Purification to Homogeneity and Reconstitution of the Individual Components of the Epoxide Carboxylase Multiprotein Enzyme Complex from Xanthobacter Strain Py2*

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Epoxide metabolism in the aerobic bacterium Xanthobacter strain Py2 proceeds by an NADPH- and NAD*-dependent carboxylation reaction that forms β-keto acids as products. Epoxide carboxylase, the enzyme catalyzing this reaction, was resolved from the soluble fraction of cell-free extracts into four protein components that are obligately required for functional reconstitution of epoxide carboxylase activity. One of these components, component II, has previously been purified and characterized as an NADPH-disulfide oxidoreductase. In the present study, the three additional epoxide carboxylase components have been purified to homogeneity and characterized. These component proteins are as follows: component I, a homohexameric protein consisting of 41.7-kDa subunits; component III, a dimeric protein consisting of 26.0- and 26.2-kDa polypeptides; and component IV, a dimeric protein consisting of a single 25.4-kDa polypeptide. Component I contained 5 mol of tightly bound zinc per mol of protein. Component I was specifically inactivated by methylepoxyp propane, a time-dependent irreversible inactivator of epoxide carboxylase activity, suggesting that this component plays an integral role in epoxide binding and activation. No metals or organic cofactors were detected for components III and IV. The molecular weights, N-terminal sequences, and amino acid compositions of the purified epoxide carboxylase components were determined and found to correlate with open reading frames within and adjacent to a cloned fragment of DNA that complements epoxide carboxylase components III and IV. The molecular weights, N-terminal sequences, and amino acid compositions of the purified epoxide carboxylase components were determined and found to correlate with open reading frames within and adjacent to a cloned fragment of DNA that complements Xanthobacter Py2 mutants defective in epoxide degradation. Using the purified epoxide carboxylase system, epoxide carboxylation was found to be stoichiometrically coupled to the transhydrogenation of pyridine nucleotide cofactors according to the following equation: epoxidepropane + CO₂ + NADPH + NAD⁺ → acetoacetate + H⁺ + NADH.

Xanthobacter strain Py2 is one of several bacteria capable of growth with short chain aliphatic alkenes as carbon and energy sources (1). The first step in alkene metabolism involves an oxidative insertion of an oxygen atom into the olefin bond in a reaction that is catalyzed by an inducible (2), multiprotein (3) alkene monoxygenase as shown for the substrate propylene and product epoxypropane in Equation 1.

\[
\begin{align*}
H_2C=C=O + O_2 + NAD^+ + H^+ &\rightarrow H_2C=O + H_2O + NAD^+ + H^+ \\
H_2C=C=O + CO_2 &\rightarrow H_2C\cdot C=CH_2 \cdot COO^- + H^+ 
\end{align*}
\]
units (11). In a separate study, this flavoprotein was shown to possess NADPH:disulfide oxidoreductase activity, suggesting that it is involved in the oxidation of the reductant necessary for epoxide carboxylation (12). Based on the specificity of the flavoprotein, NADPH rather than a cellular dithiol was proposed to serve as the physiological reductant for epoxide carboxylation (12).

With respect to catalytic mechanism, it has been proposed that redox active thiols are involved in epoxide ring opening and the further transformations that form product (9). This proposal is strengthened by the recent identification of component II as an NADPH:disulfide oxidoreductase (12). Interestingly, in the absence of CO$_2$, epoxide carboxylase catalyzes the isomerization of aliphatic epoxides to form ketones at rates comparable to those observed for carboxylation (4, 9) as shown in Equation 3.

\[
\text{H}_2\text{C} = \text{C} = \text{O} \rightarrow \text{H}_2\text{C} - \text{C} = \text{O} \quad \text{(Eq. 3)}
\]

Apparently, epoxide to ketone isomerization is a fortuitous reaction of no physiological significance (13). The observation that isomerization occurs is, however, significant, since it demonstrates that a reaction intermediate is generated that can alternatively undergo carboxylation to form a $\beta$-keto acid or protonation to form a ketone. Epoxide to ketone isomerization has not been observed in any other biological system.

