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Opposite Facial Specificity for Two Hydroquinone Epoxidases: (3-*si*,4-*re*)-2,5-Dihydroxyacetanilide Epoxidase from *Streptomyces* LL-C10037 and (3-*re*,4-*si*)-2,5-Dihydroxyacetanilide Epoxidase from *Streptomyces* MPP 3051†

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ABSTRACT: (3-*si*,4-*re*)-2,5-Dihydroxyacetanilide epoxidase (DHAE I), a key enzyme in the biosynthesis of the epoxysemiquinone antibiotic LL-C10037α by *Streptomyces* LL-C10037 [Gould, S. J., & Shen, B. (1991) *J. Am. Chem. Soc. 113*, 684-686], and (3-*re*,4-*si*)-2,5-dihydroxyacetanilide epoxidase (DHAE II) isolated from *Streptomyces* MPP 3051—which yields the (3*R*,4*S*)-epoxyquinone mirror image product of DHAE I—are described. DHAE I was purified 640-fold. Gel permeation chromatography indicated an M_r of 117 000 ± 10 000; SDS-PAGE gave a major band of 22 300 daltons, indicating that DHAE I is either a pentamer or hexamer in solution. The enzyme had a pH optimum of 6.5, a K_m of 8.4 ± 0.5 μM, and a V_{max} of 3.7 ± 0.2 μmol min^{-1} mg^{-1}. DHAE II was purified 1489-fold. The enzyme was shown to be a dimer of M_r 33 000 ± 2000, with 16 000-dalton subunits, with a pH optimum of 5.5 and a K_m of 7.2 ± 0.4 μM. Both enzymes required only O_2 and substrate; flavin and nicotinamide coenzymes had little or no effect. Neither catalase nor EDTA affected the activity of either enzyme, but complete inhibition of both was obtained with 1,10-phenanthroline. The activity of the purified DHAE I could be enhanced, but only by Mn^{2+} (relative V = 246 at 0.04 mM), Ni^{2+} (relative V = 266 at 0.2 mM), or Co^{2+} (relative V = 498 at 0.2 mM). Reconstitution from a DHAE I apoenzyme, generated by treatment with 1,10-phenanthroline followed by Sephadex G-25 chromatography, occurred only by addition of one of these three metals. It is proposed that DHAE I and DHAE II, and two other enzymes discussed, represent a hitherto unrecognized class of enzymes that should be called "hydroquinone monooxygenase (epoxidizing)".

Antibiotic LL-C10037α, produced by *Streptomyces* LL-C10037 (Lee et al., 1984) has structure **1** (Shen et al., 1990). Whole cell and cell-free studies show it is derived from the shikimate pathway via 3-hydroxyanthranilic acid, **2** (Whittle & Gould, 1987; Gould et al., 1989; Gould & Shen, 1991). As shown in Scheme I, 2,5-dihydroxyacetanilide, **3**, undergoes epoxidation catalyzed by (3-*si*,4-*re*)-2,5-dihydroxyacetanilide epoxidase (DHAE I)[1] to form epoxyquinone **4**. The desacetyl

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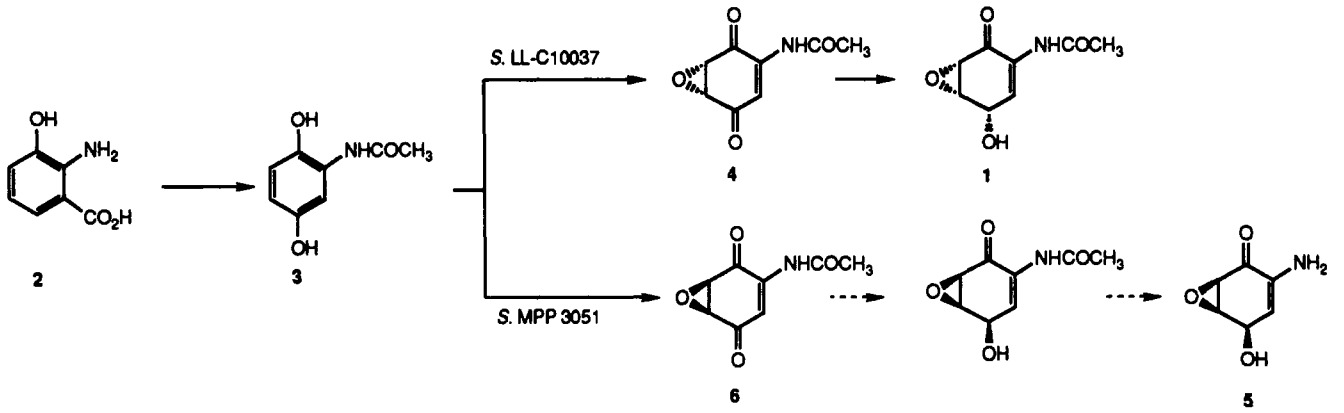
Scheme I



enantiomer of **1** is antibiotic MM 14201, **5**, produced by *Streptomyces* MPP 3051 (Box et al., 1983; Shen et al., 1990) presumably by a similar pathway but with different absolute stereochemistry. An epoxidase with the necessary properties, (3-*re*,4-*si*)-2,5-dihydroxyacetanilide epoxidase (DHAE II) has been isolated from *Streptomyces* MPP 3051 (Gould & Shen, 1990). It converts **3** to epoxyquinone **6**, the enantiomer of **4**.

Numerous epoxyquinones have been reported, and in all cases studied the oxygen of the oxirane ring was derived from molecular oxygen (Nabeta et al., 1973, 1975; Omura et al., 1981; Read et al., 1969; Thiericke et al., 1990; Whittle & Gould, 1987). However, the mechanism of epoxidation has yet to be established (Omura et al., 1981; Priest & Light, 1989; Sadowski et al., 1977). In order to gain some insight into this and to explore the active-site constraints that lead to opposite absolute facial specificity toward the planar substrate **3**, we have purified and characterized DHAE I and DHAE II. The former enzyme requires molecular oxygen but does not require any added cofactor. It is dramatically activated by Ni^{2+}, Co^{2+}, or Mn^{2+}, and the enzyme activity is totally reconstituted from its apoenzyme by the addition of Ni^{2+}, Co^{2+}, or Mn^{2+}; no other metals tested were effective. The latter enzyme also requires only molecular oxygen and substrate. It shows responses to inhibitors similar to those observed with DHAE I. It is not activated by any metal ions tested, nor can its apoenzyme be reactivated.

