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Characterization of Muconate and Chloromuconate Cycloisomerase from *Rhodococcus erythropolis* 1CP: Indications for Functionally Convergent Evolution among Bacterial Cycloisomerases

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Muconate cycloisomerase (EC 5.5.1.1) and chloromuconate cycloisomerase (EC 5.5.1.7) were purified from extracts of *Rhodococcus erythropolis* 1CP cells grown with benzoate or 4-chlorophenol, respectively. Both enzymes discriminated between the two possible directions of 2-chloro-*cis*,*cis*-muconate cycloisomerization and converted this substrate to 5-chloromuconolactone as the only product. In contrast to chloromuconate cycloisomerases of gram-negative bacteria, the corresponding *R. erythropolis* enzyme is unable to catalyze elimination of chloride from (+)-5-chloromuconolactone. Moreover, in being unable to convert (+)-2-chloromuconolactone, the two cycloisomerases of *R. erythropolis* 1CP differ significantly from the known muconate and chloromuconate cycloisomerases of gram-negative strains. The catalytic properties indicate that efficient cycloisomerization of 3-chloro- and 2,4-dichloro-*cis*,*cis*-muconate might have evolved independently among gram-positive and gram-negative bacteria.

Many chloroaromatic compounds are degraded by bacteria via chlorocatechols as central intermediates. Further catabolism involves *ortho*-cleavage of the chlorocatechols to chlorosubstituted *cis*,*cis*-muconates as well as cycloisomerization and dechlorination of the latter, yielding dienelactones (4-carboxymethylenebut-2-en-4-olides) which are hydrolyzed and finally funneled into the ubiquitous 3-oxoadipate pathway (Fig. 1). Despite much of the early work having been done with an *Arthrobacter* sp. (3, 8, 31) and despite many reports of transformation of halogenated aromatic compounds by gram-positive bacteria (recently reviewed in reference 35), the enzymology and genetics of the modified *ortho*-cleavage pathway outlined above have been elucidated almost exclusively in gram-negative strains. They usually contain separate sets of enzymes for catechol and chlorocatechol conversion, which differ from each other with respect to the affinities and turnover rates for chlorosubstituted catechols or the metabolites formed from them (6, 21, 25).

The gram-positive strain *Rhodococcus erythropolis* 1CP has previously been reported to utilize 4-chlorophenol and 2,4-dichlorophenol as sole sources of carbon and energy (10). After some adaptation, it also grows slowly with 3-chlorophenol but not with 2-chlorophenol. Like many gram-negative strains, *R. erythropolis* 1CP possesses separate catechol and chlorocatechol catabolic enzymes (14, 16). The substrate preferences of the chlorocatechol 1,2-dioxygenase (15) and of the dienelactone hydrolase (16) of *R. erythropolis* 1CP suggest that, corresponding to the growth substrates, only a 4-chlorocatechol branch and a 3,5-dichlorocatechol branch are functional in strain 1CP, but there is no 3-chlorocatechol branch (Fig. 1). In this paper, we show that the substrate preference of the *R. erythropolis* 1CP chloromuconate cycloisomerase fits well with those of the dioxygenase and of the hydrolase. Moreover, 2-chloro-*cis*,*cis*-muconate was found to be converted to only one product, 5-chloromuconolactone, by both the muconate and the chloromuconate cycloisomerase of strain 1CP, thus raising new questions about the pattern of divergence in the evolution of catechol and chlorocatechol degradative pathways. Some of the results have previously been reported in preliminary communications (29, 30).

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MATERIALS AND METHODS

Microorganism and growth conditions. *R. erythropolis* 1CP was grown in the mineral medium described by Gorlatov et al. (10) at pH 7.2 to 7.5. Growth with 4-chlorophenol was performed in a 10-liter fermenter at ca. 400 rpm and 28 to 30°C with repeated additions of 0.2 to 0.5 mM substrate (total concentration, 15 mM) and increasing aeration to keep the partial O_2 pressure at 80% saturation. With sodium benzoate, *R. erythropolis* 1CP cells were grown in shaken Erlenmeyer flasks (200 rpm at 28°C), each containing 200 ml of mineral medium and 3 mM substrate. The cells were harvested by centrifugation (8 min, 16,000 × *g*, 4°C), washed twice with 50 mM Tris-HCl (pH 7.8), and stored at −20°C until required.

