Two Short-Chain Dehydrogenases Confer Stereoselectivity for Enantiomers of Epoxypropane in the Multiprotein Epoxide Carboxylating Systems of *Xanthobacter* Strain Py2 and *Nocardia corallina* B276[†]

Jeffrey R. Allen and Scott A. Ensign*

Department of Chemistry and Biochemistry, Utah State University, Logan, Utah 84322-0300

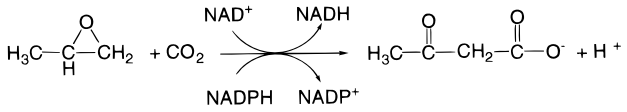
Received August 31, 1998; Revised Manuscript Received October 27, 1998

ABSTRACT: Epoxide carboxylase from the bacterium *Xanthobacter* strain Py2 is a multicomponent enzyme system which catalyzes the pyridine nucleotide-dependent carboxylation of aliphatic epoxides to β-ketoacids as illustrated by the reaction epoxypropane + CO_2 + NADPH + NAD^+ → acetoacetate + H^+ + $NADP^+$ + NADH. The combination of four distinct proteins, designated components I−IV, are required for the reconstitution of epoxide carboxylase activity with racemic mixtures of short-chain (C3−C5) terminal epoxyalkanes. In this work, components III and IV of the epoxide carboxylase system are shown to confer specificity for epoxyalkane enantiomers. Components I−III supported the carboxylation of (*R*)-epoxypropane, while components I, II, and IV supported the carboxylation of (*S*)-epoxypropane. At fixed concentrations of components I and II, the rates of (*R*)- and (*S*)-epoxypropane carboxylation saturated with increasing concentrations of component III or IV to give identical maximal rates for the two epoxide substrates. (*S*)-Epoxypropane was an inactivator of (*R*)-epoxypropane carboxylation by components I−III, while (*R*)-epoxypropane was an inactivator of (*S*)-epoxypropane carboxylation by components I, II, and IV. These inactivating effects were fully reversed upon the addition of the correct complementing dehydrogenase component. Amino acid sequence analysis of components III and IV demonstrates that they belong to the short-chain dehydrogenase/reductase (SDR) family of enzymes. Both components contain highly conserved residues within the coenzyme binding fold and catalytic regions found in SDR enzymes. Components III and IV are proposed to catalyze the NAD^+-dependent abstraction of a hydride from a chiral secondary alcohol-like intermediate bound to the active site component of the enzyme system to form the corresponding β-ketone intermediate. A multicomponent epoxide carboxylase system was purified to homogeneity from *Nocardia corallina* B276, a bacterium phylogenetically unrelated to *Xanthobacter* Py2, and found to consist of four proteins with functions identical to those of the *Xanthobacter* Py2 system. The stereoselective dehydrogenases of the *Xanthobacter* epoxide carboxylase system were able to substitute for the corresponding components of the *N. corallina* system when using (*R*)- and (*S*)-epoxypropane as substrates, and vice versa. These results provide the first demonstration of the involvement of stereospecific dehydrogenases in aliphatic epoxide metabolism and provide new insights into microbial strategies for the utilization of chiral organic molecules.

Enantiopure epoxides are highly reactive compounds that serve as valuable intermediates in the synthesis of more complex optically active or biologically active compounds, including pharmaceuticals and agrichemicals (*1*, *2*). For this reason, there is considerable interest in developing biological systems for producing enantiopure epoxides by exploiting the stereoselective and regioselective properties of epoxide-generating or epoxide-utilizing enzymes (for recent reviews, see refs *3*−*7*). These include direct stereospecific epoxidation of alkenes by alkene-oxidizing enzymes such as heme-dependent monooxygenases, ω-hydroxylases, and non-heme oxygenases (*3*). Alternatively, enantiopure epoxides can be resolved from racemic mixtures by using enzymes that degrade epoxides enantioselectively. A number of epoxide hydrolases from bacterial and fungal sources have been studied for this purpose (*3*, *4*). Although their use has been limited, biological strategies for producing enantiopure epoxides potentially offer higher enantiomeric excess (e.e.) yields at lower costs than using purely chemical methodologies.

One class of bacteria of potential interest in chiral epoxide synthesis are those that grow using aliphatic alkenes as sources of carbon and energy. The pathways of aliphatic alkene metabolism have been studied for two propylene-oxidizing bacteria, *Xanthobacter* strain Py2 and *Nocardia corallina* strain B276, and been shown to proceed via alkene epoxidation followed by epoxide carboxylation as shown in Scheme 1:

Scheme 1

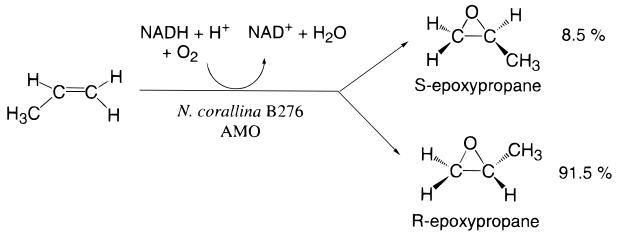


[†] This work was supported by National Institutes of Health Grant GM51805.

* Corresponding author. Phone: (435) 797-3969. Fax: (435) 797-3390. E-mail: ensigns@cc.usu.edu.

10.1021/bi982114h CCC: $18.00
Published on Web 12/05/1998

Scheme 2



The alkene monooxygenases from both *Xanthobacter* Py2 and *N. corallina* have been purified and shown to be multicomponent diiron enzymes (*8*, *9*). Studies of purified AMO[1] from *N. corallina* (*10*), and studies using whole cell suspensions of *Xanthobacter* Py2 expressing AMO (*11*), indicate that both enzymes form one enantiomer of the epoxide product in excess of the other. For example, purified AMO from *N. corallina* oxidized propylene to form 91.5% (*R*)-epoxypropane and 8.5% (*S*)-epoxypropane (Scheme 2) (*10*).

