MOPS buffer (50 mM, pH 7.2) containing 10% glycerol for 1 h at 4°C.

Purification of epoxide carboxylase component II. A portion of the pooled fractions containing component II (60 ml distributed in 15-mL aliquots) from the DEAE-Sepharose column were heat treated by incubation in a 36°C water bath, followed by centrifugation for 30 min at 17,500 g at 4°C. The supernatant was adjusted to 1.7 M ammonium sulfate and applied to a phenyl-Sepharose CL-4B column (2.5 by 12 cm) equilibrated in buffer B (50 mM MOPS [pH 7.2], 10% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM DTT) containing 1.7 M ammonium sulfate. The column was washed with 100 mL of buffer B containing 1.7 M ammonium sulfate and then washed with 100 mL of 850 mM ammonium sulfate in buffer B. Component II was eluted from the column with a 400-μL linear gradient of 850 to 0 mM ammonium sulfate in buffer B at a linear flow rate of 25 cm/h. Fractions containing component II activity were pooled and concentrated by ultrafiltration to a volume of approximately 5 mL. The protein was then applied to a Pharmacia HiPrep 26/60 Sephacryl S-100 column (2.6 by 15 cm) calibrated in 50 mM MOPS (pH 7.2) containing 0.1 M NaCl, 0.01 M EDTA, and 1 mM DTT at a linear flow rate of 11 cm/h. Fractions containing component II were collected and frozen in liquid nitrogen.

Assay of epoxide carboxylase activity. Epoxide carboxylase activity was measured by monitoring the time-dependent consumption of epoxypropane by gas chromatography as described previously (1, 10). Assays were performed in sealed vials (9 mL) containing a source of enzyme (cell extracts or column fractions), NAD+ (2 mM), DTT (5 mM), epoxidepropane (1 to 2 mM), and CO2 plus NaHCO3 (75 mM total) in 50 mM MOPS buffer, pH 7.2. A NAD+ regeneration system consisting of lactic acid dehydrogenase (10 U) and pyruvate (2 mM) was added to allow the regeneration of NAD+, which is reduced during the time course of the assay. These conditions provided concentrations of NAD+, DTT, and CO2 that were saturating throughout the course of assays. Epoxypropane degradation rates were linear over the entire time course of assays, and the initial rates of epoxypropane degradation were unchanged in assays containing initial concentrations varying from 0.25 to 2 mM. Acetoacetate was quantified by removing 1-μl samples of the liquid phase from assay vials and analyzing it by gas chromatography as previously described (1).

Assays of epoxidepropane degradation activity by whole-cell suspensions (0.33 mg of protein) were performed with shaking at 30°C in sealed 9-mL serum vials as described (previously 10).

Further resolution of component III from component II was accomplished by dilution of component III approximately fourfold with buffer A and chromatography over a Hi-Load Q-Sepharose column (2.6 by 15 cm). After loading, the column was washed with 120 mL of buffer A containing 300 mM NaCl in buffer A was applied to the column with a linear flow rate of 45 cm/h. Fractions containing component III activity were pooled, concentrated, and stored as indicated above. For assays requiring component III, only DEAE-Sepharose or Hi-Load Q-Sepharose component III fractions that contained no detectable component II activity were used.

Preparation of methylepoxypropane-treated cell extracts. Frozen cell paste (10 g) was thawed and resuspended in 210 mL of potassium phosphate (50 mM, pH 7.2) buffer. Eighty-milliliter aliquots of the cell suspension were distributed into 150-mL serum bottles and crimp sealed with gray butyl stoppers. Inactivation of epoxide carboxylase activity performed by incubation with methylepoxypropane (5 mM) with shaking for 30 min at 30°C. The cell suspensions were then centrifuged for 10 min at 8,600 g, the supernatant was decanted, and the pellets were resuspended in 2 volumes of potassium phosphate buffer (50 mM) per g of cells and then centrifuged. This procedure was repeated twice, except that the last resuspension of pellets was in buffer A. The cell suspension was then passed three times through a French pressure cell at 110,000 kPa, and the lysate was clarified by centrifugation at 54,000 × g for 1 h at 4°C. Residual methylepoxypropane was removed by dialysis against MOPS buffer (50 mM, pH 7.2) containing 10% glycerol for 1 h at 4°C.
RESULTS AND DISCUSSION

