Cis,cis-Muconate Lactonizing Enzyme from *Trichosporon cutaneum*: Evidence for a Novel Class of Cycloisomerases in Eucaryotes†,‡

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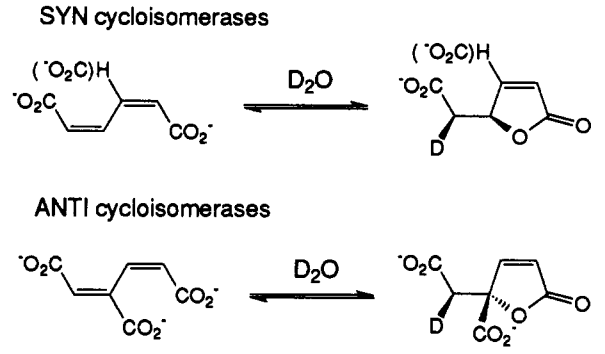
ABSTRACT: The absolute stereochemical courses of *cis,cis*-muconate lactonizing enzyme (MLE; EC 5.5.1.1) from *Trichosporon cutaneum* (TcMLE) and chloromuconate cycloisomerase (MLE II; EC 5.5.1.7) from *Pseudomonas* sp B13 have been determined from ^{1}H NMR measurements. Both cycloisomerases convert *cis,cis*-muconate to (4*S*)-muconolactone by a syn lactonization, the absolute stereochemical outcome of which is identical to that observed with MLE from *Pseudomonas putida*. The regiochemical courses of cyclization of 3-halo-*cis,cis*-muconates by TcMLE and MLE II have been characterized and shown to differ in a halogen substituent dependent manner, suggesting at least a different active site architecture of the two MLEs. Moreover, the regiochemical preferences of MLE II and TcMLE parallel results previously observed for the nonenzymatic lactonization of the 3-halomuconates at pH 1–6 and in concentrated HCl, respectively, in which alternate mechanisms of cyclization were proposed [Pieken, W. A., & Kozarich, J. W. (1990) *J. Org. Chem. 55*, 3029–3035]. Complementary DNA clones encoding TcMLE have been isolated from phenol induced *T. cutaneum* cDNA using the polymerase chain reaction. The deduced amino acid sequence does not exhibit any similarity to that of MLE from *P. putida*. It does however, exhibit moderate sequence similarity (21% residue identity, 14 gaps) with 3-carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE; EC 5.5.1.5) from *Neurospora crassa*, which catalyzes a regiochemically analogous and stereochemically identical lactonization reaction with 3-carboxymuconate. The limited data available suggest that the fungal CMLE and yeast MLE are representative of a unique class of eucaryotic cycloisomerases which have evolved convergently with the bacterial MLEs.

The cycloisomerases, *cis,cis*-muconate lactonizing enzyme (MLE)[1] and 3-carboxy-*cis,cis*-muconate lactonizing enzyme (CMLE) occur in the parallel catechol and protocatechuate branches of the β-ketoadipate pathway, respectively (Ornston & Yeh, 1982). The enzymes can be broadly classified in terms of their stereochemical outcome as either syn or anti cycloisomerases (Scheme 1). The cyclization of 3-methyl-*cis,cis*-muconate in the fungus *Aspergillus niger* occurs by a

Scheme 1



syn (4*S*) absolute stereochemical course (Cain et al., 1989b), identical to that observed with MLE from *Pseudomonas putida* (PpMLE). CMLE from the ascomycetes fungus *Neurospora crassa* (NcCMLE) has also been established as a syn cycloisomerase, with the same absolute stereochemical course (Kirby et al., 1975). This stereochemical uniformity prompted us to investigate the potential relationships of syn cycloisomerases of procaryotic and eucaryotic origin. Recent results from this laboratory have provided primary structural and mechanistic evidence which suggest that *N. crassa* CMLE is a unique cycloisomerase distinct from syn cycloisomerases found in bacteria (Mazur et al., 1994).

Several notable differences occur between bacterial and eucaryotic MLEs, suggesting that these enzymes might also be unrelated. While (4*S*)-3-methylmuconolactone (Scheme 3, **3d**) is formed by MLE in both *A. niger* (Cain et al., 1989b) and the basidiomycetes yeast *Trichosporon cutaneum*

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[1] Abbreviations: BME, 2-mercaptoethanol; BSA, bovine serum albumin; CMLE, 3-carboxy-*cis,cis*-muconate lactonizing enzyme; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; ELH, enol-lactone hydrolase; MI, muconolactone isomerase; MLE (also referred to as MLE I), *cis,cis*-muconate lactonizing enzyme (*cis,cis*-muconate cycloisomerase; EC 5.5.1.1); MLE II, *cis,cis*-muconate lactonizing enzyme II (chloromuconate cycloisomerase; EC 5.5.1.7); NcCMLE, *Neurospora crassa* CMLE (3-carboxy-*cis,cis*-muconate cyclase; EC 5.5.1.5); PCR, polymerase chain reaction; PMSF, phenylmethanesulfonyl fluoride; PpCMLE, *Pseudomonas putida* CMLE (3-carboxy-*cis,cis*-muconate cycloisomerase; EC 5.5.1.2); PpMLE, *Pseudomonas putida* MLE; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; sscDNA, single strand complementary DNA; TcMLE, *Trichosporon cutaneum* MLE.

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(Powlowski & Dagley, 1985; Cain et al., 1989a), the opposite regioisomer, (4*S*)-4-methylmuconolactone (Scheme 3, **2d**), is formed in *P. putida* (Knackmuss et al., 1976; Cain et al., 1989b). There are also distinctions between the MLEs in regard to substrate specificity. A single isoform of MLE isolated from the same strain of *T. cutaneum* used in the present work (TcMLE), and induced with either phenol or *p*-cresol, has been shown to turn over 3-methyl-*cis,cis*-muconate with approximately 1.5-fold higher relative activity compared to *cis,cis*-muconate (Powlowski et al., 1985). Gaal and Neujahr (1980) have characterized MLE from a different strain of phenol induced *T. cutaneum*, which turns over 3-substituted (halo- and methyl-) muconates with a reduced yet significant rate (ca. 25% relative activity) compared to *cis,cis*-muconate. They also reported the separation of two isoforms of MLE, although the specificity of the individual isoforms was not clarified. In contrast, two isoforms of MLE from *Pseudomonas* sp B13 with distinct substrate specificity have been isolated and individually characterized (Schmidt & Knackmuss, 1980). MLE, also designated MLE I, exhibits maximal velocity with *cis,cis*-muconate, while MLE II exhibits 2–3-fold higher relative maximal velocity with 2- and 3-substituted (methyl- and chloro-) *cis,cis*-muconates compared to MLE I. Cloning of the *catB* and *clcB* genes of *P. putida* encoding MLE I and MLE II, respectively, demonstrated significant homology between the two sequences (41% amino acid identity) and suggests that the two cycloisomerases have diverged from a common ancestral gene (Aldrich et al., 1987). An additional distinction concerns the role of metal ions in the MLEs. The preliminary characterization of TcMLE suggests that the enzyme does not require divalent metal ions for activity (Gaal & Neujahr, 1980; Powlowski et al., 1985), in contrast to the absolute requirement for divalent metal in PpMLE (Ngai et al., 1983a).

We have previously investigated the nonenzymatic lactonization of 3-halomuconates and have noted a pronounced pH and halo substituent dependency of the regiochemical course of the cyclization reactions (Pieken & Kozarich, 1990). The results were rationalized in terms of different mechanisms of addition of the carboxylic acid to the reactive double bond. In this context, the regiochemical disparity observed between procaryotic and eucaryotic MLEs in the lactonization of 3-methylmuconate suggested that analysis of the reaction of the MLEs with 3-halomuconates might not only allow for a comparison of regiochemical preferences of cycloisomerases of diverse origin but might also provide mechanistic insight into the enzymatic reaction.

In the present work we have investigated mechanistic and evolutionary relationships between *T. cutaneum* MLE and other syn cycloisomerases. We report here the determination of the absolute stereochemical courses of TcMLE and *Pseudomonas* sp B13 MLE II, the reactivity of TcMLE with 3-halomuconates in comparison to MLE II, and the isolation and sequencing of the cDNA encoding TcMLE. The results indicate that while TcMLE is also a syn cycloisomerase, it is dissimilar to the bacterial MLE in both its regiochemical course with 3-halomuconates and in its primary structure, implying that eucaryotic and bacterial MLEs have arisen by convergent evolution. However, TcMLE does exhibit moderate amino acid sequence similarity with CMLE from *N. crassa* suggesting that the two eucaryotic enzymes share a common but distant genetic origin and likely represent a unique class of eucaryotic cycloisomerases.