Materials and Methods

General Procedures. UV spectra were recorded on a IBM 9420 UV–visible spectrophotometer. HPLC analyses were performed on a Waters 600E HPLC instrument with a Kratos Spectroflow 757 UV detector or a Waters 6000A HPLC instrument with a Linear UVIS 200 detector, and an HP 3396A integrator was used with each. Radi-Pak C_{18} (Novapak, 4 μm, 8 × 100 mm, Waters Assoc.) and Versapack C_{18} (10 μm, 4.1 × 250 mm, Alltech Assoc.) columns were used.

Refrigerated centrifugations were done in an IEC B-20a centrifuge. Cell disruption was performed with a sonicator, Model W-225R made by Heat Systems-Ultrasonic, Inc. For open columns, the flow rate was controlled by peristaltic pump P-3 (Pharmacia), and fractions were monitored by a set of dual-path optical control UV-2 units (Pharmacia). A Waters 650E FPLC system was used for the enzyme purification, with a λ Max Model 481 LC spectrophotometer as detector. All FPLC columns were purchased from Waters Assoc., as was the Accell QMA anion-exchange resin. Incubations were performed in an IBM 9550 heating/cooling fluid circulator (±0.1 °C). Water used for fermentations and biochemical preparations was purified by a Milli-Q water system (Millipore Corp.). Fermentations were carried out in a rotary incubator (Lab-Line incubator–shaker). A nonrefrigerated centrifuge (IEC model HN) was used to remove proteins for enzyme assays.

Standard Culture Conditions. Streptomyces LL-C10037 and *Streptomyces* MPP 3051 were maintained as spores on sterile soil, the former at 4 °C and the latter at 20–25 °C. A loopful of this material was used to inoculate 50 mL of seed medium containing 1.0% glucose, 2.0% soluble potato starch, 0.5% yeast, 0.5% N-Z Amine A 59027, and 0.1% $CaCO_3$, adjusted to pH 7.2 with 2% KOH. The seed inoculum, contained in a 250-mL Erlenmeyer flask, was incubated for 3 days at 28 °C, 240 rpm for *Streptomyces* LL-C10037 and 2 days at 28 °C, 250 rpm for *Streptomyces* MPP 3051. Production broths (400 mL in 2-L Erlenmeyer flasks), composed of 1.0% glucose, 0.5% bactopeptone, 2.0% molasses (Grandma's famous light unsulfured), and 0.1% $CaCO_3$, adjusted to pH 7.2 with 10% HCl prior to sterilization, were subsequently inoculated 5% (v/v) with vegetative inoculum from seed broths. The production cultures were incubated under the same conditions as the seed cultures.

Protein Determination. Protein concentrations were determined by either the method of Lowry (Peterson, 1983) or the method of Bradford (1976) with bovine serum albumin as the calibration standard.

Polyacrylamide Gel Electrophoresis. Denaturing gels were run according to the Laemmli procedure (1970). The separating gel and the stacking gel were 15 and 3.5% polyacrylamide, respectively.

Molecular Weight Determination. M_r of the native enzyme was determined by gel filtration on a Protein-Pak 300 SW FPLC column. The column was eluted with 50 mM potassium phosphate buffer, pH 7.0, containing 20% glycerol and 0.2 mM EDTA at a flow rate of 0.5 mL/min. The following standards (Sigma) were used: β-amylase (200 000), alcohol dehydrogenase (150 000), bovine serum albumin (66 000), chicken egg albumin (45 000), carbonic anhydrase (29 000), and α-lactalbumin (14 000). The subunit M_r was determined by SDS–polyacrylamide gel electrophoresis using the low M_r

[1] Abbreviations: DHAE I, (3-*si*,4-*re*)-dihydroxyacetanilide epoxidase; DHAE II, (3-*re*,4-*si*)-dihydroxyacetanilide epoxidase; HPLC, high-performance liquid chromatography; FPLC, fast-protein liquid chromatography; EDTA, ethylenediaminetetraacetic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; ABQ, acetamido-1,4-benzoquinone; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; PVPP, polyvinyl polypyrrolidone; PMSF, phenylmethanesulfonyl fluoride; HIC, hydrophobic interaction chromatography; CFE, cell-free extract; NADH, dihydronicotinamide adenine dinucleotide; NADPH, dihydronicotinamide adenine dinucleotide phosphate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; PCMBA, *p*-chloromercuribenzoic acid; DEAE, diethylaminoethyl.

standards (Sigma, Catalog No. MW-SDS-70L) bovine serum albumin (66 200), chicken egg albumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), trypsin inhibitor (21 000), and α-lactalbumin (14 000) and (Bio-Rad, Catalog No. 161-0304) phosphorylase B (97 400), bovine serum albumin (66 200), chicken egg albumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), and lysozyme (14 000).

Enzyme Assay. DHAE I and DHAE II were each assayed by following the consumption of **3** and the production of **4** simultaneously by HPLC. Typically, 500 μL of assay solution, consisting of 0.1 mM **3** in 0.1 M potassium phosphate buffer (pH 6.5 for *Streptomyces* LL-C10037 and pH 5.5 for *Streptomyces* MPP 3051) in the presence of enzyme, was incubated at 30 °C. The assay was initiated by addition of the enzyme preparation and was terminated by addition of 100 μL of CH_3CN/H_2O/TFA (66/27/7 v/v). The reaction mixture was then centrifuged (800*g*, 5 min) to remove the protein, and the supernatant was analyzed for **3** and **4** either on a Waters Radi-Pak C_{18} column (H_2O/CH_3CN/TFA = 85/15/0.1%, flow rate 1.0 mL/min) or on an Alltech Versapack C_{18} column (H_2O/CH_3CN = 85/15%, flow rate 1.0 mL/min). With the former, **3**, **4**, and acetamido-1,4-benzoquinone (ABQ), which resulted from air oxidation of **3**, had retention times of 5.0, 8.0, and 9.2 min, respectively. With the latter, **3**, **4**, and ABQ had retention times of 4.2, 7.3, and 8.3 min, respectively. Eluted compounds were monitored with a UV detector set at 225 nm, and the whole system was calibrated with known quantities of **3**, **4** and ABQ (under the given conditions **3**, **4**, and ABQ showed relative responses of 1.09, 1.0, and 0.68 on a molar basis, respectively).

For the pH dependency study, complete assay solutions were prepared as above but with 0.1 M potassium phosphate buffer, pH 4.5–8.0. These were incubated at 30 °C for 5 min.

For determination of kinetic parameters, 500-μL assay solutions containing 2.5–250 μM **3** and 0.14 μg of DHAE I in 0.1 M potassium phosphate buffer, pH 6.5, were incubated at 30 °C for 5 min while for DHAE II conditions of 5.0–500 μM **3**, 0.05 μg of DHAE II, and pH 5.5 were used.

For inhibition and activation studies, unless otherwise specified, 250-μL assay solutions containing 0.1 mM **3**, 0.5 μg of DHAE I or 0.6 μg of DHAE II, and the indicated amount of either inhibitors or activators were incubated at 30 °C for 10 min for DHAE I and for 5 min for DHAE II.