Preparation of cell extracts. Frozen cells grown on 4-chlorophenol were thawed, suspended in 50 mM Tris-HCl (pH 7.5)–2 mM $MnSO_4$ (buffer A), and disrupted with an ultrasonic disintegrator (Branson sonifier B 12) for 50 min with cooling (−15°C). Cell debris and unbroken cells were removed by centrifugation for 1 h at 100,000 × *g* and 4°C. The supernatant solution after filtration through a 0.22-μm-pore-size filter was used as a crude extract for the purification of chloromuconate cycloisomerase.

Extracts of benzoate-grown cells were obtained by two different procedures. For the first procedure, washed biomass was thawed, 2 mM $MnSO_4$ was added, and disruption was performed in a Hughes press (functional pressure, 3,200 kg/cm^2). To this preparation, 50 mM Tris-HCl (pH 7.2)–2 mM $MnSO_4$ (buffer B) and DNase I were then added, and cell debris and unbroken cells were removed by centrifugation (40 min, 20,000 × *g*, 4°C). The supernatant solution was used as a crude extract for the first purification of muconate cycloisomerase described below. Alternatively the cells were thawed, resuspended in buffer B, and disrupted with an ultrasonic disintegrator as detailed above. The extract was used for the second purification described below.

Enzyme assays. Unless mentioned otherwise, the activity of muconate and chloromuconate cycloisomerase was measured spectrophotometrically at 260 nm. A published assay (12, 25) was modified to contain 50 mM Tris-HCl (pH 7.5 for chloromuconate cycloisomerase, pH 7.2 for muconate cycloisomerase), 2 mM $MnCl_2$, and a 0.1 mM substrate. The assay mixtures for 3-chloro- and 2,4-dichloro-*cis*,*cis*-muconate conversion additionally contained partially purified

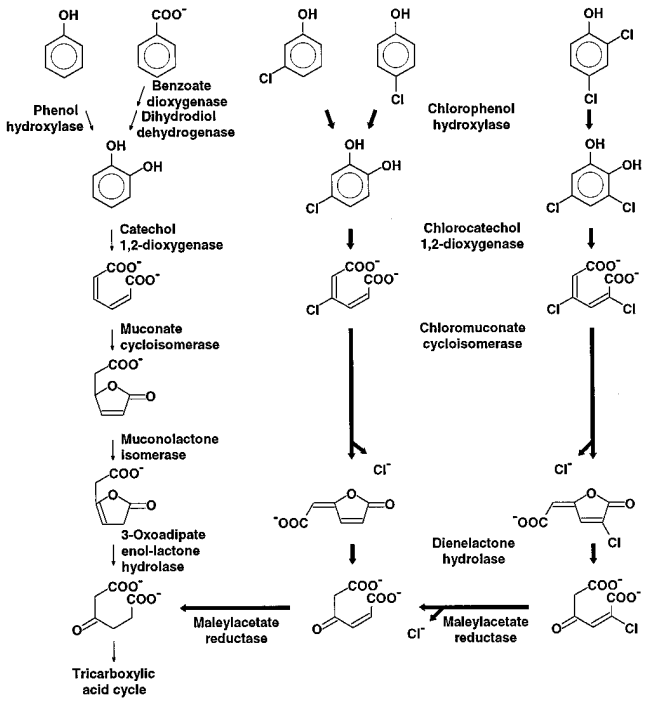

FIG. 1. The ordinary 3-oxoadipate (*ortho*-cleavage) pathway (thin arrows) and the modified *ortho*-cleavage pathway (thick arrows) as assumed to be functional in *R. erythropolis* 1CP.