Preliminary studies of purified AMO from *Xanthobacter* Py2 indicate a similar degree of stereoselectivity for epoxypropane formation (F. J. Small and S. A. Ensign, unpublished results). To date, no studies on the stereospecificity of the subsequent epoxide carboxylation reaction have been reported. This reaction represents the most recently characterized type of epoxide conversion and, in *Xanthobacter* Py2, is catalyzed by a novel four-component epoxide carboxylase system that requires cofactors (NADPH and NAD^+) not utilized by any other type of carboxylase (*12*). Epoxypropane carboxylation requires epoxide ring opening, hydrogen atom (or hydride) extraction from the C2 carbon with formation of a keto group, and addition of CO_2 to a terminal carbon atom (*13*−*15*). By some means, epoxide carboxylation is coupled to the transhydrogenation of pyridine nucleotides (Scheme 1). The biochemical properties and amino acid sequences of the four epoxide carboxylase components (designated components I−IV), together with initial mechanistic studies, have suggested possible roles for the individual components in catalysis and allowed the formulation of a plausible catalytic cycle for the overall reaction. Component I is a homohexameric (42 kDa subunit) zinc-containing protein that is believed to contain the epoxide binding and activation site(s) (*12*). Component II is a homodimeric (57 kDa subunit) flavoprotein with NADPH: disulfide oxidoreductase activity that is believed to reduce a disulfide on component I, generating a reduced thiol serving as a nucleophile for attacking and opening the epoxide ring (*16*). Components III and IV are small (26 and 25.4 kDa subunits, respectively) homodimeric proteins with no detectable organic or inorganic cofactors (*12*). The sequences of components III and IV are homologous to NAD^+-dependent dehydrogenases, suggesting that they may be involved in NAD^+ reduction and hydride abstraction from the epoxide substrate (*12*). Initial studies of the epoxide carboxylase system of *N. corallina* have shown that it is a multicomponent system also requiring NAD^+ and NADPH as cofactors (*17*).

To shed further light on the mechanism of biological epoxide carboxylation, and to investigate the possible stereoselectivity of this reaction, the utilization of *R*- and *S*-enantiomers of epoxypropane by the epoxide carboxylase system of *Xanthobacter* Py2 has been investigated in this work. Components I and II are shown to be required for the utilization of both enantiomers of epoxypropane, while components III and IV confer specificity for utilization of the *R*- and *S*-enantiomers, respectively. On the basis of these observations, and sequence analyses of components III and IV, these components are proposed to belong to the family of short-chain dehydrogenases and to impart specificity for abstraction of C2 hydrides from chiral secondary alcohol intermediates formed in the reaction cycle. Finally, the epoxide carboxylase system has been purified to homogeneity from *N. corallina* and shown to consist of four protein components with functions identical to those of the *Xanthobacter* Py2 system. These results provide the first evidence for the involvement of stereoselective dehydrogenases in epoxide metabolism and provide insights into biological strategies for the utilization of these important chiral compounds.

EXPERIMENTAL PROCEDURES

Materials. (*R*)-(+)-1,2-Epoxypropane, (*S*)-(−)-1,2-epoxypropane, (*R*)-(−)-epichlorohydrin, and (*S*)-(+)-epichlorohydrin were purchased from Aldrich Chemical Co. All other chemicals used were analytical grade.

Growth of Bacteria and Preparation of Cell-Free Extracts. *N. corallina* was grown in 15 L semicontinuous cultures in a Microferm fermentor (New Brunswick Scientific) with propylene as a carbon source as described previously (*14*, *17*). Cells were harvested when A_{600} was between 2.5 and 4.0 by tangential-flow filtration with a Pellicon system (Millipore Corp.) and stored at −80 °C. Frozen cell paste (100−200 g) was thawed and resuspended in 2 volumes of buffer [50 mM Tris-HCl (pH 8.2)] containing 1 mM DTT, 10% (v/v) glycerol, DNAse I (0.2 mg/mL), and lysozyme (0.3 mg/mL). The cell suspension was passed three times through a French pressure cell at 110 000 kPa, and the lysate was clarified by centrifugation at 137000*g* for 30 min at 4 °C. *Xanthobacter* strain Py2 was grown with propylene as the carbon source, and cell-free extracts were prepared as previously described (*14*). Tris-HCl buffer used in the preparation of cell-free extracts and in column chromatography procedures was first passed over a Bio-Rad Chelex-100 (5.0 cm × 10 cm) column to remove trace metals.

Separation of N. corallina Epoxide Carboxylase Components. The epoxide carboxylase components from *N. corallina* B276, the purifications of which are described below, were designated NcI−NcIV to correlate their activity to the respective epoxide carboxylase component from *Xanthobacter* strain Py2. For clarity, epoxide carboxylase components I−IV from *Xanthobacter* strain Py2 were abbreviated to XpI−XpIV, respectively. The clarified *N. corallina* cell-free extract was applied to a DEAE-Sepharose Fast Flow

[1] Abbreviations: AMO, alkene monooxygenase; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; DNAse I, deoxyribonuclease I; NcI−NcIV, epoxide carboxylase components I−IV, respectively, from *N. corallina* B276; XpI−XpIV, epoxide carboxylase components I−IV, respectively, from *Xanthobacter* strain Py2; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SDR, short-chain dehydrogenase/reductase; ADH, alcohol dehydrogenase; TR-I, tropinone reductase I; TR-II, tropinone reductase II.

column (5.0 cm × 20 cm) equilibrated in 50 mM Tris-HCl (pH 8.2) containing 10% (v/v) glycerol (buffer A) at a linear flow rate of 24 cm/h. After loading, the column was washed with 500 mL of buffer A containing 100 mM NaCl. The column was further developed with a 2.4 L linear gradient of 100 to 400 mM NaCl in buffer A. Each epoxide carboxylase component had the following elution profile. NcI was localized in fractions eluting between 300 and 340 mM NaCl. NcII was localized in fractions eluting between 260 and 310 mM NaCl. NcIII was localized in fractions eluting between 200 and 230 mM NaCl. NcIV was localized in fractions eluting between 225 and 250 mM NaCl. Fractions containing the desired component were pooled and stored at −80 °C.

Purification of NcI. The DEAE-Sepharose NcI pool was thawed, adjusted to 1.0 M $(NH_4)_2SO_4$, and applied to a Pharmacia HiLoad 26/15 Phenyl-Sepharose column equilibrated in buffer A containing 1.0 M $(NH_4)_2SO_4$ at a linear flow rate of 45 cm/h. The column was washed with 150 mL of 1.0 M $(NH_4)_2SO_4$ in buffer A and developed with a 480 mL reverse gradient from 1.0 M to 0 mM $(NH_4)_2SO_4$ at a linear flow rate of 45 cm/h. Fractions with component I activity were pooled and concentrated by ultrafiltration (YM30) to a volume of approximately 4 mL. The sample was applied to a Pharmacia HiPrep 26/100 Sephacryl S-300 column equilibrated in buffer A containing 200 mM NaCl at a linear flow rate of 11 cm/h. Fractions containing component I activity were pooled, diluted 4-fold with buffer A, and applied to a Pharmacia HiLoad 26/10 Q-Sepharose column equilibrated in buffer A at a linear flow rate of 57 cm/h. The column was washed with 100 mL of buffer A containing 250 mM NaCl and developed by applying a 480 mL linear gradient from 250 to 450 mM NaCl at a linear flow rate of 45 cm/h. Active fractions were pooled and dialyzed against 1 L of buffer A, concentrated by ultrafiltration (YM30), and frozen in liquid nitrogen.