Purification of DHAE I. The following buffers were used in the purification of the (3-*si*,4-*re*)-DHA epoxidase. Buffer I: 10 mM potassium phosphate, pH 7.0. Buffer II: 50 mM potassium phosphate, pH 7.0, 20% glycerol, 0.2 mM EDTA. Buffer III: 1.0 M KCl in buffer II. Buffer IV: 1.0 M $(NH_4)_2SO_4$ in buffer II. Buffer V: 50 mM Tris-HCl, pH 7.5, 20% glycerol, 0.2 mM EDTA. Buffer VI: 1.0 M KCl in buffer V. All steps were carried out at 4 °C.

Step 1: *Preparation of Cell-Free Extract.* Cells from 5.0 L of 96-h fermentations of *Streptomyces* LL-C10037 were harvested by centrifugation (4 °C, 13800*g*, 10 min) and washed sequentially with (1 L) buffer I, 1.0 M KCl, 0.8 M NaCl, and buffer I. After each wash the cells were centrifuged as above. The washed cells (179 g, wet wt) were then suspended in buffer II (550 mL); the cell suspension was subsequently brought to 3 mg/mL with polyvinyl polypyrrolidone (PVPP) and 1.0 mM with phenylmethanesulfonyl fluoride (PMSF). It was then equally distributed into six beakers, and each portion was disrupted by sonication (maximum power, 90% duty, pulsed for 4 × 20 s). Cell debris was removed by centrifugation (4 °C, 13800*g*, 20 min), and the supernatants were combined to afford a crude cell-free extract (CFE, 565 mL).

Step 2: *Protamine Sulfate Precipitate.* The CFE was brought to 0.01% with protamine sulfate by dropwise addition of a 2.0% solution. The resulting solution was stirred for 0.5 h, and centrifugation (4 °C, 38400*g*, 20 min) yielded 565 mL of supernatant.

Step 3: *$(NH_4)_2SO_4$ $(NH_4)SO_4$ Precipitation.* The protamine sulfate supernatant (565 mL) was brought to 51.3% saturation by addition of solid ammonium sulfate. The suspension was stirred for 1 h, and the precipitate was removed by centrifugation (4 °C, 13800*g*, 20 min). The resulting supernatant was brought to 71.9% saturation with solid ammonium sulfate and was stirred for an additional hour. The active pellet was collected by centrifugation (4 °C, 38400*g*, 20 min).

Step 4: *Sephacryl S-200 Column.* The 51.3–71.9% pellet was dissolved in 11.5 mL of buffer II and divided equally into two parts. Each part was applied to a Sephacryl S-200 column (2.4 × 110 cm) equilibrated in buffer II. The column was eluted with the same buffer (6.8 mL/30 min, 6.8-mL fractions). Active fractions (90 mL) from the two runs were pooled and concentrated to 42 mL (Amicon, Centriprep 30).

Step 5: *Accell QMA Anion-Exchange Column.* The concentrated material (42 mL) was divided equally into two parts, and each part was applied to an Accell QMA column (1.8 × 50 mm) equilibrated in buffer II. After the column was washed with buffer II, it was eluted with a gradient of buffer II–buffer III (2.5 mL/min, 7.5-mL fractions). Active fractions (80 mL) from the two runs were pooled and concentrated to 35 mL (Amicon, Centriprep 30).

Step 6: *Protein-Pak HIC (Phenyl 5 PW) Column.* The concentrated material (35 mL) was brought to a 1.0 M ammonium sulfate concentration by the addition of solid ammonium sulfate, and it was then divided equally into three parts. Each part was applied to an HIC (phenyl 5 PW) column (8 × 75 mm) equilibrated in buffer IV. The column was washed with buffer IV and eluted with a gradient of buffer IV–buffer V (0.7 mL/min, 2.8-mL fractions). Active fractions (100 mL) were pooled and concentrated to 40 mL (Amicon, Centriprep 30).

Step 7: *Protein-PaK DEAE 5 PW Column.* The concentrated material (40 mL) from the HIC column was dialyzed against buffer II overnight and divided equally into two parts. Each part was applied to a DEAE 5 PW column (8 × 78 mm) equilibrated in buffer II. The column was washed with buffer II and eluted with a gradient of buffer II–buffer III (1.0 mL/min, 2.0-mL fractions). Active fractions (20 mL) from two runs were pooled and concentrated to 0.6 mL (Amicon, Centriprep 30).

Step 8: *Protein-Pak 300 SW Column.* The concentrated preparation (0.6 mL) was applied to a 300 SW column (8 × 300 mm) equilibrated in buffer II. In order to obtain good resolution, only 100 μL of the sample could be loaded per run. The column was eluted with buffer II (0.5 mL/min, 0.5-mL fractions), and active fractions from each run were pooled (9.0 mL).

Step 9: *Protein-Pak DEAE 5 PW Column.* The combined active fractions (9 mL) were applied to a DEAE 5 PW column (8 × 78 mm) equilibrated in buffer V, which was then washed with buffer V and eluted with a gradient of buffer V–buffer VI (1.0 mL/min, 1.0-mL fractions). The final preparation of 4.0 mL of enzyme solution was collected and stored at −80 °C.

Purification of DHAE II. In addition to the buffers described in the previous section, the following buffers were also used in the purification of the (3-*re*,4-*si*)-DHA epoxidase. Buffer VII: 50 mM Tris-HCl, pH 7.5, 20% glycerol, 0.2 mM EDTA. Buffer VIII: 1.0 M KCl in buffer VII. Buffer IX: 1.6 M $(NH_4)_2SO_4$ in buffer II. Buffer X: 25 mM Tris-HCl, pH 7.5, 20% glycerol, 0.2 mM EDTA. Buffer XI: 1.0 M KCl in buffer X. All steps were carried out at 4 °C.

Step 1: Preparation of Cell-Free Extract. Cells from 3.3 L of 22.5-h fermentations of *Streptomyces* MPP 3051 were harvested and treated as described above except that sonication was carried out with 4 × 15 s pulses to afford a crude cell-free extract (CFE, 335 mL).

Step 2: Protamine Sulfate Precipitate. The CFE from step 1 was treated as above to yield 335 mL of supernatant.

Step 3: Accell QMA Anion-Exchange Column. The supernatant (335 mL) from step 2 was divided equally into four parts, and each part was applied to an Accell QMA column (1.8 × 50 mm) equilibrated in buffer II. The column was washed with additional buffer and then eluted with a gradient of buffer II–buffer III (3.0 mL/min, 12-mL fractions). Active fractions (360 mL) from four runs were pooled and dialyzed overnight against 6.0 L of buffer II.