The studies summarized above have revealed new transformations of an important class of xenobiotic compounds and the central role of a new type of multicomponent carboxylase in these processes. There are a number of fundamental questions that remain unanswered regarding this system, including how many separable proteins comprise the epoxide carboxylase system and what role(s) the individual components play in catalysis. It is apparent that the resolution of these questions will require the purification of each component protein. Accordingly, in this paper we report the purification to homogeneity and biochemical characterization of three proteins, designated components I, III, and IV, that, when recombined with purified component II, restore epoxide carboxylase activity. By using the purified components we demonstrate that 1 mol of NADPH is oxidized and 1 mol of NAD$^+$ is reduced for each substrate molecule carboxylated. Epoxide carboxylase is thus a four-component enzyme system coupling the transhydrogenation of pyridine nucleotides to the carboxylation of aliphatic epoxides.

MATERIALS AND METHODS

Growth of Bacteria and Preparation of Cell-free Extracts—Xanthobacter strain Py2 was grown in 15-liter semicontinuous cultures in a Microferm fermentor (New Brunswick Scientific) with propylene as a carbon source as described previously (4). Cells were harvested at an $A_{600}$ of 2.5 and 4.0 by tangential flow filtration with a Pellicon system (Millipore Corp.) and stored at $-80^\circ$C. For purifications of epoxide carboxylase components I and III, frozen cell paste (100–200 g) was thawed and resuspended in 2 volumes of buffer (50 mM MOPS (pH 7.2), 1 mM DTT, 10% (v/v) glycerol) containing 10 mg of DNase I. For the purification of component IV, frozen cell paste (100 g) was thawed and resuspended in 2 volumes of buffer (50 mM MOPS (pH 7.2)) and stored in place of MOPS. The cell suspension was centrifuged at 20,000 rpm for 30 min at $4^\circ$C. All buffers (MOPS and Tris-HCl) used in the preparation of cell-free extracts and in column chromatography procedures were first passed over a Bio-Rad Chelex-100 (5.0 × 10 cm) column to remove trace metals.

Purification of Epoxide Carboxylase Component I—Clariﬁed cell-free extract was applied to a Q-Sepharose Fast Flow column (2.5 × 15 cm) equilibrated in 50 mM MOPS (pH 7.2) containing 10% (v/v) glycerol (buffer A) at a linear flow rate of 49 cm/h. After loading, the column was washed with 150 ml of buffer A. The column was developed with a 1200-ml linear gradient of 0 to 300 mM NaCl in buffer A. Fractions containing component I activity were pooled and adjusted to 1.0 mM (NH$_4$)$_2$SO$_4$ and applied to a Pharmacia HiLoad 16/10 phenyl-Sepharose column equilibrated in buffer B containing 1 mM DTT, 10% (v/v) glycerol, and a linear flow rate of 60 cm/h. The column was developed using a reverse step gradient of buffer A containing 1.0, 0.7, 0.5, 0.25, and 0 mM (NH$_4$)$_2$SO$_4$, respectively. Fractions containing component III activity were pooled and dialyzed against 2 liters of 25 mM potassium phosphate buffer (pH 6.2) containing 10% glycerol (buffer B) for 16 h at $4^\circ$C. The protein was then clarified by centrifugation at 139,700 $g$ (Amicon column) (1.5 × 10 cm) equilibrated in buffer B at a linear flow rate of 60 cm/h. After washing the column with 120 ml of buffer B, component III was eluted with 20 ml of buffer A containing 10 mM NAD$^+$.

Fractions containing component III were then dialyzed against 1 liter of Tris-HCl (pH 8.2) containing 10% glycerol (buffer C) for 16 h, concentrated by ultrafiltration (YM30), and frozen in liquid nitrogen.

Purification of Epoxide Carboxylase Component I—I—Clariﬁed cell-free extract was heat-treated in 100-ml aliquots by incubation for 3 min in a 65 °C water bath, followed by centrifugation for 30 min at 17,500 $g$. The supernatant was applied to a Pharmacia HiLoad 26/10 Q-Sepharose column equilibrated in buffer C at 45 cm/h. The column was then washed with 150 ml of buffer C and developed with an 800-ml linear gradient from 0 to 250 mM NaCl in buffer C. Component IA was recovered in the flow-through, with a single peak of $<100$ ml (900 mM NaCl) fractions. Component IB was recovered in the fractions eluting between 140 and 160 mM NaCl. Each component was concentrated by ultrafiltration (YM30) and stored at $-80^\circ$C.