Purification of NcII. The DEAE-Sepharose NcII pool was thawed and adjusted to 1.0 M $(NH_4)_2SO_4$, and applied to a Pharmacia HiLoad 26/15 Phenyl-Sepharose column equilibrated in buffer A containing 1.0 M $(NH_4)_2SO_4$ at a linear flow rate of 45 cm/h. The column was washed with 150 mL of 0.8 M $(NH_4)_2SO_4$ in buffer A and developed with a 400 mL reverse gradient from 0.8 M to 0 mM $(NH_4)_2SO_4$ at a linear flow rate of 45 cm/h. Fractions with component II activity were pooled and concentrated by ultrafiltration (YM30) to a volume of approximately 5 mL. The sample was applied to a Pharmacia HiPrep 26/100 Sephacryl S-300 column equilibrated in buffer A containing 200 mM NaCl at a linear flow rate of 11 cm/h. Fractions containing component II activity were pooled, diluted 4-fold with buffer A, and applied to a Pharmacia HiLoad 26/10 Q-Sepharose column equilibrated in buffer A at a linear flow rate of 57 cm/h. The column was washed with 100 mL of buffer A containing 250 mM NaCl and developed by applying a 480 mL linear gradient from 250 to 350 mM NaCl at a linear flow rate of 45 cm/h. Fractions containing component II activity were dialyzed against 1 L of buffer A, concentrated by ultrafiltration (YM30), and frozen in liquid nitrogen.

Purification of NcIII. The DEAE-Sepharose pool containing component III activity was thawed and adjusted to 1.0 M $(NH_4)_2SO_4$, and applied to a Pharmacia HiLoad 16/10 Phenyl-Sepharose column equilibrated in buffer A containing 1.0 M $(NH_4)_2SO_4$ at a linear flow rate of 90 cm/h. The column was washed with 50 mL of 1.0 M $(NH_4)_2SO_4$ in buffer A and developed with a 240 mL reverse gradient from 1.0 M to 0 mM $(NH_4)_2SO_4$ at a linear flow rate of 60 cm/h. Fractions containing component III activity were pooled and dialyzed against 1 L of 25 mM MES (pH 6.2) containing 10% glycerol (buffer B) for 12 h at 4 °C. The protein was applied to a Dyematrex Red A (Amicon) column (1.5 cm × 10 cm) equilibrated in buffer B at a linear flow rate of 60 cm/h. After the column was washed with 50 mL of buffer B, the column was developed by applying a 200 mL linear gradient of buffer B to buffer A containing 1 M NaCl. Fractions containing component III activity were pooled and applied to a ceramic hydroxyapatite (Bio-Rad) column (1.6 cm × 15 cm) equilibrated in buffer A at a flow rate of 90 cm/h. After the column was washed with 60 mL of buffer A, component III was eluted by applying a 100 mL linear gradient of buffer A to 120 mM potassium phosphate buffer (pH 7.0) at a flow rate of 60 cm/h. Active fractions were pooled and dialyzed against 1 L of buffer A, concentrated by a Centricon 30 (Amicon), and frozen in liquid nitrogen.

Purification of NcIV. The DEAE-Sepharose NcIV pool was thawed and applied to a ceramic hydroxyapatite column (2.5 cm × 10 cm) equilibrated in buffer A containing 200 mM NaCl at a flow rate of 49 cm/h. The column was washed with 50 mL of buffer A containing 200 mM NaCl and developed by applying a 120 mL reverse gradient of buffer A containing 200 to 0 mM NaCl at a flow rate of 49 cm/h. Fractions containing component IV were pooled, adjusted to 1.0 M $(NH_4)_2SO_4$, and applied to a Pharmacia HiLoad 16/10 Phenyl-Sepharose column equilibrated in buffer A containing 1.0 M $(NH_4)_2SO_4$ at a linear flow rate of 60 cm/h. The column was washed with 50 mL of 1.0 M $(NH_4)_2SO_4$ in buffer A and developed with a 400 mL reverse gradient from 1.0 M to 0 mM $(NH_4)_2SO_4$ at a linear flow rate of 60 cm/h. Active fractions were pooled and dialyzed against 1 L of buffer A, concentrated by a Centricon 30, and frozen in liquid nitrogen.

Assay of Epoxide Carboxylase Activity and Preparation of Purified Epoxide Carboxylase Components from Xanthobacter Strain Py2. Epoxide carboxylase activity was measured by monitoring the time-dependent depletion of epoxypropane by gas chromatography as previously described (*14*). Assays were performed in sealed 9 mL vials containing a source of enzyme (cell-free extract, column fractions, or purified components) in 50 mM Tris-HCl (pH 8.2) containing 10% glycerol in a 1 mL assay volume using reagents and reaction conditions described previously except that NADPH (4 mM) was used as the source of reductant (*14*, *18*). Assays of epoxypropane degradation activity with whole-cell suspensions were performed with shaking at 30 °C in sealed 9 mL serum vials as described previously (*17*, *19*). Purified epoxide carboxylase components I−IV from *Xanthobacter* strain Py2 were prepared as described previously (*12*, *18*).

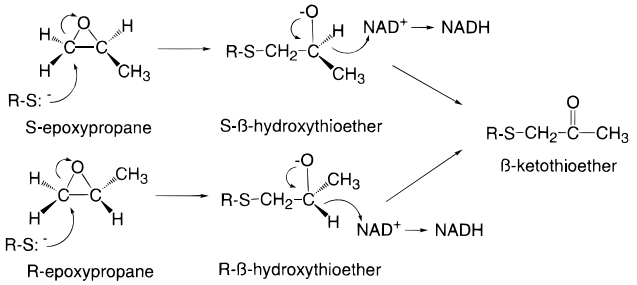
Protein Characterizations. The flavin cofactor was extracted from purified NcII and identified using high-pressure liquid chromatography as previously described (*18*). Native molecular masses were estimated by gel filtration chromatography using a Pharmacia Superose 12 HR 10/30 column equilibrated in 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), carbonic anhydrase (31 kDa), and cytochrome *c* (12.3 kDa). Polypeptide molecular masses were also determined by using matrix-assisted laser desorption ionization time-of-flight 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 (*20*). Electrophoresed proteins were visualized by staining with Coomassie blue. The apparent molecular masses of polypeptides based on the SDS−PAGE migration pattern were determined by comparison with R_f values of standard proteins. 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 (*21*). The protein concentration of XpII was routinely determined by using its reported extinction coefficient (*18*).