Step 4: Accell QMA Anion-Exchange Column. The dialyzed material (360 mL) from step 3 was applied to the Accell QMA column, which had now been equilibrated in buffer VII. The column was washed with additional buffer VII and then eluted with a gradient of buffer VII–buffer VIII (3.0 mL/min, 12.0-mL fractions), yielding active fractions (108 mL) that were pooled and concentrated to 45 mL (Amicon, Centriprep 30).

Step 5: Protein-Pak HIC (Phenyl 5 PW) Column. The concentrated material (40 mL) from step 4, brought to a 1.6 M concentration of ammonium sulfate, was applied in four portions to the HIC (phenyl 5 PW) column equilibrated in buffer VIII and eluted with a gradient of buffer VIII–buffer II (0.8 mL/min, 2.4-mL fractions). Active fractions were pooled and dialyzed overnight against 4.0 L of buffer II.

Step 6: Protein-Pak DEAE 5 PW Column. The dialyzed preparation (44 mL) from step 5 was chromatographed in three portions on the DEAE 5 PW column as described above. Active fractions (14 mL) were pooled and concentrated to 1.0 mL (Amicon, Centriprep 30).

Step 7: Protein-Pak 300 SW Column. The concentrated preparation (1.0 mL) from step 6 was applied to the 300 SW column in 100-μL aliquots as described above, and active fractions from each run were pooled (7.0 mL).

Step 8: Protein-Pak DEAE 5 PW Column. The combined active fractions (7.0 mL) from step 7 were applied to the DEAE 5 PW column, which had now been equilibrated in buffer VII. It was then washed with buffer VII and eluted with a gradient of buffer VII–buffer VIII (1.0 mL/min, 1.0-mL fractions). The final preparation of 5.0 mL of enzyme solution was collected and stored at −80 °C.

Preparation and Analysis of DHAE I and DHAE II Apoenzymes. A total of 500 μL of DHAE I (16.6 μg) in buffer II was brought to 0.2 mM 1,10-phenanthroline, and the resulting solution was kept at ice temperature for 10 min. It was then passed by centrifugation (3000*g*, 5 min, 4 °C) through a Sephadex G-25 column (1 × 5 cm) equilibrated in buffer II. The final volume of the apoenzyme was 700 μL.

For reconstitution of the DHAE I activity, a 250-μL assay solution containing the apoenzyme (1.3 μg) and the indicated amount of metal ions was preincubated at room temperature (25 ± 5 °C) for 2 min. It was then assayed for the DHAE I activity by addition of **3** (0.1 mM); the resulting solution was incubated at 30 °C for 10 min, and the terminated reaction mixture was analyzed by HPLC.

Treatment of DHAE II (20 μg) in the same manner showed total loss of the enzyme activity before passage by centrifugation (3000*g*, 5 min, 4 °C) through a Sephadex G-25 column (1 × 5 cm) equilibrated in buffer II. However, 17% of the epoxidase was detected in the filtrate. The inhibition was repeated with a second aliquot of enzyme, but 0.4 mM 1,10-phenanthroline was used. Ten percent of the epoxidase was still detected after passing through the Sephadex G-25 column. Finally, a third aliquot of DHAE II was brought to 4.0 mM 1,10-phenanthroline. No enzyme activity was found either before or after the Sephadex G-25 column.

Results

Enzymatic Formation of 4 in a Cell-Free System. Initially ABQ was thought to be the substrate, and conversion to **4** in the presence of either NADH or NADPH was readily detected with crude cell-free extract (CFE) of *Streptomyces* LL-C10037. However, initial incubations with CFE from *Streptomyces* MPP 3051 (30-min duration) showed a total disappearence of ABQ with no detection of any recognizable products. When the incubation time was cut first to 15 min, **4** was detected as the major product and, subsequently, at 7.5 min, **4** was virtually the only product observed. Subsequently, it was shown that **3** is the real substrate for both enzymes and no NADH or NADPH needed to be added.

Purification of DHAE I from Streptomyces LL-C10037 and of DHAE II from Streptomyces MPP 3051. Initially a CFE was prepared in buffer I, and the DHAE I activity was totally lost within 24 h at 4 °C. An extensive study for the stabilization of the DHAE I was then initiated (Scopes, 1987; Suelter, 1985). Protease inhibitors, such as PMSF or the comprehensive protease inhibitor cocktail of Santi (Meek et al., 1985), were tried, and PMSF appeared to improve the stability of the enzyme significantly. Sucrose and glycerol were each included in buffers to lower the water activity; data suggested that glycerol had a better effect than sucrose. Effects of metal chelating reagents on the stability of the enzyme were also investigated, and a slight improvement was observed when EDTA was present in the buffers. The optimized combination gave a CFE with maximal DHAE I activity, which was treated with protamine sulfate to remove nucleic acids and with PVPP to remove phenolic metabolites.

DHAE I activity was precipitated with $(NH_4)_2SO_4$ at 52–72% saturation. The active pellet was subsequently subjected to size-exclusion, anion-exchange, and hydrophobic interaction chromatography. The same protocol was followed for DHAE II initially, but some modifications were subsequently found to be necessary. Since DHAE II activity was spread out in most of the fractions of $(NH_4)_2SO_4$ precipitation, this step was omitted and DHAE II was purified by sequential chromatography on anion-exchange, hydrophobic interaction, and size-exclusion columns. As summarized in Table I these purification schemes gave an overall purification factor of about 640 for DHAE I and about 1489 for DHAE II.

As shown in Figure 1, SDS–PAGE of the purified DHAE I from the last step (lane 7) displayed one main band and only a faint additional doublet at 14K. Compared with the standards of known M_r, the subunit M_r of the enzyme was estimated to be 22.3K. SDS–PAGE of the most pure DHAE II obtained (not shown) displayed one major band of 16K by comparison with the standards of known M_r. There were additional minor bands of considerably higher M_r; however, because the native enzyme was determined to be 33K ± 2K,