Purification of Epoxide Carboxylase Component II—Clariﬁed cell-free extract was heat-treated in 100-ml aliquots by incubation for 3 min in a 65 °C water bath, followed by centrifugation for 30 min at 17,500 $g$. The supernatant was applied to a Pharmacia HiLoad 26/10 Q-Sepharose column equilibrated in buffer C at 45 cm/h. The column was then washed with 150 ml of buffer C and developed with a 1200-ml linear gradient of 0–250 mM NaCl in buffer C. Fractions containing component I activity were pooled, adjusted to 1.7 mM (NH$_4$)$_2$SO$_4$, and applied to a Pharmacia HiLoad 26/15 phenyl-Sepharose column equilibrated in buffer C containing 1.7 mM (NH$_4$)$_2$SO$_4$. The column was then washed with 150 ml of 1.0 M (NH$_4$)$_2$SO$_4$ in buffer C and developed with a 400-ml linear reverse gradient of buffer C containing 1.0 to 0 mM (NH$_4$)$_2$SO$_4$. Fractions containing component I were pooled and concentrated by ultrafiltration (YM30) to a volume of approximately 4 ml. The sample was then applied to a Pharmacia HiPrep 26/10 Sephacryl S-300 column equilibrated in buffer C containing 200 mM NaCl at a linear flow rate of 11.3 cm/h. Fractions containing epoxide carboxylase component I were pooled and frozen in liquid nitrogen.

Purification of Epoxide Carboxylase Component III—Clariﬁed cell-free extract was heat-treated in 100-ml aliquots by incubation for 3 min in a 65 °C water bath, followed by centrifugation for 30 min at 17,500 $g$. The supernatant was applied to a Pharmacia HiLoad 26/10 Q-Sepharose column equilibrated in buffer C at 45 cm/h. The column was then washed with 150 ml of buffer C and developed with a 1200-ml linear gradient of 0–250 mM NaCl in buffer C. Fractions containing component III were pooled and adjusted to 1.7 mM (NH$_4$)$_2$SO$_4$, and applied to a Pharmacia HiLoad 16/10 phenyl-Sepharose column that was equilibrated in buffer C containing 1.7 mM (NH$_4$)$_2$SO$_4$, and developed by applying a 200-ml reverse gradient of buffer C containing 1.0 to 0 mM (NH$_4$)$_2$SO$_4$. Fractions containing component IV activity were pooled and dialyzed against 1 liter of buffer B. The sample was then applied to a column of Reactive Green 19 (1.4 × 10 cm), equilibrated in buffer B at a linear flow rate of 78 cm/h. The column was developed by applying a 200-ml linear gradient of buffer B to buffer C containing 100 mM NaCl. Active fractions were pooled and concentrated by ultrafiltration (YM30) and frozen in liquid nitrogen.

Assay of Epoxide Carboxylase Activity—Epoxide carboxylase activity was measured by monitoring the time-dependent depletion of epoxyp propane by gas chromatography as described previously (4). Assays were performed in sealed vials (9 ml) containing a source of enzyme (cell-free extract, column fractions, or purified component) in 50 mM Tris-HCl (pH 8.2), containing 10% glycerol using reagents and reaction conditions described previously (11). Purified epoxide carboxylase component II and methylepoxyp propane-treated cell-free extract were prepared as described previously (11). Acetoacetate was quantified by removing liquid samples from assay vials and analyzing by gas chromatography as described previously (4).
Quantification of NAD\(^+\) Reduction and NADPH Oxidation—Epoxide carboxylase activity was assayed as described above except that dithiothreitol was replaced with NADPH (5 mM); the concentration of NAD\(^+\) was increased to 5 mM; the NAD\(^-\)-regenerating system was not included, and EDTA (1 mM) was added. Assays were made anoxic by repeated evacuation and flushing with argon on a vacuum manifold, and a sodium dithionite-saturated filter trap was included in the vials. NADH formation and NADPH oxidation were monitored by removing 10-μl liquid samples from assay vials, diluting 50-fold with water, and analyzing by high pressure liquid chromatography. NADH and NADPH were separated with a Supelcosil LC-18 (25 cm × 4.6 mm) column using an isocratic mobile phase consisting of 100 mM potassium phosphate (pH 6.0) and 10% methanol. Elution of nucleotides was monitored at 340 nm.