MATERIALS AND METHODS

General. The 3-methyl-*cis,cis*-muconate and 3-halo-*cis,cis*-muconates were prepared as described previously (Pieken & Kozarich, 1989). All buffers and chemicals were from commercial vendors and of reagent grade quality. The FPLC and chromatography media were from Pharmacia. Enzymes and reagents for molecular biology were obtained from commercial vendors as noted in the methods section below. The polymerase chain reaction was performed in a Temptronic thermal cycler (Barnstead/Thermolyne Corp., Dubuque, IA). Protein concentration was determined using the protein assay dye reagent (Bio-Rad) with BSA as the standard. UV spectra were recorded on a Gilford Response II spectrophotometer.

Organisms and Growth Conditions. The origin and growth conditions of *Pseudomonas* sp B13 were described previously (Dorn et al., 1974). *T. cutaneum* (ATCC 58094) was obtained from the American Type Culture Collection, Rockville, MD. Large cultures of *T. cutaneum* for MLE purification were obtained by growing a 2-L inoculum in YM broth supplemented with 0.03% phenol, which was used to inoculate 8 L of minimal medium (Sparnins et al., 1979) supplemented with 0.03% phenol. The 10-L cultures were grown at 30 °C in a Microferm fermentor (New Brunswick Scientific Co., Edison, NJ).

Purification of Cycloisomerases, Enol–Lactone Hydrolase, Muconate Isomerase, and Cycloisomerase Assays. The MLE II and TcMLE used in stereochemical and 3-halomuconate studies were partially purified from *Pseudomonas* sp B13 and *T. cutaneum*, respectively, following the published procedures of Schmidt and Knackmuss (1980) and Powlowski et al. (1985b). TcMLE and MLE II activity was assayed with 3-methyl-*cis,cis*-muconate as described by Schmidt and Knackmuss (1980) and Powlowski et al. (1985). Spectrophotometric assays with 3-halomuconate substrates were performed similarly using reported extinction coefficients (Dorn & Knackmuss, 1978). Muconate isomerase and enol–lactone hydrolase were isolated from *P. putida* according to Meagher and Ornston (1973) and McCorkle et al. (1980), respectively.

General Procedures for ^1H NMR Measurements of Cycloisomerase Reactions. Deuterated buffers were prepared by exhaustive exchange in 99.8% D_2O (Aldrich) by repeated lyophilization. When necessary, enzyme solutions were exchanged into deuterated buffer by repeated centrifugation of the enzyme in the deuterated buffer in a Centricon-10 microconcentrator (Amicon). ^1H NMR spectra were acquired on IBM AF 400 or Bruker AMX 500 spectrometers, using a presaturation pulse sequence for the suppression of the solvent H_2O peak, when necessary. Chemical shifts were standardized to the $^1H^2HO$ resonance assigned at 4.7 ppm. The general procedure for the reaction of TcMLE and MLE II with 3-halomuconates is as follows. The reaction was initiated by addition of the cycloisomerase (400 μL) to a solution of the 3-halo-*cis,cis*-muconate (5.0 mg) in 400 μL of H_2O, pH 6.8 (or D_2O, pD 7.2). ^1H NMR spectra were measured following initiation of the reaction.

^1H NMR Determination of the Absolute Stereochemical Course of cis,cis-muconate Cyclization by TcMLE and MLE II. The absolute configuration at C-4 of muconolactone was determined as follows. To a solution of *cis,cis*-muconic acid (6 mg, 42 μmol) in 500 μL of H_2O, pH 6.8, was added TcMLE (ca. 1 unit, 300 μL). After 3 h, muconolactone isomerase (100 μL) and enol–lactone hydrolase (100 μL) were added. The resulting ^1H NMR spectra are shown in Figure 1. The relative stereochemical course of the cyclization at C-5 was

determined from solvent deuterium incorporation. To a solution of *cis,cis*-muconic acid (6 mg, 42 μmol) in 600 μL of 50 mM sodium phosphate in D_2O, pD 6.0, was added TcMLE (ca. 1 unit, 300 μL). The upfield region of the ^1H NMR spectrum is shown in Figure 2. ^1H NMR (^2H_2O): (5*R*)-5-(^2H)-muconolactone; δ 7.69 (1 H, d, J = 6.0 Hz), 6.08 (1 H, d, J = 6.0 Hz), 5.40 (1 H, d, J = 8.5 Hz), 2.38 (1 H, d, J = 8.5 Hz). The absolute stereochemical course of MLE II with *cis,cis*-muconate was determined in a similar manner.

^1H NMR Analysis of the Reaction of TcMLE with 3-Fluoro-cis,cis-muconate. The reaction, which was run in completely deuterated buffer, was complete after 6 hours. ^1H NMR (^2H_2O): 5-(^2H)-4-fluoromuconolactone **2a**; δ 7.60 (1 H, d, J = 5.7 Hz), 6.31 (1 H, d, J = 5.7 Hz), 2.97 (1 H, d, $J_{H\text{-}F}$ = 15 Hz).

UV Analysis of the Reaction of TcMLE with 3-Chloro-cis,cis-muconate and 3-Bromo-cis,cis-muconate. Reactions were initiated by addition of TcMLE (10 μL) to a cuvette containing 1 mL of 3-halo-*cis,cis*-muconate, 37 μM, in 50 mM MOPS, pH 7.0. For each substrate, the decrease in absorbance at λ_{max}, 260 nm, occurred concomitantly with an increase in absorbance at 248 nm and was accompanied by isosbestic points at 238 nm and 258 nm (Figure 3).

^1H NMR Analysis of the Reaction of MLE II with 3-Fluoro-cis,cis-Muconate. The reaction, which was run in completely deuterated medium, was complete after 60 min. ^1H NMR (^2H_2O): 5-(^2H)-4-fluoromuconolactone **2a**; δ 7.61 (1 H, d, J = 6 Hz), 6.25 (1 H, d, J = 6 Hz), 3.05 (1 H, d, $J_{H\text{-}F}$ = 14 Hz).

^1H NMR Analysis of the Reaction of MLE II with 3-Chloro-cis,cis-muconate and 3-Bromo-cis,cis-muconate. The reactions were run in partially deuterated medium and were complete after 10–30 min. ^1H NMR (^2H_2O): (*E*)-dienelactone **4**; δ 8.16 (1 H, d, J = 5.7 Hz), 6.40 (1 H, d, J = 5.7 Hz), 6.00 (1 H, s). The results in completely deuterated medium were identical.

Purification of MLE from T. cutaneum. The isolation of MLE was performed following modifications of the procedure of Powlowski et al. (1985). MLE activity was monitored spectrophotometrically by observing *cis,cis*-muconate consumption at 260 nm (*cis,cis*-muconate; ϵ_{260} 16 900 M^{-1} m^{-1}). Assays were performed in 1- or 0.2-cm cuvettes and contained 100–200 μM *cis,cis*-muconate in 100 mM Na-MES, pH 6.0. The yeast paste (107 g) was resuspended in 120 mL of cold 100 mM triethanolamine hydrochloride, pH 7.0, 100 mM NaCl, 1 mM BME, 1 mM EDTA, 1 mM EGTA, 2 mM benzamidine, 0.5 mM PMSF, 1.5 μg/mL leupeptin, and 1.5 μg/mL pepstatin A. All subsequent steps were performed at 4 °C. The yeast paste was homogenized in a Bead-Beater (Biospec Inc., Bartlesville, OK), using 6 × 30 s cycles with 1–2-min cooling intervals. The crude homogenate was centrifuged (21000*g*) at 4 °C for 30 min. The supernatant (161 mL) was adjusted to 40% ammonium sulfate saturation by the addition of 107 mL of a solution of saturated ammonium sulfate, pH 7.0, stirred for 2 h, and centrifuged as above. The supernatant was collected and brought to 60% saturation by addition of 118 mL of a solution of saturated ammonium sulfate, stirred for 40 min, and centrifuged as above. The resulting pellet was dissolved in 60 mL of 20 mM histidine hydrochloride, pH 6.0, 1 mM BME, and 1 mM benzamidine (buffer B) and centrifuged as above, and the supernatant was desalted by gel filtration on a column of Sephadex G-25 equilibrated in buffer B. The sample was then applied to a DEAE-Sepharose column (2.5 × 30 cm) equilibrated in buffer B. The column was washed extensively with buffer B and eluted with a linear gradient from 0 to 0.5 M NaCl in buffer B in a volume of 700 mL at a flow rate of 2.0 mL/min, collecting 10.0-mL fractions. The active fractions (eluting at ca. 0.23–0.28 M NaCl) were pooled (89 mL), concentrated to 10.5 mL by ultrafiltration (Amicon PM-30), and loaded onto a Sephacryl S-200 gel filtration column (2.5 × 84 cm) equilibrated in buffer B. The column was eluted in buffer B at a flow rate of 0.33 mL/min, collecting 6.6-mL fractions. The active fractions were pooled (49 mL) and applied to a second DEAE-Sepharose column (1.3 × 11 cm) equilibrated in buffer B, pH 6.3. The column was eluted with a linear gradient from 0 to 0.5 M NaCl in buffer B, pH 6.3, in a volume of 60 mL at a flow rate of 0.5 mL/min, collecting 2.0-mL fractions. The active fractions were pooled (6 mL) and purified by chromatofocusing. The sample was loaded at a flow rate of 1.0 mL/min on a Mono-P 5/20 FPLC column equilibrated in 25 mM BisTris hydrochloride, pH 6.4, and pre-run with 3.0 mL of polybuffer 74 (1:10), pH 4.0. Following loading, the protein was eluted with polybuffer 74 (1:10), pH 4.0, at a flow rate of 0.5 mL/min; fractions were collected manually. Removal of the polybuffer and final purification was accomplished by gel filtration on two Superose-12 HR 10/30 FPLC columns connected in series, equilibrated in 25 mM BisTris hydrochloride, pH 6.3, 100 mM NaCl, and 1 mM BME, at a flow rate of 0.4 mL/min. The enzyme pool (0.4 mL) was stored at 4 °C and −80 °C for long-term storage. The protein so purified (ca. ≥90% homogeneity) was still susceptible to proteolysis, an observation previously noted by Powlowski et al. (1985).