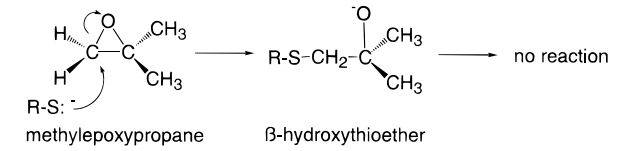
RESULTS

XpIII and XpIV Belong to the Family of Short-Chain Dehydrogenases/Reductases. As shown in Scheme 1, the four-component epoxide carboxylase system of *Xanthobacter* Py2 couples epoxide carboxylation to the transhydrogenation of pyridine nucleotides. Roles for XpI and XpII in this system have been proposed on the basis of their biochemical properties; however, the interplay of all four components and the exact role for each in catalysis remain unclear. XpI is believed to contain the epoxide binding and activation site(s) on the basis of its susceptibility to inactivation by the substrate analogue methylepoxypropane (*12*). XpII, a flavoprotein identified from its primary structure as a NADPH:disulfide oxidoreductase, is proposed to reduce a redox-active disulfide, possibly on XpI, to form a dithiol which serves as a nucleophile for attacking, and ring opening of, the epoxide substrate (*16*). Although they have no definitive roles in catalysis, the polypeptide sequences of both XpIII and XpIV are significantly homologous to each other and to a number of pyridine nucleotide-dependent dehydrogenases (*12*). This suggests possible roles for these two components in the reduction of NAD^+. To investigate this possibility in more detail, the amino acid sequences of XpIII and XpIV were analyzed by comparing their sequences to the primary sequences of other NAD^+-dependent enzymes. It was determined from this analysis that XpIII and XpIV belong to the family of short-chain dehydrogenases/reductases (SDR) originally defined for insect alcohol dehydrogenase, bacterial ribitol dehydrogenase, and bacterial glucose dehydrogenase (see refs *22* and *23*, and references therein). The SDR family is composed of about 60 enzymes that have a number of distinctive features; they are able to utilize a wide variety of substrates, do not use metals as cofactors, are approximately 250 amino acids in length, are active as dimers or tetramers, and use NAD(H) or NADP(H) as a coenzyme (*22*, *23*). Multiple sequence alignment of XpIII and XpIV (sequences available under EMBL accession number X79863) with three representative members of the SDR family (*24*−*26*) demonstrates a significant degree of homology to highly conserved residues among these enzymes. Typical of SDR enzymes, the conserved coenzyme-binding motif GlyXXXGlyXGly (Gly-14, Gly-17, and Gly-19, respectively, in the numbering system of *Drosophila melanogaster* ADH) is located near the N terminus of both XpIII and XpIV, as well as the conserved internal residues that make up the putative Ser-Tyr-Lys catalytic triad (Ser-139, Tyr-152, and Lys-156) (*22*, *23*, *27*, *28*). The substrate binding regions of SDR enzymes, which are located in the C-terminal region, share little sequence homology due to the wide variety of substrates utilized by these enzymes. Likewise, both XpIII and XpIV have little sequence homology to SDR enzymes in this region; however, they do show homology with each other in this same region.

Scheme 3



Scheme 4



XpIII and XpIV Confer Selectivity for Epoxypropane Stereoisomers. The identification of XpIII and XpIV as members of the SDR family raises the question of why two dehydrogenases, rather than one, are required by the epoxide carboxylase system. The answer may lie in the need to impart specificity for the *R*- and *S*-enantiomers of chiral epoxide substrates. The reaction mechanism proposed for epoxide carboxylation involves nucleophilic attack (e.g., cysteinyl thiol) for forming a β-hydroxythioether intermediate bound to XpI (*12*, *13*, *15*). This proposed step would be followed by oxidation of the intermediate by abstraction of the hydride from C2 and its transfer to NAD^+ to form a β-ketothioether. The β-hydroxythioether intermediate formed during the reaction resembles a secondary alcohol where C2 resides in either the *R*- or *S*-configuration (Scheme 3). Conceivably, as is the case for other NAD^+-dependent dehydrogenases (*29*, *30*), two stereospecific dehydrogenases would be needed to catalyze the oxidation of both stereoisomers formed at this step to subsequently produce the common keto intermediate (Scheme 3). This reaction scheme is consistent with the observation that methylepoxypropane is a time-dependent inactivator of epoxide carboxylase activity, since the β-hydroxythioether adduct formed with this compound cannot not react further to the β-ketothioether intermediate (Scheme 4).

Epoxypropane degradation assays were performed with the *R*- and *S*-enantiomers of epoxypropane, as well as with the racemic mixture of epoxypropane isomers used in all previous studies. As shown in Figure 1A, when either XpIII or XpIV was excluded from the assay mixture, no degradation of racemic epoxypropane was observed. However, as

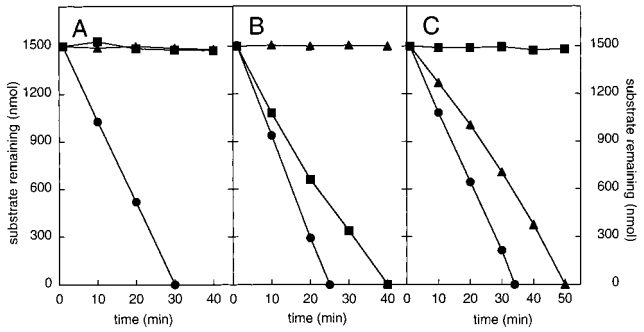


FIGURE 1: Rate of enantioselective carboxylation of epoxypropane determined by XpIII and XpIV. The concentration of XpI was 1.5 mg/mL, that of XpII 1.2 mg/mL, that of XpIII 0.16 mg/mL, and that of XpIV 0.15 mg/mL: (●) assays containing all four components, (■) assays containing XpI−XpIII, and (▲) assays containing XpI, XpII, and XpIV. (A) Assays with racemic epoxypropane as the substrate. (B) Assays with (*R*)-epoxypropane as the substrate. (C) Assays with (*S*)-epoxypropane as the substrate.

shown in panels B and C of Figure 1, the combination of XpI−XpIII alone, and the combination of XpI, XpII, and XpIV alone, were able to degrade (*R*)-epoxypropane and (*S*)-epoxypropane, respectively. These results suggest that XpIII imparts specificity for the *R*-isomer of epoxypropane while XpIV imparts specificity for the *S*-isomer. To verify these results with another chiral substrate, assays were performed as described above using (*R*)- and (*S*)-epichlorohydrin (1-chloro-2,3-epoxypropane) [epichlorohydrin has previously been shown to be a substrate for epoxide carboxylase in whole-cell studies (*31*)]. Here it was found that XpI−XpIII alone allowed (*S*)-epichlorohydrin degradation and XpI, XpII, and XpIV allowed (*R*)-epichlorohydrin degradation (data not shown). The *R*- and *S*-enantiomers of epichlorohydrin have the same steric configurations as (*S*)- and (*R*)-epoxypropane, respectively, with the difference in the nomenclature used for each enantiomer due to the priority sequence used for the atoms arranged around the chiral C2.