Continuous Spectrophotometric Assay for NADH Formation—Assays were performed in 2-ml (1-cm path length) anaerobic quartz cuvettes that had been modified by fusing a serum bottle-style quartz top (7 × 13 mm at mouth), allowing the cuvettes to be sealed with a red rubber septum and flushed with argon on a vacuum manifold, and a sodium dithionite-saturated filter trap was included in the vials. NADH formation and NADPH oxidation were monitored by removing 10-μl liquid samples from assay vials, diluting 50-fold with water, and analyzing by high pressure liquid chromatography. NADH and NADPH were separated with a Supelcosil LC-18 (25 cm × 4.6 mm) column using an isocratic mobile phase consisting of 100 mM potassium phosphate (pH 6.0) and 10% methanol. Elution of nucleotides was monitored at 340 nm.

Protein Characterizations—Native molecular weights were estimated by gel filtration chromatography using a Pharmacia Superose 12 HR 10/30 column equilibrated in 50 mM MOPS (pH 7.2) containing 200 mM NaCl. The column was calibrated using pyruvate kinase (237 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carboxic anhydrase (31 kDa), and cytochrome c (12.3 kDa). Polyepoeptide molecular weights were also determined using electrospray mass spectrometry performed by the Utah State University Biotechnology Center. SDS-PAGE (12% T, 2.7% C running gel) was performed following the Laemmli procedure (14). Electrophoresed proteins were visualized by staining with Coomassie Blue. The apparent molecular masses of polypeptides based on SDS-PAGE migration were determined by comparison with R\(_v\) values of standard proteins. Quantitative amino acid analysis was performed by the Protein/Nucleic Acid Shared Facility at the Medical College of Wisconsin, Milwaukee. N-terminal sequencing was performed by the Utah State University Biotechnology Center. Multielemental metal analysis was performed on an inductively coupled plasma atomic emission spectrophotometer at the Utah State University Soil and Plant Analysis Laboratory. Protein concentrations were determined using a modified biuret assay with bovine serum albumin as the standard (15). The protein concentration of epoxide carboxylase component II was routinely determined by using its reported extinction coefficient (11).

RESULTS

Purification of Epoxide Carboxylase Component III—Previously, the epoxide carboxylase of Xanthobacter strain Py2 was shown to be a multicomponent enzyme system composed of at least three separable proteins (11). Fractionation of cell-free extracts by DEAE-Sepharose chromatography resolved epoxide carboxylase activity into three fractions, designated components I, II, and III based on their order of elution, that were obligately required for reconstitution of epoxide carboxylase activity (11). Component II was purified to homogeneity on the basis of its ability to complement components I and III in restoring epoxide carboxylase activity (11). Similar strategies have been applied to component I and III fractions with the goal of purifying each of the required components of the epoxide carboxylase system.

No epoxide carboxylase activity could be recovered in any single fraction after further fractionation of component I by Q-Sepharose anion-exchange chromatography, suggesting that component I is either unstable, has a dissociable cofactor, or separates into more than one component upon further fractionation. In contrast, reasonable recoveries of activity could be obtained upon further chromatographic fractionation of DEAE-Sepharose-resolved component III. Therefore, the purification of component III was pursued on the basis of its ability to complement DEAE-Sepharose-resolved component I and purified component II in restoring epoxide carboxylase activity.

Component III was purified using an activity assay in which DEAE-Sepharose-resolved component I and purified component II were present at saturating levels so that component III was rate-limiting in the assays. A summary of the three-step protocol used for the purification of component III is presented in Table I. Component III was purified 96.4-fold with a 33.6% overall recovery and exhibited a specific activity of 241 milliunits per mg of protein. As shown in Fig. 1, the purification resulted in the enrichment of two proteins that migrated on SDS-PAGE with apparent molecular weights of 29,100 and 30,200. Purified component III was chromatographed over additional columns (e.g. S-100 gel filtration, hydroxyapatite, Reactive Blue) with no change in the relative intensities of the two bands. The inclusion of protease inhibitors (phenylmethylsulfonyl fluoride, leupeptin, and benzamidine) in the lysis buffers and chromatography buffers had no effect on the doublet pattern seen for component III on denaturing gels.