N-Terminal Sequence Analysis. TcMLE purified as above was subjected to N-terminal sequence analysis by Edman degradation. Automated protein sequencing was performed on an Applied Biosystems Model 473A sequencer.

Endoproteinase Lysine C Digestion. TcMLE was digested with 0.04 μg of Lys-C in 100 μL of 100 mM Tris hydrochloride, pH 8.0, at 37 °C for 16 h. Peptides were separated by reverse-phase HPLC on a C_4 Synchrom column (2 × 100 mm) equilibrated in water/0.1% trifluoroacetic acid and eluted with a 70% linear gradient of acetonitrile/0.08% trifluoroacetic acid over 40 min at a flow rate of 0.2 mL/min.

Isolation of Phenol Induced mRNA and Synthesis of cDNA. Total RNA was isolated from frozen phenol induced *T. cutaneum* which was powdered with a mortar and pestle under liquid nitrogen, and the RNA was extracted with guanidinium isothiocyanate (Choczynski & Sacchi, 1987). Polyadenylated mRNA was purified by oligo(dT)-cellulose spin column (Pharmacia) chromatography. Single strand cDNA (sscDNA) was prepared using the First strand cDNA synthesis kit (Pharmacia) primed with the accompanying *Not*I-$(dT)_{18}$ primer (5′-AAC TGG AAG AAT TCG CGG CCG CAG GAA T_{18}-3′). The cDNA:RNA hybrid was purified directly on a Chroma-spin TE-100 column (Clonetech), or the sscDNA was isolated from the cDNA:RNA hybrid by alkaline hydrolysis as follows. NaOH (4 M) was added to a final concentration of 40 mM and the mixture incubated at 65 °C for 30 min; the solution was adjusted to pH 8.0 with 0.1 N HCl and the sscDNA purified on a Chroma-spin TE-100 column.

PCR Cloning. The cDNA encoding the N-terminal region of TcMLE was obtained by PCR amplification employing degenerate primers, *Taq* DNA polymerase, and phenol induced *T. cutaneum* sscDNA as template. The primers used were 5′-*GAATTC* TA(CT) GA(CT) AT(ACT) (CT)T(ACGT) ATG GG-3′ (sense) and 5′-*GAATTC* GT(ACGT) GC(CT)

TC(AG) TT(ACGT) AC(CT) TC-3′ (anti-sense), which are the degenerate complement of TcMLE amino acid residues 7–12 (YDILMG) and 35–40 (EVNEAT), respectively (*Eco*RI restriction sites are noted in italics). The resulting 113-bp product was subcloned into pCRII (Invitrogen) and transformed into *E. coli* INVαF′ (Invitrogen). Clones encoding the full length MLE were obtained by anchored PCR amplification using a TcMLE specific (sense) primer encoding residues 1–12, 5′-*CATATG*GCG GTT GCG CCG ACC AGC TAT GAT ATT TTG ATG GG-3′ (*Nde*I restriction site is noted in italics) and the anchor (anti-sense) primer, 5′-AAC TGG AAG AAT TCG CGG CCG CAG G-3′, the sequence of which is derived from the *Not*I-$(dT)_{18}$ primer employed in cDNA synthesis. The PCR cycling parameters consisted of initial denaturation at 95 °C for 2 min and 50 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min. The first 10 cycles of amplification were performed with all necessary reagents (1.5 units *Taq* DNA polymerase and *Taq* DNA polymerase buffer, sscDNA template, 3–4 mM $MgCl_2$, 1 mM dNTPs, and 1 μM TcMLE primer) except the anchor primer, which was subsequently added with mixing, prior to initiation of the eleventh cycle. Authentic full length TcMLE clones were identified by nonisotopic Southern blot analysis (Southern, 1975) using a 99-bp digoxigenin labeled TcMLE probe corresponding to MLE base pairs 34–133. The probe was prepared by PCR (Lanzillo, 1991), substituting dTTP with a 2:1 mixture of dTTP and digoxigenin-dUTP (Boehringer-Mannheim). The resulting 1.2 kbp amplification product was subcloned directly into pCRII and transformed into *E. coli* INVαF′. Five independent TcMLE clones were isolated (pCRIIMLE 45-2, 50-1, 50-7, 50-8, 50-9) and completely sequenced using custom 17-mer oligonucleotide primers; clones 45–2 and 50–1 were sequenced on both strands.

Oligonucleotide Synthesis, DNA Manipulation, and DNA Sequencing. Oligonucleotides for PCR and DNA sequencing were obtained from Oligo's Etc. (Wilsonville, OR). Prior to all ligations, DNA fragments were purified by electrophoresis in low-melt agarose (IBI) and isolated with Geneclean or Mermaid DNA purification resins (Bio101). Ligation reactions were performed with T4 DNA ligase (Promega). Double strand plasmid DNA was isolated using Magic miniprep (Promega). DNA sequence analysis was performed on alkaline denatured plasmid by the dideoxy method (Sanger et al., 1977) using the Sequenase Version 2.0 kit (United States Biochemical Corp.) and [α-^{35}S]dATP (DuPont-New England Nuclear).

Sequence Homology Analysis. Sequence database analysis and sequence alignments were performed with the LaserGene sequence analysis software (DNAstar, Madison, WI). The DNA and deduced amino acid sequences of NcCMLE were compared with those found in GenBank/EMBL (release 75), NBRF-PIR (release 35), and SwissProt (release 24), and Prosite (release 10) databases using GeneMan. Statistical evaluation of pairwise sequence comparisons was performed with the Monte algorithm (Altschul & Erickson, 1986) using 50 randomizations of the TcMLE sequence and the Dayhoff cost matrix. The algorithm was used as presented in the Eugene sequence analysis software (Molecular Biology Information Resource, Baylor College of Medicine, Houston, TX).

RESULTS

Absolute Stereochemical Course of T. cutaneum MLE and Pseudomonas sp B13 MLE II. The absolute configuration at C-4 of muconolactone derived from *cis,cis*-muconate was

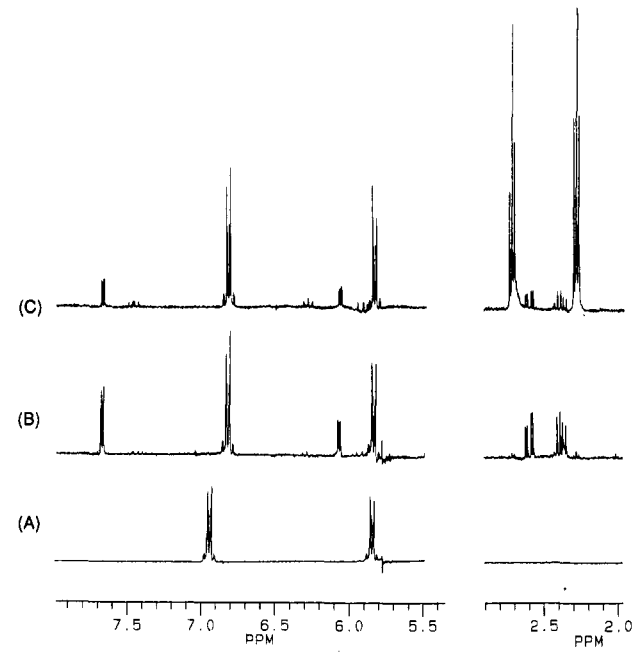
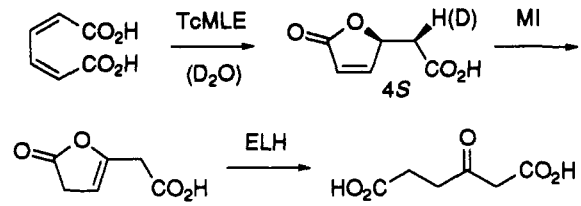


FIGURE 1: ^{1}H NMR (400-MHz) analysis of the reaction of *cis,cis*-muconate with TcMLE, muconolactone isomerase (MI), and enol–lactone hydrolase (ELH). The ^{1}H NMR spectra were acquired at the following time points: (A) prior to addition of TcMLE, (B) 200 min after addititon of TcMLE (the C-4 proton of muconolactone is not shown), and (C) 30 min after addition of MI and ELH (the C-2 protons of β-ketoadipate are not shown).