Table I: Purification of DHAE I from *Streptomyces* LL-C10037 and of DHAE II from *Streptomyces* MPP 3051

step (buffer)	vol (mL)		protein[a] (mg)		units[b]		units %		sp act.[c]		purification (*x*-fold)	
	DHAE I	DHAE II	DHAE I	DHAE II	DHAE I	DHAE II	DHAE I	DHAE II	DHAE I	DHAE II	DHAE I	DHAE II
CFE	565.0	335.0	867	1072	10.80	5.04	100.0	100.0	0.0124	0.0047	1.0	1.0
protamine sulfate	565.0	335.0	654	854	10.00	4.44	92.6	88.0	0.0153	0.0052	1.2	1.2
$(NH_4)_2SO_4$	11.5		298		6.20		57.2		0.021		1.7	
Sephacryl S-200	42.0		93.6		4.40		41.0		0.047		3.8	
Accell QMA (KP_i)	35.0	360.0	23.3	148	3.70	3.58	34.4	71.0	0.16	0.0242	12.8	5.4
Accell QMA (Tris)		45.0		68.5		3.38		67.0		0.0493		10.5
HIC/phenyl 5PW	40.0	44.0	4.6	12.6	2.50	3.08	23.2	61.0	0.54	0.244	43.8	52.0
DEAE 5 PW (KP_i)	4.0	14.0	0.83	1.76	1.70	2.31	15.6	46.0	2.05	1.31	165.0	279.0
300 SW	9.0	7.0	0.22	0.365	1.20	0.84	10.7	17.0	5.45	2.3	440.0	490.0
DEAE 5 PW (Tris)	4.0	5.0	0.112	0.070	0.86	0.49	7.9	10.0	7.68	7.0	640.0	1489.0

[a] In the early stages of the purification, total protein was determined by the method of Lowry, while in the late stages of the purification, total protein was determined by the Bradford method. [b] One unit of enzyme activity is defined as the consumption of 1 mmol of **3** per minute or the production of 1 mmol of **4** per minute. [c] sp act. is defined as 1 unit/mg of protein.

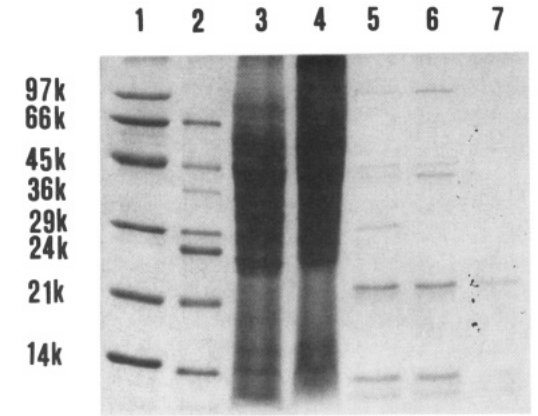


FIGURE 1: SDS–PAGE analysis of DHAE I purification on a 14% gel stained with Coomassie brilliant blue. Lanes 1 and 2: molecular weight protein standards. Lane 3: CFE. Lane 4: 51–72% $(NH_4)_2SO_4$ cut. Lanes 5 and 6: two active fractions from the 300 SW column. Lane 7: active fraction from the final DEAE 5 PW column.

this major band was assigned as the DHAE II subunit.

M_r Determination. As estimated by chromatography on a Protein-Pak 300 SW, the purified DHAE I has an M_r of 117K ± 10K (R = 0.984), indicating that the enzyme is either a pentamer or a hexamer having a subnit of 22.3K. Similarly, the purified DHAE II has an M_r or 33K ± 2K (R = 0.984) and appears to be a dimer having a subunit of 16K.

pH Dependency. The epoxidation product **4** started to decompose significantly under basic conditions (>pH 7.5). In addition, the substrate **3** was prone to air oxidation and this became predominant as the pH increased (>25% of **3** was converted to ABQ at pH 7.5 in 10 min). Therefore, when the enzymatic epoxidation was carried out in buffer above pH 7.5, these factors had to be taken into consideration. Under normal assay conditions DHAE I displayed an optimal pH of 6.5 in 0.1 M potassium phosphate buffer. In contrast, DHAE II displayed an optimal pH of 5.5 in the same buffer.

Kinetics. Assuming that the O_2 concentration was constant in the assay solution, a kinetic analysis was carried out on the basis of a pseudo-first-order treatment with a steady-state approach. The effect of the initial concentration of **3** on the formation of **4** indicated that both DHAE I and DHAE II followed classical Michaelis–Menten kinetics. Substrate inhibition was observed for the former at concentrations above 150 μM and for the latter at concentrations above 100 μM. From a Lineweaver–Burk plot, the apparent K_m and V_{max} were estimated to be 8.4 ± 0.5 μM and 3.7 ± 0.2 μmol min^{-1} mg^{-1}, respectively, for DHAE I. The apparent K_m for DHAE II was estimated to be 7.2 ± 0.4 μM.

Table II: Effect of Potential Monooxygenase Cofactors on DHAE I and DHAE II Activities

entry	assay component[a]	additive	concn (mM)	rel act.[b] (%) DHAE I	rel act.[b] (%) DHAE II
1	**3** + E	none		100	100
2	**3** + E (heat denatured)	none		0	0
3	ABQ + E	none		0	0
4	ABQ + NADH	NADH	100	40	*c*
5	**3** + E	NADH	100	101	102
6	**3** + E	NADPH	100	100	106
7	**3** + E	NAD^+	100	98	104
8	**3** + E	$NADP^+$	100	96	93
9	**3** + E	FMN	10	94	57
10	**3** + E	FAD	10	96	87
11	**3** + E	air[d]		67	69
12	**3** + E	N_2[d]		0	0

[a] Assay composition and conditions are described under Materials and Methods. [b] The control (entry 1) corresponds to 26% formation of **4** (6.5 nmol). [c] Not measured. [d] The assay tube was degased and flushed with the desired gas several times in 5 min and then assayed as usual. For N_2 the assay tube was sealed after the 5-min pretreatment and then assayed as usual.

Cofactor Requirement. Preliminary studies of DHAE I and DHAE II had demonstrated that **3** could be epoxidized in the absence of any added cofactor, requiring only molecular oxygen (Gould & Shen, 1991). To extend this, potential cofactors for monooxygenases were added to the assay solution. As shown in Table II, the reaction apparently did not involve any loosely bound cofactors: addition of NADH, NADPH, NAD^+, $NAD(P)^+$, FAD, or FMN neither stimulated nor inhibited DHAE I. Similar results were found for DHAE II, except that both FMN and FAD caused slight inhibition (entries 9 and 10). As demonstrated before, a combination of ABQ and NADH could also support the epoxidation (entry 4), but this resulted from **3**, which was generated by the chemical reduction of ABQ with NADH (Gould & Shen, 1991), and no epoxidation was observed when ABQ was used alone as the substrate (entry 3). The enzyme appeared to be very fragile, and approximately 30% of the activity was lost simply by the pretreatment used for the gas study (entry 11). Removing O_2 by exchange with nitrogen inhibited the epoxidation completely (entry 12). Finally, as expected, enzyme activity was totally lost upon heat denaturation (entry 2).