dienelactone hydrolase from *Alcaligenes eutrophus* JMP134. Generally, published extinction coefficients (7) were used: in the case of 2,4-dichloro-*cis*,*cis*-muconate transformation, the reaction coefficient was 5.8 $mM^{-1} \cdot cm^{-1}$ (12). For the conversion of 3-chloro-*cis*,*cis*-muconate by muconate cycloisomerase of *R. erythropolis* 1CP, a reaction coefficient of 4.0 $mM^{-1} \cdot cm^{-1}$ was determined. With the exception of 2-chloro- and 2-fluoro-*cis*,*cis*-muconate, substituted *cis*,*cis*-muconates were prepared in situ from the corresponding catechols by using partially purified chlorocatechol 1,2-dioxygenase from *A. eutrophus* JMP134 (12). For the determination of K_m and V_{max} values, substrate concentrations were generally between 5 and 200 μM. K_m and V_{max} values of chloromuconate cycloisomerase with *cis*,*cis*-muconate were determined at concentrations between 0.5 and 4.5 mM by high-performance liquid chromatography (HPLC [samples taken every 5 min]). All measurements were performed at 25°C. Protein concentrations were determined by the Bradford (4) procedure with bovine serum albumin as a standard. K_m and V_{max} values were calculated by nonlinear regression with the Enzfitter program (Biosoft, Cambridge, United Kingdom).

Enzyme purifications. In the course of this investigation, chloromuconate cycloisomerase was purified three times from extracts of 4-chlorophenol-grown cells by basically the same procedure, as described here. Cell extract (volume, 50 ml; protein, 368 mg; total activity, 6.8 U with 2-chloro-*cis*,*cis*-muconate) was chromatographed on a Q Sepharose Fast Flow column (HR 16/10; bed volume, 20 ml) by using buffer A and a stepwise NaCl gradient (0 to 0.2 M over 40 ml, 0.2 to 0.4 M over 260 ml, 0.4 to 1.0 M NaCl over 40 ml) for elution. The most active fractions (eluting at ca. 0.35 M NaCl) were pooled (volume, 35 ml; protein, 14 mg; total activity, 5.0 U). After addition of $(NH_4)_2SO_4$ to a final concentration of 1.6 M, the sample was centrifuged (100,000 × *g*, 30 min, 4°C), and the supernatant was loaded onto a Phenyl Sepharose Fast Flow column (HR 16/10). Proteins were eluted by a decreasing gradient of 1.6 to 0 M $(NH_4)_2SO_4$ in buffer A over 260 ml [activity peak at ca. 0.4 M $(NH_4)_2SO_4$]. The combined fractions (volume, 16 ml; protein, 3.9 mg; total activity, 2.2 U) were concentrated by ultrafiltration with Amicon Centricon 10 microconcentrators to a volume of 2 ml. The final gel filtration was performed on a Superdex 200 prep-grade column (HiLoad 16/60; bed volume, 120 ml; length, 60 cm) by using buffer A with 0.2 M NaCl (flow rate, 1 ml/min). This procedure resulted in a preparation (volume, 5.8 ml; protein, 2.6 mg; total activity, 2.1 U) with a specific activity of 0.8 U/mg, equivalent to a 43-fold purification (31% yield).

The data on muconate cycloisomerase reported here were obtained from five different preparations of the enzyme, which in all cases was purified from benzoate-grown cells. The first purifications used basically the same protocol, which is outlined here. Cell extract (volume, 225 ml; protein, 900 mg; total activity, 128 U with *cis*,*cis*-muconate) was initially subjected to an $(NH_4)_2SO_4$ fractionation at 4°C. After a cut at 35% saturation, the protein pelleted by centrifugation (20 min, 20,000 × *g*, 4°C) at 55% saturation was redissolved in buffer B and dialyzed overnight against the same buffer. The solution was then centrifuged as before, and the supernatant was chromatographed on a Q Sepharose Fast Flow column (HR 16/10; bed volume, 20 ml) by using buffer B with a stepwise NaCl gradient (0 to 0.2 M over 40 ml, 0.2 to 0.5 M over 400 ml, 0.5 to 1 M over 40 ml) for elution. To the combined fractions with activity, $(NH_4)_2SO_4$ was added to a final concentration of 1 M. After centrifugation (20 min, 20,000 × *g*, 4°C) the supernatant was subjected to a Phenyl Superose HR 10/10 chromatography (bed volume, 8 ml) employing buffer B and a decreasing gradient of $(NH_4)_2SO_4$ (1 to 0 M over 160 ml) for the elution of the proteins. The most active fractions were combined (volume, 6 ml) and directly applied to a Sephacryl S-200 Superfine column (K 26/100; bed volume, 450 ml; bed length, 85 cm). Proteins were eluted with buffer B containing 0.1 M NaCl (flow rate, 1 ml/min), and the pooled fractions from this step were dialyzed against buffer B overnight. Final purification was achieved with a Mono Q HR 5/5 column (volume, 1 ml) by using buffer B and a stepwise NaCl gradient (0 to 0.25 M over 5 ml, 0.25 to 0.5 M over 20 ml, 0.5 to 1.0 M over 5 ml). The fractions with the highest level of activity of muconate cycloisomerase (eluting at ca. 0.4 M NaCl) were pooled and dialyzed overnight against buffer B. The resulting preparation (volume, 2.5 ml; protein, 0.9 mg; total activity, 7.2 U) was used for the determination of kinetic constants of muconate cycloisomerase. Most of the other investigations concerning muconate cycloisomerase were performed with preparations that had been obtained in a similar way.