Scheme 2. Reactions of *cis,cis*-Muconate with MLE, MI, and ELH



determined by employing the known stereospecificity of muconolactone isomerase from *P. putida* (Chari et al., 1987b) which acts only on the (4*S*) isomer (Scheme 2). The muconolactone formed by both TcMLE and MLE II was converted to β-ketoadipate, as observed by ^{1}H NMR, after addition of muconolactone isomerase and enol–lactone hydrolase to the reaction mixture, establishing the absolute configuration of the resulting muconolactone as 4*S* (Figure 1). The stereochemical course of the lactonization at C-5 was determined on the basis of the previous assignment of the ^{1}H NMR resonances of the ABX system of the diastereotopic C-5 protons of (4*S*)-muconolactone (Ngai et al., 1983a), which has established that the downfield and upfield resonances arise from the 5-pro*R* and 5-pro*S* hydrogens, respectively. In the reaction of *cis,cis*-muconate with TcMLE in deuterated buffer, we observed the stereospecific incorporation of deuterium at this downfield resonance, δ 2.61 ppm, and retention of the upfield 5-pro*S* proton signal at δ 2.38 ppm (d, J = 8.5 Hz) (Figure 2). Since the resulting muconolactone was determined to be of (4*S*) configuration, the position of deuterium incorporation resulting from cyclization could be assigned as that of the 5-pro*R* proton. Consequently, TcMLE is demonstrated to catalyze a syn cycloisomerization. The results for MLE II with *cis,cis*-muconate were identical.

Activity of T. cutaneum MLE with 3-Halomuconate Substrates in Comparison with Pseudomonas sp B13 MLE II. The reaction of TcMLE with 3-fluoromuconate **1a** in

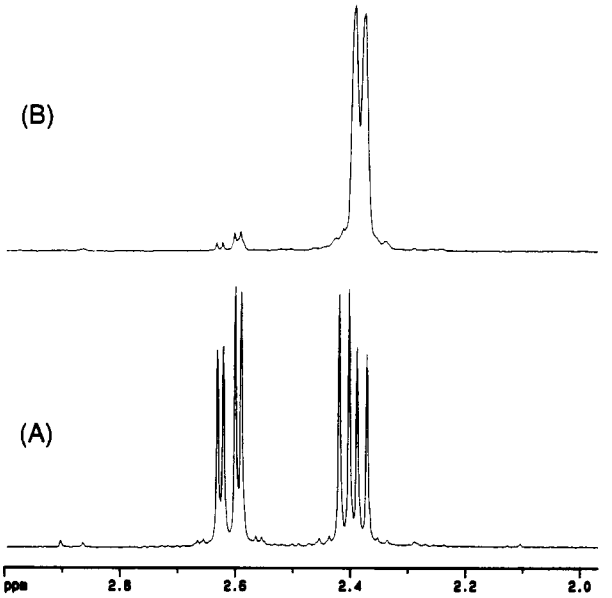
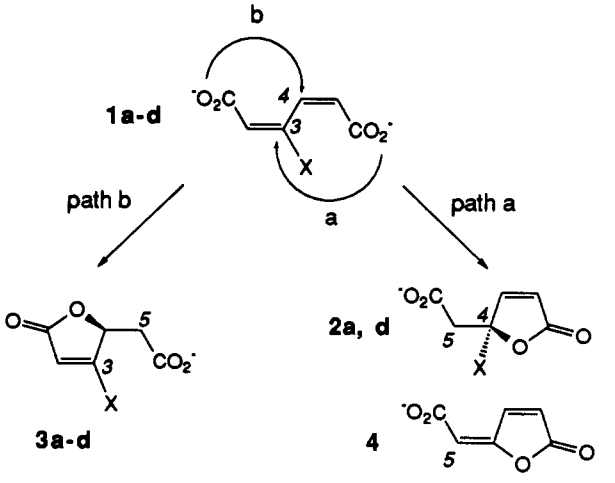


FIGURE 2: ^{1}H NMR (500-MHz) analysis of the reaction of *cis,cis*-muconate with TcMLE: (A) in H_2O; (B) in D_2O. Only the C-5 proton signals are shown.

Scheme 3. The Regiochemical Courses of Cyclization of 3-Substituted Muconates



(**a**, X = F; **b**, X = Cl; **c**, X = Br; **d**, X = CH_3)

deuterated buffer was followed by ^{1}H NMR (400 MHz). It resulted in the formation of 4-fluoromuconolactone **2a** (Scheme 3, path a), which was stereospecifically monodeuterated at the C-5 position. In contrast, the regiochemical course of the reaction of TcMLE with the chloro and bromo analogs **1b,c** deviated from that of 3-fluoromuconate and did not yield the corresponding 4-halomuconolactones **2b,c** or their elimination products, dienelactone **4**. Instead, TcMLE was found to convert 3-chloromuconate **1b** and 3-bromomuconate **1c** to the corresponding 3-halomuconolactones **3b,c** via carboxylate addition at C-4 of the 3-halomuconate (Scheme 3, path b). However, as noted previously by Evans et al. (1971) the 3-halomuconolactones undergo a rapid nonenzymatic hydrolysis under assay conditions to give 3-hydroxymuconolactone, which exhibits a maximal UV absorption at 248 nm. As a consequence, the reaction of TcMLE with **1b,c** was followed by UV spectroscopy. For both **1b** and **1c**, the reaction with TcMLE led to a decrease in absorbance at 260 nm, the λ_{max} of the 3-halomuconates, with a concomitant increase in absorbance at 248 nm, and was accompanied by isosbestic points at 238 and 258 nm (Figure 3). The TcMLE

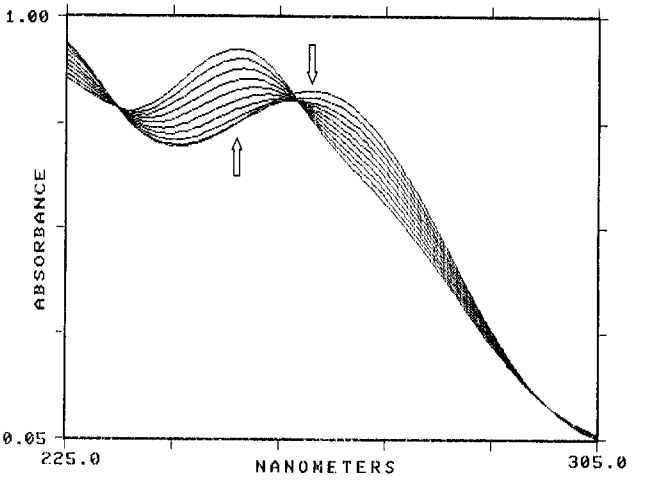


FIGURE 3: UV assay of reaction of 3-bromo-*cis,cis*-muconate with TcMLE. The spectra were measured with a 1-min delay between each acquisition.