Separation of epoxide carboxylase into three components. Cell extracts from propylene-grown cells were applied to a column of DEAE-Sepharose that was developed with a linear gradient of NaCl. In agreement with the results of Chion and Leak (2), epoxide carboxylase activity, as measured by monitoring the time-dependent degradation of epoxypropane, was not associated with any single fraction obtained from this chromatographic separation (Table 1). However, in disagreement with their results, recovery of epoxide carboxylase activity required the addition of three, rather than two, fractions that have been designated components I, II, and III based on their order of elution (Table 1). Low rates of epoxypropane degradation were associated with component I alone; however, no detectable acetoacetate (the carboxylation product) was formed during the assay time course in association with this activity. Acetoacetate did accumulate, in a stoichiometric ratio of 1 mol of acetoacetate formed to 1 mol of epoxypropane consumed, only when all three components were present. As noted in the introduction, in the absence of CO₂, epoxide carboxylase catalyzes the hydrolysis of epoxides to the corresponding epoxides. This CO₂-independent isomerase activity was also found to require the combination of all three of the resolved components.

A comparison of the elution profiles of components I, II, and III in this study and those of fractions A and B in the previous study suggests that component I approximately correlates with fraction A and component II approximately correlates with fraction B (2). In the previous study, a fraction that eluted closely after fraction A stimulated activity to some degree but was required (2). We have not observed stimulation by fraction B (2). In the previous study, a fraction that eluted in the salt gradient is required to reconstitute epoxide carboxylase activity, it seems reasonable to speculate that fraction B was actually a mixture of two separable protein components (II and III) required for epoxide carboxylase activity. This would explain the inability of the flavoprotein, purified from fraction B, to substitute effectively for DEAE-Sepharose-resolved fraction B in reconstituting epoxide carboxylase activity when added to fraction A (2).

The results of these studies demonstrate that the epoxide carboxylase system is highly complex. They also reinforce the importance of purifying the individual components on the basis of their ability to complement the other required components at each step in the purification, since other components present in a given fraction may be removed, resulting in significant loss of activity. With these thoughts in mind, DEAE-Sepharose-resolved fractions containing component II activity were further fractionated with emphasis on purifying the required protein(s) to homogeneity.

Purification and characterization of epoxide carboxylase component II from DEAE-Sepharose-resolved component II fractions. The active component of DEAE-Sepharose-resolved component II fractions was purified on the basis of its ability to complement components I and III in reconstituting epoxide carboxylase activity. Activity assays were performed under conditions in which components I and III were present at excess (rate-saturating) levels so that component II was rate limiting in the assays. A summary of the four-step protocol used for the purification is presented in Table 2. The active component was purified 5.7-fold with a recovery of 32% and exhibited a specific activity for epoxide carboxylation of 41.8 μU/mg of protein. Purification resulted in the specific enrichment of a flavoprotein that migrated on SDS-PAGE with an apparent subunit molecular mass of 57 kDa (Fig. 1). The molecular mass of the polypeptide was determined to be 57.5 kDa by using time-of-flight mass spectrometry. As can be seen from the gel electrophoretic banding patterns of proteins in the cell extract, the 57-kDa protein represents a sizable percentage of the total cell protein, resulting in a low-fold overall purification. The 68% loss of activity during the purification can be largely attributed to the need to quantitatively separate the flavoprotein from the other epoxide carboxylase fractions. For example, recovery of component II activity after the initial DEAE-Sepharose column chromatography can be increased to more than 90% if all fractions containing the flavoprotein are combined. However, the fractions pooled in this manner have measurable levels of component I and III activities. Significantly, component II activity throughout the purification was present only in the fractions that contained the 57-kDa flavoprotein, indicating that it is the only epoxide carboxylase

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**TABLE 1.** Reconstitution of epoxide carboxylase activity after fractionation by anion-exchange chromatography

<table>
<thead>
<tr>
<th>Component(s) added to assay</th>
<th>Epoxypropane degradation (nmol/min)</th>
<th>Acetoacetate formation (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.6 ± 0.0</td>
<td>ND</td>
</tr>
<tr>
<td>II</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>III</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>I + II</td>
<td>1.1 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>I + III</td>
<td>1.4 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>II + III</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>I + II + III</td>
<td>25.2 ± 0.4</td>
<td>25.6 ± 0.1</td>
</tr>
</tbody>
</table>