The staining intensities of the two polypeptides on SDS-PAGE gels gave relative ratios of 1.5 to 1.0 for the larger and smaller molecular weight bands, respectively. Mass spectrometric analysis also revealed the presence of two polypeptides in component III preparations and provided more accurate molecular mass estimates of 26,124 Da and 26,025 Da for these proteins. Gel filtration chromatography provided a molecular weight estimate of 64,774 for the native protein, indicating that component III is a dimer composed of two subunits. No metals or organic cofactors were detected in component III preparations.
Identification of a Fourth Epoxide Carboxylase Component—As mentioned above, further fractionation of DEAE-Sepharose-resolved component I by a Q-Sepharose chromatography salt gradient resulted in loss of component I activity. To determine whether two or more additional components had been resolved by this fractionation, various combinations of fractions from the Q-Sepharose step were recombined with purified components II and III and assayed for epoxide carboxylase activity. As shown in Table II, the simultaneous, but not separate, addition of two Q-Sepharose fractions restored epoxide carboxylase activity. These fractions were designated fractions IA and IB for the earlier and later eluting components, respectively.

Methyleneoxypropane has been shown to be a time-dependent, irreversible inactivator of epoxide carboxylase activity that was proposed to act as a mechanism based inactivator of the enzyme (11). The addition of DEAE-resolved component I, but not that of DEAE-resolved components II or III, to methylepoxypropane-inactivated cell extracts restored epoxide carboxylase activity (11). Fractions IA or IB from above were added to methylepoxypropane-treated cell-free extract to determine whether one or both of these fractions could restore epoxide carboxylase activity in a fashion similar to DEAE-resolved component I. The addition of fraction IB alone, but not that of fraction IA, restored activity in methylepoxypropane-treated cell-free extract from propylene-grown cells (35). Assays contained 0.11 mg of fraction IA.

FIG. 2. SDS-PAGE analysis of epoxide carboxylase component I. Lane 1, molecular weight standards (2 μg of each); lane 2, cell-free extract from propylene-grown cells (35 μg); lane 3, heat-treated fraction (20 μg); lane 4, Q-Sepharose fraction (10 μg); lane 5, phenyl-Sepharose fraction (5 μg); lane 6, Sephacryl S-300 fraction (5 μg).

TABLE II
Separation of epoxide carboxylase DEAE-resolved component I into fractions IA and IB by Q-Sepharose chromatography

| Fraction(s) | Epoxidepropane degradation<sup>a</sup> | Added to purified components IA and IB | Added to methylepoxypropane-treated cell-free extract |
|-------------|--------------------------------------|--------------------------------------|-----------------------------------------------------|
| No addition | 0                                    | 0                                    | 0                                                   |
| DEAE-component I<sup>b</sup> | 13.6 | 0 | 9.7 |
| IA<sup>c</sup> | 0 | 0 | 10.3 |
| IB<sup>d</sup> | 0 | 13.9 | 13.6 |

<sup>a</sup> In addition to the indicated fractions, assays contained either 1.2 mg of purified component II plus 0.2 mg of purified component III or 8.6 mg of methylepoxypropane-treated cell-free extract.

<sup>b</sup> Assays contained 4.6 mg of DEAE-resolved component I.

<sup>c</sup> Assays contained 0.11 mg of fraction IA.

<sup>d</sup> Assays contained 0.25 mg of fraction IB.

<sup>e</sup> Assays contained 0.11 mg of fraction IA plus 0.25 mg of fraction IB.

Purification of Epoxide Carboxylase Component IV—Component IV was purified to homogeneity on the basis of its ability to complement purified components I, II, and III in restoring epoxide carboxylase activity. A summary of the four-step protocol is presented in Table IV. Component IV was purified 90.3-fold with an overall recovery of 16% and a specific activity of 3,684 milliunits per mg of purified component IV. The purification resulted in the enrichment of a single polypeptide with an apparent molecular mass of 28.4 kDa on SDS-PAGE (Fig. 3). Mass spectrometry provided a more accurate molecular mass estimate of 25.4 kDa for purified component IV. Gel filtration chromatography provided a molecular mass estimate of 49.9 kDa for the native protein, indicating that component IV is a homodimer. No metals or organic cofactors were detected in component IV preparations.

Analysis of N-terminal Sequences and Amino Acid Compositions of Purified Epoxide Carboxylase Components and Their Relation to Sequenced Xanthobacter DNA—Previously, Swav-}

Analysis of N-terminal Sequences and Amino Acid Compositions of Purified Epoxide Carboxylase Components and Their Relation to Sequenced Xanthobacter DNA—Previously, Swav-
TABLE IV

| Purification step          | Total protein | Volume | Total activity* | Specific activity | Purification factor | Recovery |
|----------------------------|---------------|--------|----------------|------------------|---------------------|----------|
| Cell-free extract          | 7,014         | 227    | 296,020        | 40.8             | 1                   | 100      |
| Heat treatment             | 2,606         | 200    | 244,060        | 93.6             | 2.3                 | 85       |
| Q-Sepharose-hydroxyapatite| 385           | 120    | 134,400        | 368              | 9.0                 | 47       |
| Phenyl-Sepharose           | 55.5          | 150    | 81,000         | 1,459            | 35.8                | 28       |
| Reactive Green             | 12.5          | 50     | 46,050         | 3,684            | 90.3                | 16       |

* Assays contained 0.75 mg of component I, 1.24 mg of purified component II, 0.10 mg of purified component III, and a limiting amount of the component IV fraction. One unit of activity is defined as 1 μmol of epoxypropane degraded per min.