In later purifications of muconate cycloisomerase, the cell extract (volume, 70 ml; protein, 139 mg; total activity, 34 U) was initially incubated at 60°C for 20 min and then centrifuged (40 min, 100,000 × *g*, 4°C). The supernatant (volume, 67 ml; protein, 77 mg; total activity, 22 U) was subjected to Q Sepharose chromatography (HR 16/10; bed volume, 20 ml) with buffer B and a stepwise NaCl gradient (0 to 0.3 M over 40 ml, 0.3 to 0.45 M over 400 ml, 0.45 to 1 M over 40 ml) for elution. Fractions containing the highest levels of activity (eluting at ca. 0.4 M NaCl) were pooled (volume, 29 ml; protein, 2.0 mg; total activity, 17 U). The final hydrophobic interaction chromatography was performed as described above and resulted in a preparation (volume, 6.5 ml; protein, 0.69 mg; total activity, 5.5 U) with a specific activity of 8.0 U/mg (33-fold purification; 16% yield). This preparation was used for N-terminal amino acid sequencing and investigation of 2-chloro-*cis*,*cis*-muconate turnover.

Protein purification was performed at room temperature by using BioPilot and fast protein liquid chromatography systems as well as columns and chromatography media from Pharmacia. Unless mentioned otherwise, purified enzymes were stored at 4°C.

HPLC. For HPLC analyses, Grom SIL 100 C_8 reversed-phase columns (Grom, Herrenberg, Germany) with a 4.6-mm internal diameter and 125- or 250-mm length were used. The flow rates were 1 ml/min for the short column and 0.6 ml/min for the long one. The detection of compounds was performed at 210 nm. The mobile phase was an aqueous solution of 20 or 40% (vol/vol) methanol and 0.1% (wt/vol) H_3PO_4 (33).

Estimation of subunit and total molecular masses. The purity of protein preparations was evaluated and subunit molecular masses were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by a modified Laemmli (13) procedure on slab gels with 10, 12, or 15% (wt/vol) acrylamide in the separation gel. Gels were stained by a modification of the method of Merril et al. (17) by using the Bio-Rad silver-staining kit. For the determination of molecular masses, Sigma Low and High Molecular Weight Standards were used, which contained *Escherichia coli* β-galactosidase (116 kDa), rabbit phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), porcine fumarase (48.5 kDa), bovine carbonic anhydrase (29 kDa), bovine β-lactoglobulin (18.4 kDa), and bovine α-lactalbumin (14.2 kDa).

The molecular mass of native proteins was determined by PAGE under nondenaturing conditions and by gel filtration chromatography on a Superose 6 column (HR 10/30; length, 30 cm). For nondenaturing electrophoresis, Bio-Rad Mini-Protean II Ready Gels with a 4 to 20% polyacrylamide gradient in 0.375 M Tris-HCl (pH 8.8) were used. The following proteins (Sigma) served as standards for the electrophoresis: α-lactalbumin (14.2 kDa), chicken ovalbumin (45 kDa), bovine serum albumin monomer (66 kDa) and dimer (132 kDa), and jack bean urease trimer (272 kDa) and hexamer (545 kDa). The Superose 6 column was calibrated with the Bio-Rad gel filtration standards with molecular masses and Stokes radii as reported previously (24).

N-terminal amino acid sequencing. The N-terminal amino acid sequence of chloromuconate cycloisomerase was determined after running an SDS-PAGE (12% polyacrylamide) and electroblotting the protein onto a Millipore polyvinylidene difluoride Immobilon P membrane. The membrane was stained with Coomassie blue R-250 (18). The protein band was excised and subsequently sequenced in an Applied Biosystems model 473A protein sequencer. The N-terminal amino acid sequence of muconate cycloisomerase was determined by using a solution of the enzyme which had been partially desalted by ultrafiltration (Centricon 10 microconcentrators) and repeated dilution with 10 mM potassium phosphate buffer (pH 7.8).