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**TABLE 2.** Activity-based purification of epoxide carboxylase component II from *Xanthobacter* strain Py2

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Vol (ml)</th>
<th>Total activity (μU)*</th>
<th>Sp act (μU/mg)</th>
<th>Purification (fold)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract*</td>
<td>3,416</td>
<td>135</td>
<td>25,353</td>
<td>7.4</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>1,101</td>
<td>60</td>
<td>15,720</td>
<td>14.3</td>
<td>1.9</td>
<td>62</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>514</td>
<td>50</td>
<td>14,700</td>
<td>28.6</td>
<td>5.9</td>
<td>58</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>293</td>
<td>106</td>
<td>9,888</td>
<td>33.7</td>
<td>4.6</td>
<td>39</td>
</tr>
<tr>
<td>Sephacryl S-100</td>
<td>192</td>
<td>12</td>
<td>8,016</td>
<td>41.8</td>
<td>5.7</td>
<td>32</td>
</tr>
</tbody>
</table>

* Assays contained 8 mg of component I, 3.8 mg of component III, and a limiting amount of the component II fraction. One unit of activity is defined as 1 nmol of epoxypropane degraded per min.

* Component II was purified from a portion (1,101 mg of the 3,671 mg available) of the pooled component II fractions resolved by DEAE-Sepharose. The total amount of protein in the cell extract has been normalized to 30% of the actual amount of protein in the cell extract to reflect this.
component present in DEAE-Sepharose-resolved component II fractions.

The biochemical properties of the purified flavoprotein confirm that it is the NADPH:disulfide oxidoreductase independently characterized by Swaving et al. (11) and Chion and Leak (2). Gel filtration chromatography on Superose 12 confirmed that the protein is homodimeric. Quantitative amino acid analysis was performed on the purified flavoprotein and used as a basis for reporting analytical data. Based on these results, and using the subunit molecular mass of 57,488 Da determined by mass spectrometry, the biuret protein assay was found to overestimate the protein concentration by a factor of 1.3. The flavin content of the protein was determined to be 0.95 mol of flavin adenine dinucleotide (FAD) per mol of protein. This is higher than the 0.78 mol of FAD per mol of protein reported by Chion and Leak; the discrepancy is probably due to colorimetric protein assays overestimating the protein concentration relative to BSA standards.

Swaving and coworkers have previously cloned a fragment of DNA that complements Xanthobacter mutants unable to degrade epoxides (12). One of the open reading frames in this fragment of DNA encodes a 57,315-Da protein that has an N-terminal sequence which is identical to the sequence of the NADPH:disulfide oxidoreductase (11). The results of our quantitative amino acid analysis agree with the amino acid composition deduced from the gene sequence of the open reading frame, further confirming that it is the same protein.

Immunoblot analysis of epoxide carboxylase component II. In a previous study, the alkene- and epoxide-metabolizing system of Xanthobacter strain Py2 was shown to be repressed during growth with other carbon sources (e.g., glucose and acetone) and induced upon the introduction of a range of aliphatic or chlorinated alkenes and epoxides to the cultures (5). In that study, a polypeptide of 57 kDa, presumably the flavoprotein, was shown to be newly synthesized upon induction of epoxide carboxylase in the cultures (5). The inductive nature of this protein was further investigated by raising polyclonal antibodies to the purified flavoprotein and examining the cross-reactivity in cell extracts of Xanthobacter strain Py2 grown under various conditions. As shown in the immunoblot in Fig. 2A, the antibodies cross-reacted with a protein band in cell extracts of propylene-grown cells that migrated at the same apparent molecular weight as the purified flavoprotein. Interestingly, cross-reaction was observed to a lesser degree at this same position in cell extracts of glucose- and acetone-grown cells in which no epoxide carboxylase activity could be detected (5), suggesting that the flavoprotein is expressed at low levels in these cells.