FIG. 3. SDS-PAGE analysis of epoxide carboxylase component IV. Lane 1, molecular weight standards (2 μg each); lane 2, cell-free extract from propylene-grown cells (50 μg); lane 3, heat-treated fraction (20 μg); lane 4, Q-Sepharose-hydroxyapatite fraction (15 μg); lane 5, phenyl-Sepharose fraction (5 μg); lane 6, Reactive Green fraction (1.5 μg).

The two additional ORFs in the sequenced DNA (orf6 and orf7) would encode for polypeptides of 26,011 and 16,742 molecular weights, respectively. Clearly, orf7 has no relation to the four-epoxide carboxylase components. However, the similarity in molecular weight of orf6 to those of components III and IV warranted its further analysis. The N-terminal sequence of orf6 was DRPGAPPLHA, which has no similarity to the N-terminal sequences of either component III or IV (Table V). In addition, the deduced amino acid analysis of orf6 is substantially different than that of components III and IV (Table VI). These results indicate that orf6 has no relation to either component III or IV.

The concentration of component IV was varied. Significantly, no component (III or IV) was varied in individual assays. As shown in Table V, does not match that of orf4 (Table V). After accessing the sequence of the complementary DNA fragment as deposited by Swaging and coworkers (16) in the European Molecular Biology Library (EMBL accession number X79863), we found that an additional 2.2 kb of DNA beyond the initial 4.8 kb reported in the paper had been sequenced. The additional 2.2 kb of DNA contains three additional ORFs (orf5-7). orf5 encodes for a polypeptide with molecular mass of 24,941 Da which has extremely high sequence homology (41% identity) to that of orf4. The N-terminal sequence of orf5 matches closely to the experimentally determined N-terminal sequence of component 4 (Table V). In addition, the experimentally determined amino acid analysis of component 4 matches closely to the deduced amino acid analysis of orf5 (Table VI). These results strongly indicate that component 4 is the product of the orf5 gene.

Obligate Requirement of Components III and IV for Reconstitution of Epoxide Carboxylase Activity—The data presented above demonstrate that components III and IV are the products of two genes that are highly homologous. This raises the question of why two polypeptides with similar molecular weights and amino sequences are both required by the epoxide carboxylase system. To verify the essential nature of both components in epoxide carboxylation, activity assays were performed in which components I, II, and either III or IV were held at fixed concentrations, whereas the concentration of the other component (III or IV) was varied in individual assays. As shown in Table V, at fixed concentrations of components I, II, and IV, the rate of epoxypropane carboxylation was dependent on the amount of component III added to the assay. The rate saturated within the range of component III concentrations included in the assays. Significant rate dependencies were observed when components I, II, and III were held constant, and the concentration of component IV was varied.
Roles of Reductant and NAD\(^+\) in Epoxide Carboxylation—
The in vitro carboxylation of epoxides was previously shown to require a reductant (DTT or NADPH) and NAD\(^+\) (4, 9). These same cofactors are required for reconstituting epoxide carboxylase activity using the purified protein components. NADPH has been proposed to serve as the physiological reductant for the system based on the characterization of component II as a specific NADPH:disulfide oxidoreductase (12). It is important to note that there is no net redox chemistry involved in the carboxylation of aliphatic epoxides to \(\beta\)-keto acids. Presumably, NADPH (or DTT) is oxidized in the course of epoxide carboxylation, and NAD\(^+\) is concomitantly reduced, although this possibility, as well as the entire reaction stoichiometry, has not been verified experimentally. To resolve these points, the concentrations of substrates and products were determined in epoxide carboxylase degradation assays using the purified epoxide carboxylase components and NADPH and NAD\(^+\) as cofactors. As shown in Fig. 5, an exact 1:1:1:1 stoichiometry was observed at each time point for the consumption of epoxypropane and NADPH and production of acetoacetate and NADH. This stoichiometry demonstrates definitively that epoxide carboxylation is coupled to the transhydrogenation of the pyridine nucleotide cofactors as shown in Equation 4.