Inhibition Studies. Potential inhibitors were added to assay solutions of each enzyme. Table III summarizes the results. Addition of catalase, a hydrogen peroxide scavenger, did not inhibit the epoxidation (entries 2 and 3). Partial inhibition of DHAE I was observed from addition of CN^-, but the effect on DHAE II was much less (entry 4). CO had no more effect

Table III: Effect of Potential Monooxygenase Inhibitors on DHAE I and DHAE II Activities

entry	addition[a]	concn	rel act.[b] (%) DHAE I	DHAE II
1	none		100	100
2	catalase	10 units	90	87
3	catalase	50 units	91	89
4	KCN	1.0 mM	44	81
5	PCMBA	0.5 mM	60	82
6	PCMBA	1.0 mM	46	29
7	EDTA	1.0 mM	93	108
8	EDTA	5.0 mM	92	106
9	1,10-phenanthroline	1.0 mM	0	0
10	1,10-phenanthroline	0.2 mM	0	0
11	air		67[c]	69[c]
12	CO		70[c]	43[c]
13	1,10-phenanthroline	0.2 mM		
	$CuSO_4$	0.4 mM	61[d]	65[d]
14	1,10-phenanthroline	0.2 mM		
	CuCl	0.4 mM	67[d]	74[d]
15	1,10-phenanthroline	0.2 mM		
	$FeSO_4$	1.0 mM	65[d]	80[d]

[a] Assay composition and conditions are described under Materials and Methods. [b] The control (entry 1) corresponds to 26% formation of 4 (6.5 nmol). [c] The assay tube was degased and flushed with air or CO several times in 5 min and then assayed as usual. [d] In the presence of these metal ions the air oxidation of 3 became significant even at pH 6.5.

Table IV: Effect of Metal Ions on DHAE I and DHAE II Activities

entry	addition[a]	concn (mM)	rel act.[b] (%) DHAE I	DHAE II
1	none		100	100
2	$FeSO_4$	1.0	71[c]	71[c]
3	$CuSO_4$	0.2	63[c]	40[c]
4	CuCl	0.2	79[c]	60[c]
5	$HgCl_2$	0.2	41[c]	54[c]
6	$MgSO_4$	0.2	94	101
7	$ZnSO_4$	0.2	89	105
8	$CaCl_2$	0.2	95	88
9	$MnCl_2$	0.2	108[c]	60[c]
10	$MnCl_2$	0.04	246	*d*
11	$NiCl_2$	0.2	266	100
12	$CoSO_4$	0.2	498	95

[a] Assay composition and conditions are described under Materials and Methods. [b] The control (entry 1) corresponds to 26% formation of 4 (6.5 nmol). [c] In the presence of these metal ions the air oxidation of 3 became significant even at pH 6.5. [d] Not measured.

on DHAE I than did air, but it did partially inhibit DHAE II (entries 11 and 12). While as high a concentration as 5.0 mM EDTA had no significant effect (entries 7 and 8), both reactions were completely inhibited by addition of as little as 0.2 mM 1,10-phenanthroline (entries 9 and 10). This inhibition by 1,10-phenanthroline could be reversed by addition of Cu^{2+}, Cu^{1+}, or Fe^{2+} to the assay mixture (entries 13–15). Thus, enzyme was first treated with 1,10-phenanthroline, leading to completely loss of activity. To this solution was added the indicated amount of Cu^{2+}, Cu^{1+}, or Fe^{2+}, and the resulting mixture was reassayed for epoxidase activity. As shown in entries 13–15, most of the enzyme activity was recovered. Finally, epoxidation was significantly inhibited by addition of the sulfhydryl reagent *p*-chloromercuribenzoic acid (PCMBA) (entries 5 and 6).

Metal Ion Effect. Since it was possible that during the multistep purification the enzyme could lose some of its metal ions if they were loosely bound, metal ions were added to the assay solution and their effect on the enzymatic epoxidation was examined. Table IV presents the data. As previously described, the substrate 3 was prone to air oxidation and this process was found to be accelerated in the presence of Cu^{2+}, Cu^{1+}, Hg^{2+}, and Mn^{2+}. The relative rates in these cases were, therefore, probably a bit low since substrate concentration was simultaneously being depleted. This was demonstrated for DHAE I in the case of Mn^{2+}. When 0.2 mM was added, less than 5.0% of 3 had been left at the end of the incubation period and 3 was converted either to ABQ or to 4 with a relative reactivity of 108% (entry 9). However, when the concentration of Mn^{2+} was decreased to 0.04 mM, less than 5% of ABQ was formed and substantially more 4 was produced with a relative activity of 246% (entry 10). Therefore, if competition between the air oxidation and enzymatic epoxidation was corrected, it appeared that the addition of 0.2 mM Cu^{1+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Zn^{2+}, or Ca^{2+} or 1.0 mM Fe^{2+} had neither activated nor inhibited the epoxidation reactions (entries 2–8). Remarkably, however, Co^{2+} and Ni^{2+} as well as Mn^{2+} accelerated the epoxidation catalyzed by DHAE I as much as 5-fold (entries 10–12). Enhancement of DHAE II activity was not observed.

Table V: Effect of Metal Ions on the Apoenzyme of DHAE I

entry	addition[a]	concn (mM)	rel act.[b] (%)
1	holoenzyme		100
2	apoenzyme		0
3	$FeSO_4$	0.2	0
4	$CuSO_4$	0.2	0
5	CuCl	0.2	0
6	$HgCl_2$	0.2	0
7	$MgSO_4$	0.2	0
8	$ZnSO_4$	0.2	0
9	$CaCl_2$	0.2	0
10	$MnCl_2$	0.2	53[c]
11	$NiCl_2$	0.2	484
12	$CoSO_4$	0.2	518

[a] Assay composition and conditions are described under Materials and Methods. [b] The control (entry 1) corresponded to 18% formation of 4 (5.5 nmol). [c] Most of 3 was air oxidized to ABQ (14.8 nmol, 59%).

Reconstitution of DHAE I from an Apoenzyme. The apoenzyme of the DHAE I was next prepared by 1,10-phenanthroline treatment of the holoenzyme followed by removal of the complexed metal ions and excess 1,10-phenanthroline by Sephadex chromatography (Penefsky, 1977; Maret, 1986; Wagner, 1988). Complete loss of the epoxidase activity resulted from this treatment. Various metal ions were then added to this preparation in order to see if the metal extraction process was reversible and the enzyme activity could be reconstituted. Table V summarizes the results. Remarkably, although monooxygenases requiring metal ions have been well-known and iron and copper have been found to be the predominant (in fact, almost exclusive) metal ions involved, DHAE I was reconstituted only by addition of Co^{2+}, Ni^{2+}, or Mn^{2+}. In each of these cases, the reconstituted enzyme showed even substantially better activity then the control (entries 9–11). On the other hand, no epoxidation was observed upon addition of either 0.2 mM Cu^{1+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, or Fe^{2+} (entries 3–8). In contrast to DHAE I, no epoxidase activity could be reconstituted from a preparation of DHAE II apoenzyme similarly generated.