To further investigate the stereospecificity imparted by XpIII and XpIV, activity assays were performed in which XpI and XpII were held at fixed concentrations while the concentration of XpIII or XpIV was varied in individual assays using (*R*)- or (*S*)-epoxypropane as the substrate. As shown in Figure 2, at fixed concentrations of XpI and XpII, the rates of (*R*)- and (*S*)-epoxypropane carboxylation were dependent on the amounts of XpIII and XpIV added to the assay. The maximal rates of (*R*)- and (*S*)-epoxypropane degradation were nearly identical when extrapolated to saturating concentrations of XpIII and XpIV [calculated rates of 35.0 ± 1.5 nmol min^{-1} with (*R*)-epoxypropane and XpI−XpIII and 33.0 ± 0.9 nmol min^{-1} with (*S*)-epoxypropane and XpI, XpII, and XpIV]. However, the concentrations of XpIII or XpIV required to obtain half-maximal rates with their respective substrates were significantly different (43.0 ± 5.3 μg for XpIII vs 11.7 ± 1.3 μg for XpIV). These results suggest that, under the assay conditions used, XpIV has a higher affinity for the epoxide carboxylase system than does XpIII. In both cases, the concentrations of XpIII or XpIV required to obtain half-maximal rates were significantly lower that the concentrations of XpI and XpII in the assays (1.7 μM XpIII or 0.5 μM XpIV with 36.0 μM XpI and 20.9 μM XpII).

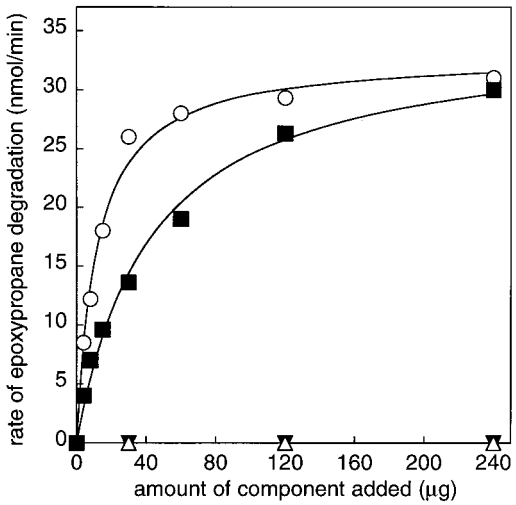


FIGURE 2: Effect of XpIII and XpIV concentrations on the degradation of (*R*)- and (*S*)-epoxypropane in the presence of fixed XpI and XpII concentrations. Assays contained 1.5 mg of XpI, 1.2 mg of XpII, and the indicated amounts of XpIII and XpIV: (■ and △) assays using variable amounts of XpIII and (○ and ▼) assays using variable amounts of XpIV. Black symbols represent data from assays with (*R*)-epoxypropane as the substrate; white symbols represent data from assays with (*S*)-epoxypropane as the substrate.

Inhibition of Epoxide Carboxylation by Epoxypropane Enantiomers. When using a racemic mixture of epoxypropane as the substrate, one might expect to observe 50% degradation in the presence of XpI−XpIII or XpI, XpII, and XpIV, on the basis of the imparting stereospecificity of XpIII and XpIV. However, as shown in Figure 1A, racemic epoxypropane is only degraded in the presence of all four epoxide carboxylase components. The lack of any detectable activity with only three components suggests that the second epoxypropane enantiomer exerts an inhibitory or inactivating effect in the absence of its complementing dehydrogenase. To investigate this possibility in more detail, epoxide carboxylase assays were performed using (*R*)- or (*S*)-epoxypropane as the substrate and in the presence of the required three components. A small amount (50 nmol) of the other isomer was added to the assay when approximately one-half of the substrate had been depleted, such that the ratio of the second isomer to substrate isomer was 1:10. As shown in Figure 3A, the addition of (*S*)-epoxypropane to an assay containing XpI−XpIII prevented any further degradation of (*R*)-epoxypropane. Epoxide carboxylase activity was restored upon addition of XpIV to the assay to a rate comparable to the rate observed before inhibition (Figure 3A). As shown in Figure 3B, a similar inhibitory effect was observed for (*S*)-epoxypropane carboxylation when (*R*)-epoxypropane was added to an assay containing XpI, XpII, and XpIV. Activity was restored in this assay upon addition of XpIII. The complete loss of activity exerted in each case by the addition of the opposing enantiomer, at a concentration only $^1/_{10}$ of that of the substrate enantiomer, suggests that the opposing isomer is an inactivator of epoxide carboxylase activity rather than a rapid equilibrium inhibitor. These results reinforce the idea that a covalent complex is formed between the epoxide substrate and the active site-containing component, and that the further reactivity of this complex requires the presence of the correct stereoselective dehydrogenase (Scheme 3).

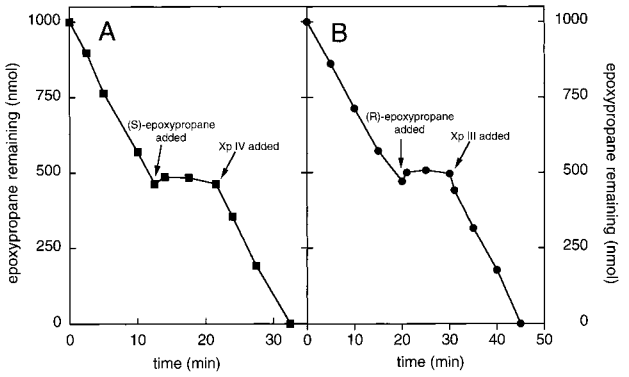


FIGURE 3: Inhibition of epoxide carboxylation by epoxypropane enantiomers. (A) Inhibition of (*R*)-epoxypropane carboxylation by addition of (*S*)-epoxypropane. (■) The assay was initiated with (*R*)-epoxypropane as the substrate containing XpI (1.5 mg), XpII (1.2 mg), and XpIII (0.16 mg), where at $t = 12.5$ min, (*S*)-epoxypropane (50 nmol) was added, and at $t = 22.5$ min, XpIV (0.15 mg) was added. (B) Inhibition of (*S*)-epoxypropane carboxylation by addition of (*R*)-epoxypropane. (●) The assay was initiated with (*S*)-epoxypropane as the substrate containing XpI (1.5 mg), XpII (1.2 mg), and XpIV (0.15 mg), where at $t = 21$ min, (*R*)-epoxypropane (50 nmol) was added, and at $t = 31$ min, component III (0.16 mg) was added.