Table 1: Relative Velocities for Turnover of 3-Halomuconates by *T. cutaneum* MLE[a]

substrate	relative velocity[b]	substrate	relative velocity[b]
3-methyl-*cis,cis*-muconate	1.0	3-chloro-*cis,cis*-muconate	0.11
3-fluoro-*cis,cis*-muconate	0.17	3-bromo-*cis,cis*-muconate	0.09

[a] Assays were performed with 150 μM substrate in 50 mM MOPS, pH 7.0, at 25 °C. [b] Velocity was determined by spectrophotometric assay. The value represents the mean of four determinations.

catalyzed formation of 3-halomuconolactones was verified by a comparison of the UV time course of the enzymatic reaction with 3-chloromuconate with the UV absorbance changes observed with synthetic 3-chloromuconolactone[2] in the assay buffer, which were identical. The relative velocities for the turnover of 3-halomuconates **1a–c** by the partially purified TcMLE were found to be approximately 10–20% of that observed with the optimal substrate, 3-methyl-*cis,cis*-muconate (Table 1). In contrast to the results observed with TcMLE, the regiochemical course of cyclization of 3-halomuconates catalyzed by MLE II was one which followed consistently from carboxylate addition to C-3 of the 3-halomuconate (Scheme 3, path a). We monitored the reaction of 3-halomuconates **1a–c** with MLE II in deuterated medium by ^{1}H NMR. The 3-fluoromuconate **1a** was converted to 4-fluoromuconolactone **2a**, which was stereospecifically monodeuterated in the same C-5 position observed in the reaction with TcMLE. The reaction of MLE II with 3-chloro- and 3-bromomuconates **1b,c** led to formation of the (*E*)-dienelactone **4** without any detectable incorporation of solvent deuterium at C-5. Furthermore, we were unable to detect by ^{1}H NMR the formation of the corresponding 4-halomuconolactones **2b,c** as discrete intermediates in the MLE II catalyzed formation of (*E*)-dienelactone **4**.

Isolation of MLE from T. cutaneum. TcMLE has been purified to near homogeneity from phenol induced *T. cutaneum* following modifications of the procedure described by Powlowski et al. (1985b). Inclusion of a chromatofocusing chromatography step was paramount to obtaining the purified protein. The six-step protocol, summarized in Table 2, provided TcMLE in an approximately 600-fold purification, albeit with an exceptionally poor recovery. SDS–PAGE of the freshly

[2] 3-Chloromuconolactone was synthesized by the method of Neunhofer (1935).

Table 2: Purification of *T. cutaneum* Muconate Lactonizing Enzyme

step	volume (mL)	protein (mg)	total activity (units)[a]	specific activity (units/mg)	purification (x-fold)	recovery (%)
crude extract	161	3900	820	0.21	1	100
ammonium sulfate (40–60%)	69	1070	630	0.59	2.8	76
DEAE-Sepharose	89	82	420	5.1	24	51
Sephacryl S-200	49	22	256	11.9	57	31
DEAE-Sepharose	6	11	206	18.7	89	25
chromatofocusing	4.5	nd[b]	156	nd	nd	19
Superose-12	0.4	~0.2[c]	26	nd	~625[c]	3

[a] One unit of CMLE activity is defined as the consumption of 1 μmol of 3-carboxymuconate per minute at 25 °C. [b] Not determined. [c] Values estimated using the previously reported specific activity of 126 units/mg (Powlowski et al., 1985).

purified enzyme showed a single band with an estimated subunit molecular mass of 43 kDa. The native molecular mass was estimated as 169 kDa from gel filtration and suggests that TcMLE is a homotetramer. The isoelectric point of the protein was estimated as 4.5 from chromatofocusing chromatography. Duplicate runs of sequence analysis by automated Edman degradation identified the N-terminal amino acid sequence of TcMLE as AVAPTSYDIL MGTFRSPYLY TLTFDVLA(R)K LQV(R)EVN(E)AT G. Two peptides were also isolated from an endoproteinase lysine C digest by reverse-phase HPLC and sequenced. The sequence of LysC-18 was partially determined as [K]XXI(M)XATTV and that of LysC-31 was [K]XXAXEV(R)LX(P)M. Previous characterization of TcMLE from the same strain of *T. cutaneum* used in this work noted a low pH optimum at pH 4–5.6 and the lack of a requirement for divalent metals (Powlowski et al., 1985). We have observed these same characteristics in the course of this work. Furthermore, we have observed small amounts of MLE activity in a separately eluting fraction of the inital DEAE chromatography step, suggesting the possibility of a minor MLE isoform, although additional purification of this activity has not been pursued.

Cloning of the cDNA Encoding MLE by the Polymerase Chain Reaction. Initial PCR amplification of a small TcMLE cDNA fragment was performed using degenerate oligonucleotide primers and phenol induced *T. cutaneum* cDNA as the template. The amino acid sequences of TcMLE residues 7–12 (YDILMG) and 35–40 (EVNEAT) were of relatively low codon degeneracy and were used to design degenerate 5′-sense and 3′-antisense oligonucleotide primers, respectively, as detailed under Materials and Methods. The resulting PCR product was subcloned directly into the pCRII vector, and seven independent clones containing a 113-bp PCR product were identified and subjected to nucleotide sequence analysis. Each clone was found to encode TcMLE amino acid residues 7–40 as determined from amino acid sequencing, verifying the authenticity of the PCR products. Furthermore, this sequence information enabled us to establish a consensus DNA sequence for the degenerate 5′-sense PCR primer which encoded TcMLE residues 7–12. A cDNA clone encoding the entire amino acid sequence of mature TcMLE was obtained by anchored PCR methods. This was accomplished using a 41-bp 5′-sense primer encoding TcMLE residues 1–12 (AVAPTSYDILMG) and a 3′-antisense anchor primer corresponding to the oligonucleotide originally employed in the cDNA synthesis. The TcMLE specific oligonucleotide primer was designed incorporating the consensus sequence established above for residues 7–12. The remaining TcMLE N-terminal residues (1–6) were known only by amino acid sequencing and were encoded in the primer using codons based on optimal usage in *E. coli* (Chen et al., 1982). Additionally, an ATG initiation codon within an *Nde*I restriction site was incorporated into the primer, allowing for subsequent subcloning into expression vectors. The use of a linear preamplification sequence (by which specificity was enhanced by using only the MLE specific primer) prior to conventional PCR was essential in generating a sufficient amount of amplified products (Rother, 1992). Authentic TcMLE bands were identified by Southern blot analysis, and the largest PCR product, approximately 1.2-kbp, was subcloned into pCRII. Thus, while the cDNA clones so obtained encoded the entire protein sequence of TcMLE, the authentic cDNA sequence encoding residues 1–6 was not determined.

Nucleotide Sequence and the Deduced Amino Acid Sequence of MLE. Our cloning of the cDNA encoding TcMLE was performed with PCR methodology, and it has been established that *Taq* DNA polymerase has a relatively high misincorporation rate due to a lack of proofreading function (Keohavong & Thilly, 1989). As a consequence, we have completely sequenced five independent TcMLE clones so as to establish a consensus sequence for the cDNA. The TcMLE cDNA sequence so derived is presented in Figure 4 and was deduced from the cDNA oligonucleotide sequence which encoded amino acid residues 7–373 and the N-terminal sequence information obtained by Edman degradation. As indicated by the underlined sequences in Figure 4, the deduced amino acid sequence is in complete agreement with the results from sequence analysis of the TcMLE N-terminus and internal peptides LysC-18 and LysC-31. Furthermore, the calculated molecular mass of 41 136 Da and the isoelectric point of 4.95 are in good agreement with the observed values of 43 kDa and pI 4.5, respectively.

Structural Comparisons of T. cutaneum MLE with Other Cycloisomerases. Sequence alignment of TcMLE with *N. crassa* CMLE (Mazur et al., 1994) yielded the highest similarity score (20.6) of all protein sequences examined. Attempts at generating alignments between TcMLE and any of the known homologous procaryotic MLEs from *P. putida* (Aldrich et al., 1987), *Acinetobacter calcoaceticus* (Neidle et al., 1989), and *Alcaligenes eutrophus* (Perkins et al., 1990) failed to identify any significant sequence similarity between the eucaryotic and bacterial MLEs. In addition, we were unable to identify any significant similarity with known protein or DNA sequences upon screening current versions of the NBRF-PIR, SwissProt, or GenBank/EMBL databases. The sequence comparison of TcMLE and NcCMLE is shown in Figure 5 and reveals approximately 21% residue identity distributed throughout the length of 366–373 amino acid residues, including 14 gaps which vary in length from 1 to 6 residues. An analysis of the statistical significance of the alignment was performed with a Monte Carlo algorithm which compares the similarity score of TcMLE and NcCMLE with a distribution of random similarity scores obtained by comparing NcCMLE and 50 randomized versions of the TcMLE sequence. The alignment of TcMLE and NcCMLE scores 5.0–8.2 standard deviation units from the mean of the

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ATG GCG GTT GCG CCG ACC AGC TAT GAT ATT TTG ATG GGC ACG TTC CGC TCG CCC TAC CTC TAC ACG CTC ACC TTT GAT GTG 81
    A  V  A  P  T  S  Y  D  I  L  M  G  T  F  R  S  P  Y  L  Y  T  L  T  F  D  V  26