Discussion

The isolation of DHAE II gives strong support to the hypothesis that the biosynthesis of 5 follows a pathway parallel to the biosynthesis of 1 (Gould & Shen, 1991) but with an additional deacetylation as the last step (Scheme I). Although no complementary in vivo study has been performed to correlate this enzyme with the biosynthesis of 5, the product 4 was very readily metabolized in the presence of the CFE, suggesting that some other activities in the CFE could very

efficiently consume **4**. In the presence of CFE almost 50% of the **4** formed was further metabolized during the second 7.5 min of incubation. It has been reported that **5** decomposed readily upon concentration (Box et al., 1983).

Numerous epoxyquinones and epoxysemiquinones have been reported (in the 1,4-benzoquinone family alone more than 25 have been isolated), but very little has been known about the formation of the epoxyquinone functionality. Although the oxygen of the oxirane ring (epoxide oxygen) in all cases studied has come from molecular oxygen (Nabeta et al., 1973, 1975; Omura et al., 1981; Read et al., 1969; Thiericke et al., 1990; Whittle & Gould, 1987), presumably via an enzymatic process involving a monooxygenase (Hubbard et al., 1989; Omura et al., 1981; Priest & Light, 1989; Sadowski et al., 1977), the nature of the epoxidation remained a matter of speculation. Evidence was obtained that supported either a quinone (Gould et al., 1989; Nabeta et al., 1973, 1975; Omura et al., 1981; Read et al., 1969) or a hydroquinone (Gould & Shen, 1991; Priest & Light, 1989; Read et al., 1969; Sadoski et al., 1977) substrate for epoxidations.

The purification of DHAE I from *Streptomyces* LL-C10037 and DHAE II from *Streptomyces* MPP 3051 and the subsequent study of their cofactor requirements unambigiously established the stoichiometry of the biosynthesis of the enantiomeric epoxyquinones. Hydroquinone can be epoxidized directly to form epoxyquinone, and only molecular oxygen is required for this process. Unlike most monooxygenases, which require a reduced cofactor such as NAD(P)H, in these cases the substrate hydroquinone itself apparently serves as the reducing equivalent. This has also been observed with amine oxidases; this type of enzyme has been named an internal monooxygenase. In addition to DHAE I and DHAE II, the mammalian dihydrovitamin K epoxidase (Hubbard et al., 1989; Sadowski et al., 1977) and a particulate preparation from the fungus *Penicillium patulum* (Priest & Light, 1989) have also been shown to utilize a hydroquinone without added coenzyme. However, those working with microbial systems apparently overlooked results from cell-free preparations of rat livers that could epoxidize dihydrovitamin K in the absence of added coenzyme (Sadowski et al., 1977; Suttie et al., 1981) and vice versa for those working on the mammalian enzyme (Ham & Dowd, 1990; Hubbard et al., 1989; Suttie et al., 1981).

It seems likely that in all cases the hydroquinone is the true substrate for an internal monooxygenase, defining a hitherto unrecognized class that we now refer to as "hydroquinone monooxygenase (epoxidizing)". The reported epoxidation of nanaomycin A (a naphthoquinone) with a crude cell-free extract requiring NAD(P)H as the coenzyme (Omura et al., 1981) is strikingly similar to early results with DHAE I and DHAE II (Gould & Shen, 1990) and dihydrovitamin K epoxidase (Sadowski et al., 1977), and it is likely that the actual substrate had been the hydroquinone of nanaomycin A, generated in situ from chemical or enzymatic reduction by NAD(P)H.

Table VI lists all the known features of DHAE I and DHAE II. The two enzymes, although differing in the number of subunits and their size and displaying opposite facial specificity with respect to their common substrate, share many similarities in catalyzing the epoxidation reaction.

DHAE I and DHAE II belong to none of the previously defined groups of monooxygenases. Cofactors such as NADH, NADPH, NAD^+, $NADP^+$, FAD, or FMN neither stimulate nor significantly inhibit the epoxidation. The partial inhibition of DHAE I only with CN^- and of DHAE II only with CO suggests that a cytochrome P_{450} system is not likely involved. The epoxidase activities are substantially inhibited by PCMBA, suggesting an involvement of a sulfhydryl group. Although EDTA had no effect, complete inhibition of the DHA epoxidases by addition of 0.2 mM 1,10-phenanthroline clearly indicated a requirement for a metal ion, which selectively formed a very tight complex with this latter chelator. Recovery of these activities from 1,10-phenanthroline inhibition by in situ addition of Cu^{2+}, Cu^{1+}, and Fe^{2+} could have resulted from reconstitution by either Cu^{2+}, Cu^{1+}, or Fe^{2+} directly, or by the natural metal ion(s), which had been freed from its 1,10-phenanthroline complex upon addition of Cu^{2+}, Cu^{1+}, or Fe^{2+}, which have very high stability constants for complexes with 1,10-phenanthroline (Schilt, 1969; Sillen & Martell, 1964, 1971). The latter possibility is supported by the enhancement of DHAE I activity observed upon addition of only Mn^{2+}, Ni^{2+}, or Co^{2+} *and* the demonstration that the DHAE I apoenzyme could be reconstituted only by these three metal ions.

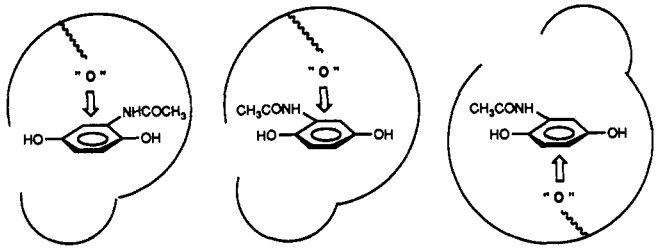
Unlike DHAE I, the DHAE II apoenzyme was unstable and was rapidly and irreversibly denatured upon removal of the metal ion. This would also explain the lack of activity enhancement when metals were added to the purified enzyme preparation: if the metal ion for this enzyme was lost during the purification, the enzyme activity could not be reconstituted by providing the right metal. The reversal of 1,10-phenanthroline inhibition by in situ addition of Fe^{2+}, Cu^{2+}, or Cu^{1+} could, therefore, suggest that 1,10-phenanthroline may initially inhibit the epoxidase activity by forming a transient ternary complex with the metal ion and enzyme, but this could be dissociated upon addition of external metals (Wagner, 1988).