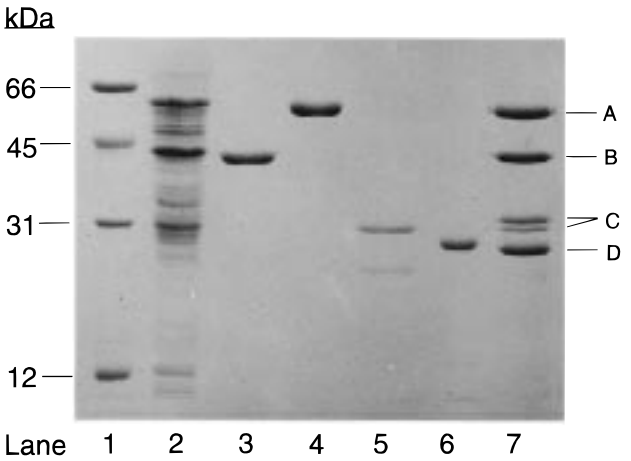


FIGURE 4: SDS−PAGE analysis of purified epoxide carboxylase components from *N. corallina* B276 and *Xanthobacter* strain Py2: lane 1, molecular mass standards (2 μg each); lane 2, propylene-grown cell extract from *N. corallina* B276 (20 μg); lane 3, purified NcI (2 μg); lane 4, purified NcII (2 μg); lane 5, purified NcIII (2 μg); lane 6, purified NcIV (2 μg); and lane 7, purified epoxide carboxylase components from *Xanthobacter* strain Py2. (A) XpII (2 μg), (B) XpI (2 μg), (C) XpIII (2 μg), and (D) XpIV (2 μg).

Purification of Epoxide Carboxylase Components from N. corallina B276. In a previous study (*17*), aliphatic epoxide metabolism in the Gram-positive actinomycete *N. corallina* was shown to proceed by a carboxylation reaction analogous to that of *Xanthobacter* Py2. Initial in vitro studies of the epoxide carboxylase activity of *N. corallina* provided evidence that carboxylation was catalyzed by a multiprotein system requiring the same cofactors (NADPH and NAD^+) as the *Xanthobacter* Py2 system (*17*). In light of the present identification and characterization of stereospecific dehydrogenases as components of the *Xanthobacter* Py2 epoxide carboxylase system, it was of interest to determine whether a similar or distinct mechanism is used to discriminate epoxide enantiomers in the *N. corallina* system. Toward this end, the epoxide carboxylase system of *N. corallina* was purified to homogeneity and the constituent components were compared to those of the epoxide carboxylase from *Xanthobacter* Py2.

Initial fractionation of the epoxide carboxylase system of *N. corallina* revealed that, as for the *Xanthobacter* system, four proteins were required for reconstitution of activity with racemic epoxypropane as the substrate. Each of these four protein fractions could substitute for one of the *Xanthobacter* components when combined with the other three purified *Xanthobacter* components. These results indicate that the *N. corallina* system consists of four proteins with functions analogous to those of the *Xanthobacter* system. To expedite and simplify the purification of the *N. corallina* components, each component was purified on the basis of its ability to reconstitute activity with racemic epoxypropane as the substrate when added to three of the *Xanthobacter* components.

The purification protocols for each of the four epoxide carboxylase components from *N. corallina* are listed in Table 1, and SDS−PAGE analysis of each purified component is shown in Figure 4. It should be noted that it was necessary to apply purification strategies (i.e., nature and order of columns, eluents, and gradients) for the *N. corallina* components different from those conditions giving optimal purifications of the *Xanthobacter* components. The biochemical properties for each of the *N. corallina* epoxide carboxylase components, with direct comparisons to each of the epoxide carboxylase components of *Xanthobacter* strain Py2, are presented in Table 2.

The levels of expression, molecular masses (monomeric and native), and cofactor contents of the four epoxide carboxylase components from *N. corallina* were found to be very similar to the corresponding components of *Xanthobacter* strain Py2 (Table 2). These similarities are not surprising, since the *N. corallina* components are fully functional when recombined with the appropriate *Xanthobacter* components. Despite these similarities, there were some notable differences in the two systems. For example, the elution profiles of the epoxide carboxylase components using anion exchange chromatography were substantially different. The *Xanthobacter* components eluted in the following order: XpIV > XpI > XpII > XpIII (*12*); on the other hand, the *N. corallina* components eluted in the following order: NcIII > NcIV > NcII > NcI. In addition, NcIII and NcIV appeared to be less stable than their *Xanthobacter* counterparts on the basis of the losses of activity experienced during purification (Table 1). An additional notable difference between the two systems lies in the N-terminal sequences determined by Edman degradation (Table 2). Of the N-terminal sequences compared, NcI has the highest sequence similarity to XpI, where (excluding the initial methionine) four amino acid residues DVxxPT correlated with the N-terminal amino acid sequence of XpI. The N-terminal sequence of NcIV is somewhat similar to XpIV where residues LxxxVxxI of NcIV are identical to those of XpIV. While the complete amino acid sequences of the four epoxide components of *Xanthobacter* Py2 have been deduced from the gene sequences (*32*), there is no information currently available for the *N. corallina* system beyond the N-terminal regions. Therefore, it remains to be determined how much overall sequence homology exists between the two systems.

Table 1: Purification of the Four Epoxide Carboxylase Components from *N. corallina* B276

step	volume (mL)	total protein (mg)	total activity[a] (milliunits)	specific activity (milliunits/mg)	yield (%)	purification (*x*-fold)
NcI						
extract	290	3120	39481	12.7	100	1
DEAE-Sepharose	190	429	28772	67	73	5.3
Phenyl-Sepharose	125	288	26865	93	68	7.3
S-300	30	128	12634	99	32	7.8
Q-Sepharose	22	109	11130	102	28	8.0
NcII						
extract	290	3120	22458	7.2	100	1
DEAE-Sepharose	130	559	15873	28	71	3.9
Phenyl-Sepharose	112	224	11648	52	52	7.2
S-300	25	138	9347	68	42	9.4
Q-Sepharose	23	101	7107	70	32	9.7
NcIII						
extract	220	5016	63518	12.7	100	1
DEAE-Sepharose	180	99	36468	368	57	29.0
Phenyl-Sepharose	24	10	7127	713	11	56.1
Dyematrex Red A	50	4	3772	943	6	74.3
hydroxyapatite	21	2.8	2974	1062	5	83.6
NcIV						
extract	220	5016	49073	9.8	100	1
DEAE-Sepharose	118	138	45567	330	93	33.7
hydroxyapatite	40	22	10438	474	21	48.4
Phenyl-Sepharose	20	2.9	3345	1153	7	117.7

[a] One unit of activity is defined as 1 μmol of epoxypropane degraded per minute at 30 °C.