Chemicals. 3-Methylcatechol was obtained from Aldrich, 4-methylcatechol was obtained from Sigma, and *cis*,*cis*-muconic acid was obtained from L. N. Ornston, New Haven, Conn. 2-Chloro-*cis*,*cis*-muconic acid, (+)-2-chloromuconolactone, (+)-5-chloromuconolactone (33), 4-fluorocatechol (23), 4-chlorocatechol, and 3,5-dichlorocatechol (7) were available from previous syntheses. 2-Fluoro-*cis*,*cis*-muconic acid was prepared by the procedure of Schmidt and

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CatB   P.putida PRS2000:      MTSALIERIDAIIV-DLPTIRP
CatB   P.putida RB1:          MTSVLIERIEAIIVHDLPTIRP
CatB   A.calcoaceticus:       ----MYKSVETILV-DIPTIRP
TfdD   pJP4:                  ---VKIDAIEAVIV-DVPTKRP
ClcB   pAC27:                 ---MKIEAIDVTLV-DVPASRP
TcbD   pP51:                  ---MKIEAISTTIV-DVPTRRP
CMCI   R.erythropolis 1CP:    -PDLTVSGVRTTIV-DLPILRP
MCI    R.erythropolis 1CP:    -TDLSIVSVETTIL-DVPLVRP
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FIG. 2. Comparison of the N-terminal amino acid sequences of chloromuconate cycloisomerase (CMCI) and muconate cycloisomerase (MCI) from *R. erythropolis* 1CP with the sequences predicted from DNA sequences for muconate cycloisomerases (CatB) from *P. putida* and *A. calcoaceticus* and for chloromuconate cycloisomerases (TfdD, ClcB, and TcbD) from *A. eutrophus* JMP134(pJP4), *P. putida* AC866(pAC27), and *Pseudomonas* sp. strain P51 (pP51), respectively (1, 9, 11, 20, 28, 32). Positions identical in all sequences are marked by asterisks. Position 2 of *R. erythropolis* 1CP chloromuconate cycloisomerase could be an N instead of a D. Positions 21 to 24 of *R. erythropolis* 1CP muconate cycloisomerase were found to be H, K, F, and A, respectively.

Knackmuss (26), employing 3-fluorobenzoate conversion by 3-chlorobenzoate-grown cells of *Pseudomonas* sp. strain B13. A ^{1}H nuclear magnetic resonance spectrum of the product agreed well with published data (27).

RESULTS AND DISCUSSION

Enzyme purifications and stabilities. Chloromuconate cycloisomerase was purified 43-fold from cell extracts of 4-chlorophenol-grown cells in three steps (yield, 31%) resulting in a preparation that on an SDS-polyacrylamide gel appeared to be homogeneous (additional proof from N-terminal sequencing). Only one peak of cycloisomerase activity was found after each step. Chloromuconate cycloisomerase was relatively stable at slightly alkaline pH, showing 77 to 100% residual activity after incubation of the enzyme (0.03 mg/ml) for 36 h at 4°C in 50 mM Tris-HCl, MOPS [3-(*N*-morpholino)propanesulfonate], or HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonate buffer at pH 7.2 to 8.2. Long-term storage of chloromuconate cycloisomerase (0.45 mg/ml) in 50 mM Tris-HCl buffer (pH 7.5)–2 mM $MnSO_4$–0.2 M NaCl at 4°C yielded half of the original activity after 3.5 months.

While both purification strategies for muconate cycloisomerase resulted in preparations with similar specific activities, fewer chromatographic steps were necessary in the one making use of the enzyme's relatively high stability at 60°C (in preliminary experiments, 83% residual activity after incubation of cell extract [2 mg/ml] for 30 min in 50 mM Tris-HCl [pH 7.2]–2 mM $MnSO_4$). The final preparations on SDS-polyacrylamide gels usually showed one major band of ca. 40 kDa and two very faint ones whose masses added up to that of the major band. The faint bands did not disappear in several subsequent chromatographic steps, and the N-terminal sequencing did not indicate the presence of a contaminating protein. Low specific activities of muconate cycloisomerase observed during some steps of the purification were found to be due to an inhibitory effect of NaCl. Its inclusion in assays (0.5 μg of protein per ml, 50 mM MOPS [pH 6.6]) reduced the activity almost linearly with the added concentration (at 100 mM NaCl, 13% of the activity without salt [22.4 U/mg]). Purified muconate cycloisomerase (3.5 μg/ml) was stable in 50 mM MOPS (pH 6.5) at 4°C (in 2 months, no loss of activity), while at −20°C and +20°C losses were 26 and 69%, respectively.