CTG GCG CGC AAG CTC CAG GTG CGC GAG GTC AAC GAG GCG ACG GGC GGG CAC AAC TGG CTC GAC GTG AGC CCC GAC GGT AAC 162
 L  A  R  K  L  Q  V  R  E  V  N  E  A  T  G  G  H  N  W  L  D  V  S  P  D  G  N  53

ACC CTG TAC GCG ACA GTG TGG GGC GAG CCG CCC AAG CTC ACG AGC TAC GAC ATT GTG CGC GGC GGC GAG TAC GCG ACG ACC 243
 T  L  Y  A  T  V  W  G  E  P  P  K  L  T  S  Y  D  I  V  R  G  G  E  Y  A  T  T  80

AAG CTC TCG CGC AAC GTC GCC TCG CAG TAC ATG TCG GGG TAC GTG TGC AGC AAC AAC AAG GCC ATG TAC TCG GCC TGT GGG 324
 K  L  S  R  N  V  A  S  Q  Y  M  S  G  Y  V  C  S  N  N  K  A  M  Y  S  A  C  G  107

CCG CAG GTG GAC ACG TTC CTC GTC GAC GAC AAC GGC ACC CTC CTC GAC CAG CCG GCG GTG CAG AGC TTT AAC CTC CTC CAG 405
 P  Q  V  D  T  F  L  V  D  D  N  G  T  L  L  D  Q  P  A  V  Q  S  F  N  L  L  Q  134

GGG CAG GAG AAG AAC AAG GCG AAC GGG ACG CTC GAC TTT GGT GGC CTG CGG CAC GGT GGG CAC TCT GCC GAC CTC TCG CCA 486
 G  Q  E  K  N  K  A  N  G  T  L  D  F  G  G  L  R  H  G  G  H  S  A  D  L  S  P  161

GAC GGC ACC AAG CTC TAC GTC GCC GAC ATT GGG CGC AAC TGC GTG TGG ATG TAC CAC GTC GAC CGG GAG ACG GGC CTG CTC 567
 D  G  T  K  L  Y  V  A  D  I  G  R  N  C  V  W  M  Y  H  V  D  R  E  T  G  L  L  188

ACC GAG GCG TCC AAG AAC ATC GCG ACG CGC CCG CAC GAC GGC CCG CGT CAC GCA TGG CCC CAC CCC AAC GGC CGC ATC GTC 648
 T  E  A  S  K  N  I  A  T  R  P  H  D  G  P  R  H  A  W  P  H  P  N  G  R  I  V  215

TAC TCG CTC CAG GAG CAC TCG TCG TAT GTC GAC GCG TTC CGC CTT ACC GAC GAC AAC AAG CTC GAG TTC CTC GAG GGC GGC 729
 Y  S  L  Q  E  H  S  S  Y  V  D  A  F  R  L  T  D  D  N  K  L  E  F  L  E  G  G  242

TGC ATC ATC CCC GAC GAG AAG GAC CAC GAC AAG TAC TGG GCC GAC GAG GTG CGC CTC TCG CCC ATG GCT GAT GTG GTG TTC 810
 C  I  I  P  D  E  K  D  H  D  K  Y  W  A  D  E  V  R  L  S  P  M  A  D  V  V  F  269

GGA TCG ACC CGC GGC CTC GAG GAG GGC ACG CCT GGC TTC GTG ACT GCC TGG AAC CTC CGC CCA GAC GGC ACC TTT GCC AGC 891
 G  S  T  R  G  L  E  E  G  T  P  G  F  V  T  A  W  N  L  R  P  D  G  T  F  A  S  296

ACC GAG GCC ACC CAC CGC TTC CAG ACC AAG ACG TCG GGC GGG TGG GCC AAC GCC ATC GCC GTC TGC CCC AAC CTC GGG CCC 972
 T  E  A  T  H  R  F  Q  T  K  T  S  G  G  W  A  N  A  I  A  V  C  P  N  L  G  P  323

AAT GGC GAG GTC TTC ATG ACC CTC ACC GAC TCG GAG GTC GGC TTC ATC CAG ATC CTT GCA TAC ACT AGC GAC AAG GGC TTC 1053
 N  G  E  V  F  M  T  L  T  D  S  E  V  G  F  I  Q  I  L  A  Y  T  S  D  K  G  F  350

GAG GTC GTC GAC GAG CTC AAG ATC AGC ACC GAG AAG GAG CAC ATT ATG CCC GCC ACC ACT GTC TGG TTG TAG ATT TAG GTA 1134
 E  V  V  D  E  L  K  I  S  T  E  K  E  H  I  M  P  A  T  T  V  W  L  end              373

CAT CAT GCA CAT CAC ATT AGC ATC GGA AAA AAA 1167
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FIGURE 4: Partial nucleotide sequence of the TcMLE cDNA and the complete deduced amino acid sequence of the protein (nucleotides denoted in italics, bp 1–21, were not derived from the authentic cDNA and were incorporated by PCR; see Results for a detailed explanation). The N-terminal and peptide sequences (partially) determined by sequence analysis are underlined.

```
TcMLE   TSYDILMGTFRSP-YLYTLTF--DVLARKLQVREVNEATGGHNWLDVSPDGNTLYATVWGEPPKLTSYDIVRGGEYATTK 81
          ::::GT  :P  ::T: F  :  L: KL  R          :W:     :  :  :Y::       K : S: :     E
NcCMLE  PLHHLMIGTWTPPGAIFTVQFDDEKLTCKLIKRTEIPQDEPISWMTFDHERKNIYGA---AMKKWSSFAVKSPTEIVHEA 77

TcMLE   LSRNVASQYMSGYVCSNNKAMYSACGPQVDTFLVDDNGTLLDQPAVQSFNLLQGQEKNKANGTLDFGGLRHGGHSADLSP 161
         S: :::: :    :N : A:: :  Q  : V  N          : F:: :    :K     ::      G H:  : P
NcCMLE  -SHPIGGHPRANDADTNTRAIFLLAAKQP-PYAVYANPFYKFAGYGNVFSVSETGKLEKNVQNYEYQE-NTGIHGMVFDP 154

TcMLE   DGTKLYVADIGRNCVWMYHVDRETGLLTEASKNIATRPHDGPRHAWPHPNGRIVYSLQEHSSYVDAFRLTDDNKL----- 236
          T LY AD:  N : W  H    : G :   :   A  P D PR    HP G  : Y: L  E :: :  : :      :
NcCMLE  TETYLYSADLTANKLWT-HRKLASGEVELVGSVDAPDPGDHPRWVAMHPTGNYLYALMEAGNRICEYVIDPATHMPVYTH 233

TcMLE   -EF-LEGGCIIPDEKDHDK--YWADEVRLSPMADVVFGSTRGLEEGTPGFVTAWNLRPDGTFASTEATHRFQTKTSGGWA 312
          F L    I   : : :K  Y: AD   L:  :   : F: S: R:       G: : : :LR  G: :           T TSGG :
NcCMLE  HSFPLIPPGIPDRDPETGKGLYRADVCALTFSGKYMFASSRANKFELQGYIAGFKLRDCGSI--EKQLFLSPTPTSGGHS 311

TcMLE   NAIAVCPNLGPNGEVFMTLTDSEVGFIQILAYTSDKGF-EVVDELKISTEKEHIMPATTVW 372
        NA:: CP     ::  M: : TD :  G : : I  Y     F :  V  : : I:      : :
NcCMLE  NAVSPCP----WSDEWMAITDDQEGWLEI--YRWKDEFLHRVARVRIPEPGFGMNAIWYD. 366
```

FIGURE 5: Sequence alignment of TcMLE and NcCMLE. Conservative substitutions are represented by (:).

randomized similarity scores, depending on the gap penalty used. A guideline to evaluating the scores suggests that those between 3 and 6 indicate a possible ancestral evolutionary relationship between the sequences, while a score larger then 6 suggests a probable relationship (Pearson, 1990). In comparison, the scores obtained for alignments between TcMLE and either MLE I or MLE II are 0.3 and −0.4, respectively.