Table 2: Biochemical Properties of the Epoxide Carboxylase Components from *N. corallina* B276 and *Xanthobacter* Strain Py2

analysis[a]	component I	component II	component III	component IV
molecular mass (Da)				
SDS−PAGE	43800	59300	29200, 23200	27000
	41500	*57000*	*30200, 29100*	*26400*
mass spectroscopy	40568	58679	30037, 25459	25867
	41713	*57488*	*26124, 26025*	*25354*
Superose 12	246700	104700	50100	72400
	256283	*90000*	*64774*	*49877*
quaternary structure	α_6	α_2	α_2	α_2
	α_6	α_2	α_2	α_2
organic cofactors	N[b]	2 FAD per dimer	N	N
	N	*2 FAD per dimer*	*N*	*N*
metals	0.8 zinc per monomer	N	N	N
	0.83 zinc per monomer	*N*	*N*	*N*
N-terminal sequence	MRVGSQDVVLPT	MDLRNTVLSMDE	MEEHVSIRGDLA	TGFLQGRVAMI
	MLIRGEDVTIPT	*MKVWNARNDH*	*SRVAIVTGAS*	*MLDNEVIAIT*

[a] The biochemical properties for each epoxide carboxylase component from *N. corallina* B276 are listed with the comparative biochemical properties of each analogous epoxide carboxylase component from *Xanthobacter* Py2 in italicized text directly below. The epoxide carboxylase component properties from *Xanthobacer* strain Py2 are as previously reported in ref *12*. [b] N, none detected.

It should be noted that a protein band, with an apparent molecular mass of 23 200 Da on SDS−PAGE gels, copurified with NcIII. We were unable to separate this band from the 29.2 kDa band using additional chromatographic techniques. Whether this band is a contaminating protein or a degradation product of NcIII remains unclear. Interestingly, XpIII also runs as a doublet of major (M_r = 30 200) and minor (M_r = 29 100) bands on SDS−PAGE gels. The fact that both proteins exhibit an additional substoichiometric band on SDS−PAGE gels may be significant, but it is not understood at present.

Stereoselectivity of NcIII and NcIV. As described above, both NcIII and NcIV were purified on the basis of their abilities to complement *Xanthobacter* epoxide carboxylase components in restoring activity using racemic epoxypropane as the substrate. The catalytic roles of NcIII and NcIV in epoxide carboxylation are presumably identical to those of XpIII and XpIV, respectively. To verify the stereoselective roles of NcIII and NcIV in epoxide carboxylation, assays were performed using (*R*)-epoxypropane, (*S*)-epoxypropane, or racemic epoxypropane as the substrate in the presence of homologous or heterologous combinations of three epoxide carboxylase components purified from either *N. corallina* or *Xanthobacter* Py2. As shown in Table 3, the combination of NcI−NcIII degraded (*R*)-epoxypropane and the combina-

Table 3: Stereospecific Epoxypropane Degradation by Epoxide Carboxylase Components III and IV from *N. corallina* B276

epoxide carboxylase components added[a]	rate of epoxypropane degradation (nmol/min)		
	R[b]	*S*[c]	racemic[d]
NcI−NcIII	15.0	0	0
NcI, NcII, and NcIV	0	11.7	0
XpI, XpII, and NcIII	27.0	0	0
XpI, XpII, and NcIV	0	21.2	0

[a] Where indicated, assays contained 1.5 mg of XpI, 1.2 mg of XpII, 0.13 mg of NcIII, 0.15 mg of NcIV, 2.6 mg of NcI, and 1.2 mg of NcII. [b] (*R*)-Epoxypropane. [c] (*S*)-Epoxypropane. [d] Racemic epoxypropane.

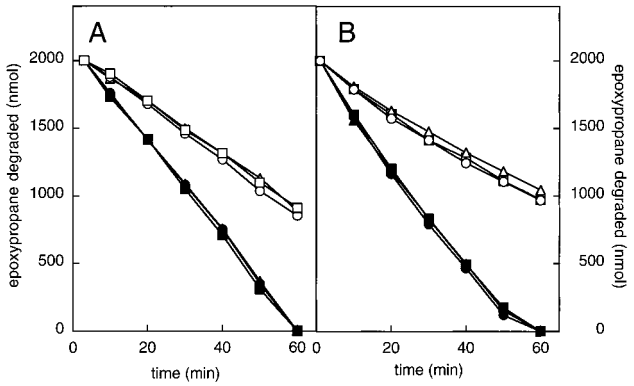


FIGURE 5: Degradation of a racemic mixture, and pure enantiomers, of epoxypropane by whole cells of *Xanthobacter* strain Py2 and *N. corallina* B276. (A) Assays with *Xanthobacter* Py2. White symbols represent data from assays in which 0.15 mg of protein was used; black symbols represent data from assays in which 0.30 mg of protein was used. (B) Assays with *N. corallina* B276. White symbols represent data from assays in which 0.35 mg of protein was used; black symbols represent data from assays in which 0.70 mg of protein was used. (□ and ■) Racemic epoxypropane, (○ and ●) (*R*)-epoxypropane, and (△ and ▲) (*S*)-epoxypropane.

tion of NcI, NcII, and NcIV degraded (*S*)-epoxypropane. No degradation of racemic epoxypropane was observed with either combination. Analogous results were obtained for the heterologous combinations of components (Table 3). These results demonstrate that the four-component epoxide carboxylase from *N. corallina* utilizes the same strategy for enantioselective epoxide carboxylation as the epoxide carboxylase from *Xanthobacter* strain Py2.

Enantioselectivity of Epoxypropane Carboxylation in Vivo. On the basis of the stereoselectivity of epoxyalkane formation catalyzed by alkene monooxygenase from *N. corallina* (Scheme 2), it has been proposed that the subsequent epoxide conversion reaction might preferentially utilize one epoxide enantiomer over the other [e.g., (*R*)-epoxypropane would be used more efficiently than (*S*)-epoxypropane in vivo] (*10*). The results of this work demonstrate that both the *Xanthobacter* Py2 and *N. corallina* epoxide carboxylase systems can use either enantiomer of epoxypropane as the substrate through the mediation of the correct short-chain dehydrogenase (component III or IV). This result is not surprising in light of the data presented in Figure 3; i.e., even a small amount of the second enantiomer will inactivate epoxide carboxylase activity in the absence of the complementing dehydrogenase. Even with alkene monooxygenase exhibiting a selectivity of 90:10 or greater for the production of one epoxyalkane enantiomer, the cells will still need to maintain some level of expression of the second dehydrogenase to prevent this inactivation phenomenon in vivo. The levels of purification (*x*-fold) required to obtain homogeneous preparations of components III and IV suggest that both proteins are expressed at levels approximately equal to 0.5−1% of that of the cell protein for the *Xanthobacter* Py2 (*12*) and *N. corallina* (Table 2) systems. Since components III and IV appear to be the sole determinants in imparting stereoselectivity for epoxyalkane enantiomers, these observations would not support enantioselectivity for the epoxide carboxylase systems in vivo. To further investigate this, the rates of degradation of (*R*)-, (*S*)-, and racemic epoxypropane were compared for whole cell suspensions of *Xanthobacter* Py2 and *N. corallina*. As shown in Figure 5, the rates of degradation were identical in both bacteria for both the enantiomers and the racemic mixture. To verify that the assay rates were true steady-state rates (i.e., the assays were not saturated with enzyme), the concentration of cells was doubled and the degradation assays were repeated. As shown in Figure 5, doubling the concentration of cells doubled the degradation rates in all cases, demonstrating that the assays conform to steady-state kinetics, and confirming that no enantioselectivity for epoxypropane carboxylation occurs in vivo.