The DNA sequence of the NADPH:disulfide oxidoreductase revealed that it has a high degree of sequence homology to a family of pyridine nucleotide:disulfide oxidoreductases and most notably to dihydrolipoamide dehydrogenase (11, 12). Given the strong sequence homology to dihydrolipoamide dehydrogenase, studies comparing these two enzymes were performed. As shown in Fig. 2B, dihydrolipoamide dehydrogenase from porcine heart has the same apparent monomeric molecular weight as the NADPH:disulfide oxidoreductase from Xanthobacter strain Py2 as determined by SDS-PAGE. However, the antibodies raised to Xanthobacter NADPH:disulfide oxidoreductase do not cross-react with porcine heart dihydrolipoamide dehydrogenase (Fig. 2A). No detectable epoxide carboxylase activity was observed when porcine heart dihydrolipoamide dehydrogenase was substituted for Xanthobacter NADPH:disulfide oxidoreductase in the epoxide carboxylase assay (data not shown).

Relationship of the purified flavoprotein to components I and III. At fixed concentrations of the flavoprotein, epoxide carboxylase activity could be saturated by fractions I and III, allowing the calculation of a specific activity for epoxide degradation of 41.8 mU/mg of flavoprotein (Table 2). The interrelationships between the epoxide carboxylase components were further examined by varying the concentration of one component while holding the concentrations of the other two components constant. For these experiments, the purified flavoprotein was used as the source of component II activity while component I and III fractions from anion-exchange chromatography were used without further purification. At fixed concentrations of components I and III, there was a hyperbolic relationship between the amount of flavoprotein added to the assay and the rate of propylene oxidation (Fig. 3A). At fixed concentrations of flavoprotein and component III, a similar concentration dependence for component I was observed (Fig. 3B). A linear relationship between rate and component I con-
centration was observed when a relatively large amount of flavoprotein was included in these assays. At a fourfold lower amount of flavoprotein, the rate saturated within the range of component I concentrations included in the assays (Fig. 3B). Similar rate dependencies were observed when flavoprotein and component I were held constant and the concentration of component III was varied (Fig. 3C). Since components I and III have not been purified to homogeneity, no conclusions can be drawn about the stoichiometric relationship between the flavoprotein and the other components. These results do, however, demonstrate the concentration dependence, relative to the concentrations of the other components, for each component of the epoxide carboxylase system.

**Inhibition of epoxide carboxylase activity with methylepoxyp propane.** The results presented in Fig. 3 establish the requirement of the flavoprotein (component II) and at least two additional components for functional reconstitution of epoxide carboxylase activity. As mentioned in the introduction, the flavoprotein may function to reduce a disulfide bond to free sulfhydryls that function as nucleophiles in covalent catalysis. Whether the flavoprotein or one of the other components contains the active site for epoxide carboxylation is not clear. To date, no specific inhibitor of the epoxide carboxylase system has been identified. A time-dependent inactivator (e.g., tight-binding or slow-binding inhibitor) or a mechanism-based inactivator could serve as an invaluable probe for identifying and characterizing the active-site-containing component of the epoxide carboxylase system. One excellent candidate for such an inactivator is methylepoxyp propane, which differs from epoxyp propane in containing a methyl rather than a hydrogen substituent on the C-2 carbon atom. Methylepoxyp propane was previously shown to serve as an inducer of the epoxide carboxylase but was incapable of serving as a substrate for the enzyme (5). As mentioned in the introduction, Weijers and coworkers have proposed a catalytic mechanism for epoxide activation that involves nucleophilic attack of a sulfhydryl on the C-1 carbon atom to form a \( \beta \)-hydroxythioether, followed by abstraction of a hydride from the C-2 carbon, yielding a \( \beta \)-keto-thioether intermediate (Fig. 4A). As shown in Fig. 4B, reaction of methylepoxyp propane in a similar fashion would lead to the formation of a methyl-substituted \( \beta \)-hydroxythioether that could not react further due to the presence of the methyl group on the C-2 carbon.

**FIG. 3.** Dependence of epoxide carboxylation rates on component I and III concentrations. Epoxypropane degradation rates were determined in a 0.7-ml reaction volume. Assays contained purified component II and components I and III resolved by DEAE-Sepharose chromatography. (A) Saturation of epoxide carboxylase activity by purified component II. Assays contained 8 mg of component I and 3.8 mg of component III. (B) Saturation of epoxide carboxylase activity by component I. Assays contained 3.8 mg of component III and either 1.32 (●) or 0.33 (○) mg of purified component II. (C) Saturation of epoxide carboxylase activity by component III. Assays contained 8 mg of component I and either 1.32 (●) or 0.33 (○) mg of component II.