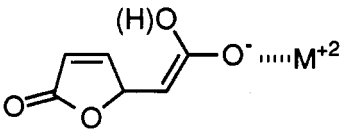
DISCUSSION

Previous studies of cycloisomerases have demonstrated the significance of the stereochemical course of cyclization with regard to mechanistic and evolutionary interpretations. Our current results show that cyclization of *cis,cis*-muconate by TcMLE, a eucaryotic cycloisomerase, occurs by the same relative (syn) and absolute (4*S*) stereochemical course as

determined for its bacterial counterparts, MLE I (Avigad & Englard, 1969) and MLE II from *Pseudomonas*. These results are consistent with previous stereochemical studies of the cyclization of 3-methyl-*cis,cis*-muconate in *A. niger* (Cain et al., 1989b) and in *T. cutaneum* (Cain et al., 1989a). The chemical identity of the catechol branch of the β-ketoadipate pathway in both procaryotes and eucaryotes (Ornston & Yeh, 1982) and the identity of the stereochemical course of the constituent MLEs suggest two likely interpretations: (i) a significant example of convergent evolution, demonstrating perhaps the chemical efficiency of the resulting catabolic route, or (ii) an indication of a common evolutionary origin. Our present results suggest that TcMLE and bacterial MLEs do not share a common genetic origin, therefore favoring the notion of convergent evolution of eucaryotic and bacterial MLEs. In addition, an identical relative and absolute stereochemical course is maintained in CMLE from *N. crassa* (Kirby et al., 1975); the characterization and cloning of this enzyme have also revealed a complete lack of similarity to the bacterial MLEs (Mazur et al., 1994).

The noted distinction between TcMLE and MLE II from *Pseudomonas* sp B13 with regard to the regiochemical course of cyclization of 3-methylmuconate prompted us to investigate the reactions of the above cycloisomerases with 3-halomuconates. The regiochemical outcome with 3-halomuconates was equally distinct. MLE II was found to have an absolute preference for cyclization of 3-substituted muconates via carboxylate addition at C-3 of the substrate (Scheme 3, path a). In contrast, TcMLE catalyzed lactonization proceeds via carboxylate addition to the unsubstituted double bond (C-4) for the larger chloro-, bromo-, and methyl-substituted muconates (Scheme 3, path b), while the smaller analog, 3-fluoromuconate, is cyclized in the opposite regiochemical course (Scheme 3, path a). The likely explanation of the contrasting regiochemical courses between TcMLE and MLE II are differences in the active site architecture which are manifested in distinct orientations of substrate binding, although an alternate mechanistic explanation will be discussed shortly. Different active site structures would suggest that the eucaryotic and bacterial MLEs are not likely to be related, consistent with the observed sequence dissimilarity of TcMLE and bacterial MLE's. Fluorine is nearly isosteric with hydrogen and is not likely to have a major steric influence on the orientation of binding of 3-fluoromuconate. Hence, the anomalous regiochemistry observed with this analog and TcMLE might be explained as a consequence of the strong electron withdrawing effects of the fluorine substituent, making the double bond particularly susceptible to nucleophilic addition. In contrast to the regiochemical distinctions between eucaryotic and procaryotic MLEs as discussed above, the regiochemical outcome of cyclization of 3-substituted muconates in both MLE (3-chloro-, 3-bromo-, and 3-methylmuconate) and CMLE (3-carboxymuconate) of eucaryotic origin occurs consistently by carboxyl group addition to the unsubstituted double bond of the substrate (Scheme 3, path b), with the noted exception of 3-fluoromuconate. This common regiochemical preference suggests the possibility of a similar active site in eucaryotic MLE and CMLE, resulting in the ability to accomodate 3-substituted muconates in a common binding orientation.

The currently accepted mechanism of PpMLE invokes the formation of a metal stabilized anionic (enol/enolate) intermediate as a consequence of carboxylate addititon (Ngai et al., 1983b; Gerlt & Gassman, 1992). The MLE II catalyzed formation of the (*E*)-dienelactone **4** from 3-chloro- and



3-bromomuconates **1b,c** is generally consistent with this mechanistic scenario as follows. The observed lack of solvent deuterium incorporation into the dienelactone product at C-5 and the inability to detect 4-halomuconolactones **2b,c** as intermediates discredit the generation of **4** by chemical elimination of HX from **2b,c**. Rather, a rapid elimination of the halide from an anionic (enol/enolate) intermediate is more consistent with these and previous observations regarding the nonenzymatic cyclization reactions. The chemical lactonization of halomuconates **1b,c** between pH 1 and 6 also generates dienelactone **4** (as a 9/1 mixture of the *E/Z* geometric isomers) and is thought to proceed by nucleophilic addition of the carboxylate group (Pieken & Kozarich, 1990). In analogy to the enzymatic lactonization, the dienelactone products resulting from chemical cyclization do not incorporate solvent deuterium at C-5, discrediting any mechanism involving obligatory protonation at C-5 and interpreted as evidence of rate-limiting formation of an anionic intermediate and a subsequent rapid elimination of the halide. However, one cannot completely discount the enzymatic formation of dienelactone **4** via the intermediacy of **2b,c** (not necessarily formed by an anionic mechanism), whereby an active site base catalyzes fast elimination of the solvent derived C-5 proton and the halide.

In our previous investigations of the chemical lactonization of 3-halomuconates, we have noted that the regiochemical course of cyclization exhibited a pronounced dependency on both the pH and the nature of the halogen substituent and suggested that the regiochemistry of lactone formation was directed by alternate mechanisms of activation of the double bond (Pieken & Kozarich, 1990). In a reaction medium at pH 1–6, the results were consistent with cyclization via nucleophilic addition by the carboxylate anion, while in concentrated HCl an electrophilic mechanism was invoked to explain the observed changes in regiochemistry and other data. Thus, it is of some interest to note that the different regiochemical courses of enzymatic lactonization of 3-halomuconates **1a–c** catalyzed by MLE II and TcMLE, respectively, are identical to the outcomes observed for the chemical cyclizations in phosphate buffer, pH 1–6, and in concentrated HCl, respectively. This observation, in addition to the results discussed above, is consistent with a nucleophilic/anionic mechanism for MLE II. The correlation between TcMLE and acid-catalyzed cyclization suggests the intriguing possibility of an alternate electrophilic/cationic mechanism for TcMLE. However, it should be pointed out that unsubstituted *cis,cis*-muconate is extremely recalcitrant toward acid-catalyzed electrophilic lactonization and requires prolonged reaction in 75% sulfuric acid to affect the reaction (Elvidge et al., 1950). Furthermore, in the absence of binding constraints, one would expect formation of 4-methylmuconolactone from 3-methylmuconate if cyclized via an electrophilic mechanism, based on the enhanced stability of a tertiary carbocation. However, the dissimilarity of TcMLE and bacterial MLEs with respect to primary amino acid sequence and metal dependency is not inconsistent with an alternate mechanism for TcMLE nor is the unusually low pH optimum (4–5.6) observed for TcMLE (Powlowski et al., 1985). Benner and co-workers have offered an explanation of the syn versus anti dichotomy of addition/elimination enzymes based in part

on the stability of potential carbocation intermediates (Benner et al., 1989). Furthermore, the regiochemistry observed in TcMLE catalyzed cyclization of 3-chloro- and 3-bromomuconate does not correlate with the electron withdrawing capability of the halide substituent and the expected favorable influence on the reactivity of the double bond toward nucleophilic addition. Of course, as previously discussed, one can invoke overriding steric constraints imposed by the enzyme active site to explain the counterintuitive regiochemical course observed with these substrates.

Stereochemical and cloning studies have demonstrated the dissimilarity of MLE and CMLE in bacteria (Chari et al., 1987; Williams et al., 1992). In contrast, the sequence alignment between TcMLE and NcCMLE suggests that the two eucaryotic cycloisomerases are at least distantly related, consistent with their noted regio- and stereochemical uniformity. The comparison is complicated by the fact that *N. crassa* and *T. cutaneum* belong to distinct taxonomic classes, ascomycetes and basidiomycetes, respectively, the evolutionary relationship of which is unclear (Hori & Osawa, 1987; Smith, 1989). Moreover, we have suggested that the β-ketoadipate pathway arose in response to the bioavailability of lignins in terrestrial plants (Mazur et al., 1994). As a consequence of the uncertainty regarding the timing of the divergence of ascomycetes and basidiomycetes with respect to the emergence of land plants, it is unclear whether TcMLE and NcCMLE have arisen from a common ancestral cycloisomerase concurrently with the divergence of the two classes or, alternatively, have been independently recruited from similar precursors, perhaps a conserved metabolic enzyme, after the divergence of the two classes.