DISCUSSION

The epoxide carboxylase systems of *Xanthobacter* Py2 and *N. corallina* constitute a new class of multicomponent enzyme with unprecedented cofactor requirements and catalytic mechanism. By some means, the interplay of the four epoxide carboxylase components and the associated transhydrogenation of pyridine nucleotides facilitate epoxide ring opening and carboxylation to form the β-keto acid product (Scheme 1). The complexity of these systems, the novelty of the reactions they catalyze, and the potential biotechnological applications associated with them [e.g., in stereospecific epoxide transformations and bioremediation (*31*, *33*, *34*)] warrant further investigations into their molecular properties and modes of action.

The work described in this paper has defined roles for two components of these systems as dehydrogenases belonging to the SDR family. Presumably, these components catalyze the abstraction of a hydride from C2 of a secondary alcohol-like intermediate, a required step in converting the ring-opened intermediate to one containing a keto group. Perhaps the most interesting feature of these dehydrogenases is the selectivity they confer for epoxide enantiomers, with component III being required for conversion of one enantiomer and component IV being required for the other (Scheme 3). The confirmatory studies of the epoxide carboxylase system of *N. corallina*, an organism that is morphologically and phylogenetically unrelated to *Xanthobacter* Py2, demonstrate that stereoselective epoxide ring opening and dehydrogenation represent a general and microbiologically important mode of aliphatic epoxide conversion.

Comparison of the amino acid sequences of components III and IV of the *Xanthobacter* Py2 system to those of other enzymes of the SDR family provide some important insights into their probable functions. For example, sequence alignments suggest that XpIII and XpIV possess the same folding topology as observed for other SDR enzymes. Comparison of the available three-dimensional structures of enzymes belonging to the SDR family reveals that they share highly similar tertiary structures and catalytic mechanisms (*28*, *35*, *36*). The coenzyme binding domain is in the N-terminal region of SDR enzymes, where the GlyXXXGlyXGly motif constitutes the Rossmann fold (*23*, *37*, *38*). This element is completely conserved in both XpIII and XpIV. Both XpIII and XpIV possess an aspartate at position 37 (in numbering of *D. melanogaster* ADH) which is indicative of their preference for NAD^+ over $NADP^+$ and is in agreement with the cofactor requirements for epoxide carboxylase (i.e., NAD^+, not $NADP^+$, is the required oxidant). SDR enzymes possessing an aspartate in this region, rather than positively

charged side chain residues, distinguish NAD^+ from $NADP^+$ by the favorable hydrogen bonding that occurs between the negative charge of aspartate and the 2′-OH of NAD^+ rather than by the electrostatic repulsion that would occur with the 2′-ribophosphate of $NADP^+$ (*23*, *36*, *39*). On the basis of numerous site-directed mutagenesis studies (*40*−*43*), corroborated by three-dimensional structures (*28*, *35*, *36*), the substrate binding pockets of SDR enzymes orient their respective substrates in close proximity to the nicotinamide moiety of NAD^+ and the conserved catalytic residues Ser, Tyr, and Lys. These three amino acids, conserved in both XpIII and XpIV, constitute the "catalytic triad" which plays a key role in the reaction mechanism of the SDR enzymes (*22*, *23*, *28*). Tyrosine, with its functional hydroxyl group deprotonated, acts as a catalytic base to extract a hydrogen from the hydroxyl group of the substrate (going in the oxidative direction of alcohol to aldehyde or ketone). This facilitates the concomitant abstraction of a hydride from the substrate and its transfer to NAD^+. Lysine interacts with the ribose ring of the nicotinamide, and its positive charge lowers the pK_a of the hydroxyl group of tyrosine. Serine forms a stabilizing hydrogen bond with the reaction intermediate. It is easy to envisage this same reaction mechanism and substrate−residue interactions for the stereospecific reactions catalyzed by both XpIII and XpIV as illustrated in Scheme 3.

The highly variable C-terminal regions of SDR enzymes provide the divergence necessary for the enzymes to recognize and distinguish substrates. Presumably, the differences in the C-terminal regions of XpIII and XpIV allow for the enantioselectivity exhibited by these enzymes. Of potential relevance in this regard, Nakajima and co-workers recently published (*35*) the crystal structures of two highly homologous plant SDR enzymes, tropinone reductase I and II (TR-I and TR-II, respectively). These enzymes catalyze stereospecific reductions of the carbonyl group of the alkaloid intermediate tropinone (*41*, *44*). It was determined that the presence of differently charged residues in the substrate binding regions of the enzymes created different electrostatic environments on TR-I and TR-II. The electrostatic environment was proposed to control the binding orientation of tropinone so a specific face of the prochiral carbonyl group is closest to the catalytic site. By this means, the stereospecificity of the reaction product is believed to be controlled. In contrast to tropinone, which has a positive charge at physiological pH, epoxypropane, or the putative nucleophile-bound 2-hydroxy intermediate (assuming the ring-opened oxygen atom is protonated to the hydroxyl form in Scheme 3), is not charged. Apparently, the stereospecificity of XpIII and XpIV is controlled by other means, perhaps by polar and/or hydrophobic interactions, or specific surface interactions with the XpI protein where the substrate intermediate is believed to be bound. The C-terminal regions (the proposed substrate binding region) of XpIII and XpIV show similar sequence topologies interspersed with nonidentities. Further structural and biochemical analyses are needed to define the important features imparting enantioselectivity in these two enzymes.

The studies described in this paper allow the refinement of the previous mechanistic proposals for the epoxide carboxylase system to include probable roles for XpIII and XpIV. To summarize, XpI is believed to contain a nucleophilic thiolate that binds and activates the epoxide substrate. XpII is an NADPH:disulfide oxidoreductase that is believed to regenerate the reduced form of the thiol on XpI, which is oxidized during the catalytic cycle. While this reaction mechanism has by no means been rigorously established, it serves as a starting point for designing additional experiments for identifying and characterizing reaction intermediates, identifying the critical amino acid residues involved in catalysis, and characterizing the protein−protein interactions required for the reaction cycle. These studies, we are hopeful, will provide further insights into the structure and function of multicomponent enzyme systems and biological strategies controlling the enantioselectivity for the transformation of an important class of organic molecules.

ACKNOWLEDGMENT

We thank Mark Harris for technical assistance.

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BI982114H