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\text{epoxypropane + CO}_2 + \text{NADPH} + \text{NAD}^+ \rightarrow \text{acetoacetate} + \text{H}^+ + \text{NADP}^+ + \text{NADH} \quad (\text{Eq. 4})
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The roles of oxidant and reductant in epoxide carboxylation were further investigated by developing an assay where the production of NADH could be monitored in real time by following the resulting increase in absorbance at 340 nm. For this assay it was necessary to use DTT as the reductant, since no net change in \(A_{340}\) will be seen if NADPH and NAD\(^+\) are used (the reduced forms of both cofactors have the same extinction coefficient at 340 nm). With all four epoxide carboxylase components present, a low rate of DTT-dependent NADH formation was observed that increased approximately 9-fold upon addition of epoxypropane (Fig. 6, trace I). The low rate of epoxidepropane-independent NADH formation was dependent upon the addition of component II and independent of the other epoxide carboxylase components. This result is consistent with the assignment of component II as an oxidoreductase and the previous demonstration that component II exhibits diaphorase activity (12). As shown in Fig. 6, the single exclusion of either component I, III, or IV from the assay mixture decreased the rate of epoxidepropane-dependent NADH formation to the rate observed with all four components but in the absence of epoxypropane. When component II was excluded, the rate of NADH formation decreased to nearly undetectable levels (Fig. 6, trace I).

### Summary of Biochemical Properties of Epoxide Carboxylase Components

The biochemical properties of the four purified epoxide carboxylase components were summarized in Table V. As part of the analyses, specific activities were recalculated for components I, II, and III using purified sources of the other components in the assay (component IV specific activity was already calculated on this basis). The specific activities of components I and II increased approximately 2-fold by using this optimized assay system, whereas the specific activity for component III increased nearly 9-fold (Table V).

### Roles of Reductant and NAD\(^+\) in Epoxide Carboxylation—

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DISCUSSION

The results of the present work demonstrate that epoxide carboxylase activity can be reconstituted by the simultaneous presence of four distinct proteins resolved from cell-free extracts of *Xanthobacter* strain Py2. These results verify the multicomponent nature of epoxide carboxylase suggested by the results of recent studies (10–12) and demonstrate that activity can be obtained using purified sources of the epoxide carboxylase component proteins. The four proteins required for activity are as follows: component I, a homohexameric protein consisting of 41.7-kDa subunits; component II, a dimeric FAD-containing protein consisting of 57-kDa subunits; component III, a dimeric protein consisting of 26.0- and 26.1-kDa polypeptides; and component IV, a dimeric protein consisting of a single 25.4-kDa polypeptide. As observed for cell-free extracts, epoxide carboxylase activity using the purified protein components is dependent upon the addition of NADH and a reductant (NADPH or DTT).

Components III and IV, the two additional proteins required for epoxide carboxylation, are remarkably similar in terms of molecular weight and amino acid composition (Tables V and VI). These proteins appear to be the products of two highly homologous genes (orf4 and orf5). Only a portion of orf5 is located...
within the 4.8-kb fragment of DNA that complemented Xanthobacter mutants incapable of degrading epoxides (16). These results would suggest that a wild type copy of orf5 was still present in the mutant strains that could be complemented. Notably, not all of the epoxide degradation minus mutants could be complemented with the 4.8-kb fragment of DNA; possibly, these mutants had mutations in orf5 as well (16).

Despite the remarkable homology of components III and IV, they are both obligately required for reconstitution of epoxide carboxylase activity (Fig. 4). Even very high concentrations of either component III or IV were unable to compensate for the lack of the other component in epoxide carboxylase assays. Apparently, there is some difference in the molecular properties of the two components that allow them to assume distinct roles in epoxide carboxylation. In this context, there are a number of differences in the biochemical properties of components III and IV that should be noted. One of these is the different banding patterns of the two proteins on SDS-PAGE. Component III migrates as a doublet at a higher than expected apparent molecular mass value of approximately 30 kDa on SDS-PAGE. In contrast, component IV migrates as a single band at an apparent molecular mass of 26 kDa, which is much closer to the true molecular masses of components III and IV determined by mass spectrometry (Table V). Possibly, either or both components have undergone some form of posttranslational modification (e.g., C-terminal processing, phosphorylation, methylation, acetylation, glycosylation, covalent addition of a cofactor, etc.) that gives rise to the distinguishing banding patterns on SDS-PAGE. Such modification might also activate the components for their respective functions in epoxide carboxylation.