The natural metal ion of DHAE I has not yet been clearly identified, since it has not yet been determined whether the apoenzyme, as prepared, is indeed free from any metals. However if this were the case, DHAE I would appear to be an enzyme that requires Mn^{2+}, Ni^{2+}, or Co^{2+}, representing a new type of monooxygenase. Since the apoenzyme was quite stable, this could explain the activation of DHAE I by addition of Mn^{2+}, Ni^{2+}, or Co^{2+}, possibly replacing equivalents lost during the purification. At this point, it is also possible, but less likely, that neither Mn^{2+}, Ni^{2+}, nor Co^{2+} is the natural metal ion, but each can substitute for the natural one to reconstitute the enzyme activity. With both carboxypeptidase A (Coleman & Vallee, 1960, 1961) and thermolysin (Holmquist & Vallee, 1974), Zn^{2+} could be replaced by various metals, including Mn^{2+}, Ni^{2+}, and Co^{2+}. In fact, Co^{2+}-sub-

Table VI: Characteristics of DHAE I and DHAE II

characteristic	*Streptomyces* LL-C10037	*Streptomyces* MPP 3051
substrate	**3** + O_2	**3** + O_2
product	**5**	**5**
molecular weight	117K ± 10K	33K ± 2K
subunit	22.3K	16K
number of subunit	5 or 6	2
apoenzyme	stable	unstable
optimal pH	6.5	5.5
K_m for **3**	8.4 mM	7.2 mM
substrate inhibition (**3**)	150 mM	100 mM
V_{max}	3.7 mmol min^{-1} mg^{-1}	undetermined
cofactor	none	none
PCMBA	inhibited	inhibited
1,10-phenanthroline	inhibited	inhibited
KCN	inhibited	inhibited
CO	no	inhibited
Mn^{2+}	activated	no
Ni^{2+}	activated	no
Co^{2+}	activated	no

Scheme II



6 7 8

stituted carboxypeptidase A showed better activity (215%) than its natural enzyme, as did the Co^{2+}-substituted thermolysin (200%). However, neither Zn^{2+} nor any of six other metals could reactivate DHAE I apoenzyme.

Priest and Light (1989) proposed a mechanism for the *P. patulum* epoxidation of the hydroquinone substrate gentisyl alcohol (patulin biosynthesis) involving the formation of a hydrogen peroxide intermediate by attack at C-3; catalase had no effect on the enzymatic epoxidation, hence ruling out the possibility of free hydrogen peroxide as an intermediate. A non-enzymic model without a divalent metal for the action of the dihydrovitamin K dependent carboxylase/epoxidase was proposed (Ham & Dowd, 1990) in which an organic peroxide intermediate generated at C-5 was involved. Glutathione peroxidase, which can reduce organic peroxides, showed an inhibitory effect on the dihydrovitamin K dependent microsomal carboxylase (Suttie et al., 1981), which catalyzes both the peptide carboxylation and dihydrovitamin K epoxidation (Hubbard et al., 1989). No inhibitory effect was observed upon addition of catalase to DHAE I or DHAE II, again clearly eliminating the involvement of free hydrogen peroxide as an intermediate.

Since a metal ion is essential to the DHAE I activity, an enzyme-bound metal–oxygen species is most likely required for activation of molecular oxygen, with the reaction proceeding via either an ionic or a radical process, and transfer of the activated molecular oxygen to an enzyme-bound hydroquinone anion or radical would result in the formation of epoxyquinone **4**. A number of mechanisms have been proposed for the activation of molecular oxygen by monooxygenases. These include simple flavin proteins (Muller, 1985; Walsh, 1979) or enzymes having complicated electron transfer mechanisms from molecular oxygen to the oxygenated substrates. Among these latter, the iron-containing monooxygenases, either heme or non-heme systems, are those that have been most studied (Guengerich & MacDonald, 1984; Holland, 1982; Matsuura & Nishinaga, 1981). While model studies containing metals other than iron to activate molecular oxygen have been developed (Battioni et al., 1988; Collman et al., 1985; Kimura & Machida, 1984; Kushi et al., 1985; Maruyama et al., 1989; Nolte et al., 1986; Powell et al., 1984, and references cited therein), metal ions other than iron and copper have only very rarely been found in enzymes activating molecular oxygen. Of the three metals that can reconstitute DHAE I, only manganese has previously been reported to play any role in oxygenase enzymes (Paszczynski et al., 1985; Que et al., 1981; Rutherford, 1989). DHAE I is the first example in which nickel or cobalt may be associated with the enzymatic activation of molecular oxygen. In fact, to date only two cobalt-containing enzymes not involving vitamin B_{12} (Moura et al., 1980; Northrop & Wood, 1969) and four nickel-containing enzymes (Ankel-Fuchs & Thauer, 1988) have been reported. Since the metabolism of molecular oxygen plays such a vital role in biological systems, further study of the mechanism of the DHAE I epoxidation not only may lead to the detailed elucidation of the biosynthesis of epoxyquinones but also may provide fundamental information for the understanding of molecular oxygen activation in other biological systems.

Enantiomers are relatively rare in nature. In only a few such situations have complementary pairs of enzymes been isolated and shown to utilize the same achiral substrate (Anderson & Hammes, 1984; Croteau et al., 1987; Croteau & Karp, 1979; Croteau & Shaskus, 1985; Dennis & Kaplan, 1960; Gambliel & Croteau, 1984; Huang & Tang, 1970; Lenz et al., 1971; Levis, 1970; Martinez-Carrion & Jenkins, 1965a,b; Saier & Jenkins, 1967a,b; Saito et al., 1981; Wunderwald et al., 1971; Yoshida, 1965). Approximately 20 other enzymes each catalyze a racemization and, therefore, proceed through an achiral intemediate. [Since enzyme-catalyzed reactions are in principle reversible, the D- and L-amino acid oxidases, the D- and L-lactate/cytochrome dehydrogenases, the D- and L-serine dehydratases, and the 6-hydroxy-L- and D-nicotine oxidases (Decker et al., 1972; Bruhmuller et al., 1972) could be construed to fit this description, but they each almost certainly catalyze only the opposite process under cellular conditions (i.e., conversion of a single enantiomer to an achiral product), so the required stereochemical recognition would appear to be more facile.] Little is known about the enantiomeric specificity of such enzymes (Shen et al., 1983; Cardinale & Abeles, 1968); recently an X-ray structure of mandelate racemase at 2.5-Å resolution was reported (Neidhart et al., 1990). The opposite facial specificities of the two epoxidases DHAE I and DHAE II could result from controlling the orientation of the substrate with the activated oxygenating species delivered from the same side (Scheme II, **6** and **7**). Alternatively, the two enzymes could deliver the activated oxygenating species from opposite sides of the substrate bound in the same orientation (Scheme II, **7** and **8**). Further studies on the reaction mechanism and determination of the three-dimensional features of the active sites will provide an understanding of the factors that control the selective formation of this pair of enantiomeric natural products.

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