**FIG. 4.** Proposed mechanisms for epoxide carboxylase-catalyzed epoxide conversions. A, epoxypropane; B, methylepoxyp propane.

**FIG. 5.** Concentration- and time-dependent inactivation of epoxide carboxylase in whole-cell suspensions by methylepoxyp propane. Assays were performed with whole-cell suspensions (0.33 mg of protein per assay) prepared from cultures grown with propylene as the carbon source in 1-ml assay volumes. Each assay contained 10 mM total CO\(_2\) and NaHCO\(_3\). Assays were initiated by addition of 2 \( \mu \)mol of epoxypropane. Symbols: ■, no methylepoxyp propane; ○, 0.5 mM methylepoxyp propane; ▲, 1 mM methylepoxyp propane; ●, 2 mM methylepoxyp propane; ▼, 5 mM methylepoxyp propane.
indicated components in 0.9-ml reaction volumes. The epoxypropane degrada-
mg) prepared from methylepoxypropane-treated whole cells and the additional
in methylepoxypropane-treated cell extracts. Assays contained cell extract (12.2
component I (25.2 mg/ml) from DEAE-Sepharose chromatography; component II (6.6 mg/ml).

The possibility that methylepoxypropane would serve as an
inactivator of epoxide carboxylase activity was investigated by moni-
toring the degradation of epoxypropane over time in as-
says that contained various concentrations of methylepoxypro-
pane. As shown in Fig. 5, the presence of methylepoxypro-
pane and epoxypropane were removed and fresh epoxypro-
pane was added to the cells. If methylepoxypropane is a mechanism-based or tight-bind-
ing inactivator, it should specifically inactivate the epoxide
 carboxylase component containing the epoxide binding and
activation site and not affect the activity of the other com-
ponents. To test this possibility, cell extracts were prepared from
methylepoxypropane-treated cells and assayed for epoxide car-
boxylase activity in the absence and presence of anion-ex-
change chromatography-resolved components I and III and
purified flavoprotein. As shown in Fig. 6, no epoxide carbox-
ylase activity was present in the cell extract, confirming the
irreversibility of methylepoxypropane inactivation. Addition of
component I to extracts of methylepoxypropane-treated cells
restored epoxide carboxylase activity, while addition of com-
ponent II or III had no effect on activity (Fig. 6). These data
suggest that component I contains the catalytic site for epoxide
activation, isomerization, and carboxylation.

Conclusion. The results presented in this report identify
three components obligately required for functional reconsti-
tution of the epoxide carboxylase complex in Xanthobacter
strain Py2. It has been shown that one of these components,
the FAD-containing NADPH:disulfide oxidoreductase, can be
purified to homogeneity on the basis of its ability to comple-
ment the other two components of this system. It is hoped that
similar strategies will allow the other components to be puri-
ified to homogeneity, the ultimate goal being to elucidate the
role of each component in the novel mechanism of epoxide and
CO₂ activation catalyzed by this enzyme system.

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REFERENCES

of two components of epoxyp propane isomerase/carboxylase from Xan-
5. Ensign, S. A. 1996. Aliphatic and chlorinated alkenes and epoxides as in-
ducers of alkene monooxygenase and epoxide activity in Xanthobacter
264.
iron-sulfur flavoprotein from Methanosarcina thermophila. J. Biol. Chem.
271:24023–24028.
of an ATP-dependent carboxylase in a CO₂-dependent pathway of acetone
olism of propylene and propylene oxide by Xanthobacter strain Py2. J. Bac-
of pyridine nucleotide-disulfide oxidoreductase is essential for NAD⁺-
and NADPH-dependent degradation of epoxylkines by Xanthobacter strain
1995. Complementation of Xanthobacter Py2 mutants defective in epoxyl-
kane degradation, and expression and nucleotide sequence of the comple-
menting DNA fragment. Microbiology 141:477–484.
J. A. M. de Bont. 1995. Dithiol- and NAD-dependent degradation of epoxy-