The remaining ORF (orf2) of the complementary DNA fragment encodes a polypeptide of 7.4 kDa molecular mass (16). The position of orf2 among the four ORFs required for epoxide carboxylation would suggest that the Orf2 protein may play some role in epoxide metabolism. At present, we have not observed detectable stimulation of epoxide carboxylase activity by the addition of any side fractions to the purified components nor have we isolated a side fraction containing a polypeptide that may be the Orf2 protein. If orf2 is involved in some aspect of epoxide metabolism, it is not obligately required for the carboxylation reaction, since the combination of components I–IV alone reconstitutes activity with the required cofactors.

The discovery that epoxide carboxylase is a multicomponent enzyme raises the important questions of what role(s) the individual components play in catalysis and how the transhydrogenation of pyridine nucleotides is coupled to epoxide carboxylation. Based on studies of epoxide isomerization (the reaction catalyzed by epoxide carboxylase in the absence of CO2) in cell-free extracts, Weijers and coworkers (9) proposed a hypothetical catalytic mechanism for the enzyme. This mechanism proposes that a sulfhydryl (e.g., cysteine residue) at the enzyme active site is a nucleophile that attacks the C-1 carbon atom of the epoxide substrate to form an enzyme-bound β-hydroxythioether intermediate. The C-2 hydrogen atom of the bound substrate is then proposed to undergo abstraction as a hydride and transfer to NAD+ resulting in the oxidation of the β-hydroxythioether to a β-ketothioether. The next proposed step is a heterolytic cleavage of the C=S covalent bond of the substrate-enzyme complex which is promoted by formation of a disulfide bond between the thioether sulfur and a second sulf-hydryl group. This step would result in the formation of the ketone carbanion which could alternatively be protonated to form the corresponding ketone or carboxylated to form the corresponding β-keto acid (11). Finally, the disulfide formed in the next to last step would be re-reduced before the next round of catalysis.

A logical role for component II in this hypothetical mechanism would be to catalyze the reduction of the active site disulfide since component II has been shown to possess NADP:disulfide oxidoreductase activity (12). It is unclear whether the active site disulfide resides on component II or another of the epoxide carboxylase components. An argument for the latter possibility stems from our identification of methylepoxypropane as a time-dependent, irreversible inactivator of epoxide carboxylase activity (11). Methylepoxypropane differs from epoxypropane in containing a methyl rather than hydrogen substituent on the C-2 carbon. The lack of an abstractable hydride at the C-2 carbon would trap the substrate on the enzyme at the level of the β-hydroxythioether covalent intermediate. As shown previously for DEAE-resolved fractions (11), and in Table II for component I-and IV-resolved fractions, methylepoxypropane specifically and irreversibly inactivates component I. This suggests that component I may contain the epoxide binding and activation site(s). Interestingly, component I contains approximately one tightly bound zinc per monomeric unit (Table V). This zinc could conceivably act as a Lewis acid in the stabilization of the hypothetical β-hydroxythioether intermediate or perhaps in the activation of CO2.

Currently, it is unclear what roles components III and IV may play in catalysis. Possibly, these proteins are involved in the reduction of NAD+ or formation and stabilization of the required protein-protein complexes. orf4 and orf5, the genes which apparently encode components III and IV, show strong sequence similarity to a number of 3-ketoacyl reductases, including acetocacetyl-CoA reductases from several sources (17). This similarity is interesting since 3-keto acids are the products of epoxysalane carboxylation. However, it is unclear how this sequence similarity might relate to enzymatic or other roles for components III and IV in epoxide carboxylation.

In summary, this paper provides the first reported reconstitution of epoxide carboxylase activity using a purified enzyme system. The physiological role of epoxide carboxylase is to convert epoxides formed during alkene metabolism to β-ketoacids which undergo further metabolic transformations by well-characterized biochemical pathways (5). The properties of purified epoxide carboxylase suggest a novel catalytic mechanism unprecedented among epoxide- and CO2-activating enzymes. Further experimentation is necessary to elucidate the mechanistic details of this multicomponent enzyme and the roles the individual components play in catalysis.

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