The likely significance of the homology observed between TcMLE and NcCMLE (ca. 21% identity with 14 gaps) is strengthened when one considers the sequence comparison between *P. putida* MLE and mandelate racemase (Tsou et al., 1990). The alignment reveals approximately 25% sequence identity with five minor gaps, yet in spite of the relatively moderate and scattered sequence identity, the backbone structures of both proteins are virtually superimposable (Neidhart et al., 1990). The alignment of NcCMLE and TcMLE does not reveal any regions of extensive homology that might be indicative of the active site. However, it is of interest to note that key active site and catalytic residues which are conserved between PpMLE and mandelate racemase are distributed throughout a relatively large sequence span (Neidhart et al., 1991). In addition, mechanistic similarities suggest that the two eucaryotic cycloisomerases are likely related. We have recently shown NcCMLE to possess MLE activity (Mazur et al., 1994). The considerable reduction observed in k_{cat}/K_M ($\sim 8 \times 10^5$-fold) for muconate as substrate can be explained in large part by loss of the normal 3-carboxylate substrate binding interaction. Furthermore, both TcMLE and NcCMLE appear to be metal independent, in contrast to *P. putida* MLE. One notable distinction between TcMLE and NcCMLE is the significant difference in the reported pH optima: 4–5.6 for TcMLE and 6.5–7.5 for NcCMLE.

The present work suggests that both eucaryotic cycloisomerases, MLE and CMLE, have arisen from a common ancestral precursor. However, we have not been able to identify CMLE activity in *T. cutaneum* nor is the strain viable with *p*-hydroxybenzoate as its primary carbon source, indicating the apparent lack of a functional protocatechuate branch of the β-ketoadipate pathway in this strain. It is interesting then, that Anderson and Dagley (1980) have identified a different strain of *T. cutaneum* which can readily metabolize protocatechuate and other aromatic acids, converting them to β-ketoadipate by a different sequence of reactions which are also involved in the catabolism of tyrosine in this organism (Sparnins et al., 1979). Thus it appears that in the *T. cutaneum* strain used in our present studies, MLE but not CMLE, was recruited from an ancestral cycloisomerase precursor, while in other strain(s) a unique pathway of protocatechuate catabolism was recruited from a preexisting tyrosine catabolic route.

The apparent relatedness of TcMLE and NcCMLE and their obvious dissimilarity with bacterial cycloisomerases suggest that the eucaryotic enzymes represent a third class of cycloisomerases. The procaryotic MLEs are a group of manganese dependent syn cycloisomerases, the evolutionary origin of which is ambiguous, but which are structurally and mechanistically related to mandelate racemase (Neidhart et al., 1990; Tsou et al., 1990; Gerlt & Gassman, 1992). The bacterial CMLEs are anti cycloisomerases recruited from an ancestral member of the class II fumarase family (Williams et al., 1992). Our current results indicate that the eucaryotic cycloisomerases, exemplified by TcMLE and NcCMLE (Mazur et al., 1994), appear to represent a novel class of metal independent syn cycloisomerases. A better understanding of the relationship between TcMLE and NcCMLE, and the structural and mechanistic consequences of the convergent evolution of the eucaryotic cycloisomerases and the bacterial MLEs, awaits the detailed characterization of these enzymes. Crystallographic studies are in progress.

REFERENCES

Aldrich, T. L., Frantz, B., Gill, J. F., Kilbane, J. J., & Chakrabarty, A. M. (1987) *Gene 52*, 185–195.

Altschul, S. F., & Erickson, B. W. B. (1986) *Bull. Math. Biol. 48*, 603–616.

Anderson, J. J., & Dagley, S. (1980) *J. Bacteriol. 141*, 534–543.

Avigad, G., & Englard, S. (1969) *Fed. Proc. 28*, 345, Abstr. 486.

Benner, S. A., Glasfeld, A., & Piccirilli, J. A. (1989) in *Topics in Stereochemistry*, (Eliel, E. L., & Wilens, S. H., Eds.) Vol. 19, pp 188–193, John Wiley & Sons, New York.

Cain, R. B., Freer, A. A., Kirby, G. W., & Rao, G. V. (1989a) *J. Chem. Soc., Chem. Commun.*, 202–203.

Cain, R. B., Kirby, G. W., & Rao, G. V. (1989b) *J. Chem. Soc., Chem. Commun.*, 1629–1631.

Chari, R. V. J., Whitman, C. P., Kozarich, J. W., Ngai, K.-L., & Ornston, L. N. (1987a) *J. Am. Chem. Soc. 109*, 5514–5519.

Chari, R. V. J., Whitman, C. P., Kozarich, J. W., Ngai, K.-L., & Ornston, L. N. (1987b) *J. Am. Chem. Soc. 109*, 5520–5521.

Chen, H. R., Dayhoff, M. O., Barker, W. C., Hunt, L. T., Yeh, L.-S., George, D. G., & Orcutt, B. C. (1982) *DNA 1*, 365–374.

Choczynski, P., & Sacchi, N. (1987) *Anal. Biochem. 162*, 156–159.

Dorn, E., & Knackmuss, H.-J. (1978) *Biochem. J. 174*, 85–94.

Dorn, E., Hellwig, M., Reineke, W., & Knackmuss, H.-J. (1974) *Arch. Microbiol. 99*, 61–70.

Elvidge, J. A., Linstead, R. P., Orkin, B. A., Sims, P., Baer, H., & Pattison, D. B. (1950) *J. Chem. Soc.*, 2228–2235.

Evans, W. C., Smith, B. S. W., Moss, P., & Fernley, H. N. (1971) *Biochem. J. 122*, 509–517.

Gaal, A., & Neujahr, H. Y. (1980) *Biochem. J. 191*, 37–43.

Gerlt, J. A., & Gassman, P. G. (1992) *J. Am. Chem. Soc. 114*, 5928–5934.

Hori, H., & Osawa, S. (1987) *Mol. Biol. Evol. 4*, 445–472.

Keohavong, P., & Thilly, W. G. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9253–9257.

Kirby, G. W., O'Laughlin, G. J., & Robins, D. J. (1975) *J. Chem. Soc., Chem. Commun.*, 402–403.

Knackmuss, H.-J., Hellwig, M., Lackner, H., & Otting, W. (1976) *Eur. J. Appl. Microbiol. 2*, 267–276.
Lanzillo, J. J. (1991) *Anal. Biochem. 194*, 45–53.
Mazur, P., Henzel, W. J., Mattoo, S., & Kozarich, J. W. (1994) *J. Bacteriol.* (in press).
Meagher, R. B., & Ornston, L. N. (1973) *Biochemistry 12*, 3523–3530.
McCorkle, G. M., Yeh, W.-K., Fletcher, P., & Ornston, L. N. (1980) *J. Biol. Chem. 255*, 6335–6341.
Neidhart, D. J., Kenyon, G. L., Gerlt, J. A., & Petsko, G. A. (1990) *Nature 347*, 692–694.
Neidhart, D. J., Howell, P. L., Petsko, G. A., Powers, V. M., Li, R., Kenyon, G. L., & Gerlt, J. A. (1991) *Biochemistry 30*, 9264–9273.
Neidle, E. L., Hartnett, C., & Ornston, L. N. (1989) *J. Bacteriol. 171*, 5410–5421.
Neunhofer, O. (1935) *Ber. Dtsch. Chem. Ges. 68*, 1778.
Ngai, K.-L., Ornston, L. N., & Kallen, R. G. (1983a) *Biochemistry 22*, 5223–5230.
Ngai, K.-L., Ornston, L. N., & Kallen, R. G. (1983b) *Biochemistry 22*, 5231–5236.
Ornston, L. N., & Yeh, W.-K. (1982) in *Biodegradation and Detoxification of Environmental Pollutants* (Chakrabarty, A. M., Ed.) pp 105–126, CRC Press, Miami, FL.
Pearson, W. R. (1990) *Methods Enzymol. 183*, 63–98.
Perkins, E. J., Gordon, M. P., Caceres, O., & Lurquin, P. F. (1990) *J. Bacteriol. 172*, 2351–2359.
Pieken, W. A., & Kozarich, J. W. (1989) *J. Org. Chem. 54*, 510–512.
Pieken, W. A., & Kozarich, J. W. (1990) *J. Org. Chem. 55*, 3029–3035.
Powlowski, J. B., & Dagley, S. (1985) *J. Bacteriol. 163*, 1126–1135.
Powlowski, J. B., Ingebrand, J., & Dagley, S. (1985) *J. Bacteriol. 163*, 1136–1141.
Rother, R. P. (1992) *Biotechniques 13*, 524–527.
Sanger, F., Nicklenm S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schmidt, E., & Knackmuss, H.-J. (1980) *Biochem. J. 192*, 339–347.
Smith, T. L. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7063–7066.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Sparnins, V. L., Burbee, D. G., & Dagley, S. (1979) *J. Bacteriol. 138*, 425–430.
Tsou, A. Y., Ransom, S. R., Gerlt, J. A., Buechter, D. D., Babbitt, P. C., & Kenyon, G. L. (1990) *Biochemistry 29*, 9856–9862.
Williams, S. E., Woolridge, E. M., Ransom, S. C., Landro, J. A., Babbitt, P. C., & Kozarich, J. W. (1992) *Biochemistry 31*, 9768–9776.