Similarities to other cycloisomerases in basic properties. SDS electrophoresis gave subunit molecular masses for chloromuconate and muconate cycloisomerase from *R. erythropolis* 1CP of 40 to 42 kDa. The molecular masses of the native enzymes as determined by nondenaturing electrophoresis and gel filtration were about 230 to 250 kDa. These data are similar to those found for the *Pseudomonas putida* muconate cycloisomerase and for the pJP4-encoded chloromuconate cycloisomerase (2, 12). The N-terminal sequences of the cycloisomerases from *R. erythropolis* 1CP were identical to those of other muconate or chloromuconate cycloisomerases in 6 to 9 of 20 positions (Fig. 2), thus implying homology. With each other, they share 10 identities in 20 positions, indicating that their evolutionary relationship might be closer than that to the other cycloisomerases. The pH optima of both cycloisomerases as measured with *cis*,*cis*-muconate or 2-chloro-*cis*,*cis*-muconate, respectively, in 50 mM Tris-HCl and 50 mM Bis-Tris-HCl (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane) were around pH 7. They are thus similar to the pH optimum of the *P. putida* muconate cycloisomerase (19).

Substrate specificities. The two cycloisomerases of *R. erythropolis* 1CP had distinctly different substrate preferences (Tables 1 and 2), although the relatively low degree of specificity of the muconate cycloisomerase and the high degree of specificity of the chloromuconate cycloisomerase were remarkable. 3-Chloro-*cis*,*cis*-muconate, 2,4-dichloro-*cis*,*cis*-muconate, and 3-fluoro-*cis*,*cis*-muconate clearly were the preferred substrates for the latter enzyme, while the turnover numbers (k_{cat}) for methyl-substituted muconates were so low and the apparent K_m ($K_{m,app}$) for 2-chloro-*cis*,*cis*-muconate were so high that the respective specificity constants ($k_{cat}/K_{m,app}$) were lower than those found with muconate cycloisomerase for these substituted substrates.

Conversion of 2-chloro-*cis*,*cis*-muconate, (+)-2-chloromuconolactone, and (+)-5-chloromuconolactone. The addition of dienelactone hydrolase from *A. eutrophus* JMP134 as an auxiliary enzyme in the assay for chloromuconate cycloisomerase activity resulted in the expected stronger decrease in A_{260} with

TABLE 1. Substrate specificity of chloromuconate cycloisomerase from *R. erythropolis* 1CP

Substrate	$K_{m,app}$ (μM)[a]	V_{max} (U/mg)[a]	k_{cat} (1/min)[b]	$k_{cat}/K_{m,app}$ ($\mu M^{-1} \cdot min^{-1}$)
cis,*cis*-Muconate	6,997 ± 134	6.5 ± 0.1	260	0.037
2-Chloro-*cis*,*cis*-muconate	312 ± 35	3.3 ± 0.3	132	0.42
3-Chloro-*cis*,*cis*-muconate	173 ± 12	90.9 ± 3.3	3,640	21.0
2,4-Dichloro-*cis*,*cis*-muconate	119 ± 26	33.5 ± 5.6	1,340	11.3
2-Methyl-*cis*,*cis*-muconate	47 ± 11	0.084 ± 0.007	3.4	0.072
3-Methyl-*cis*,*cis*-muconate	66 ± 0.1	1.2 ± 0.01	48	0.73
3-Fluoro-*cis*,*cis*-muconate	52 ± 10	9.1 ± 0.7	364	7.0
2-Fluoro-*cis*,*cis*-muconate	—[c]	(<0.03)[d]		

[a] Calculated by nonlinear regression with the program Enzfitter. Standard deviations are given as calculated by this program.
[b] The k_{cat} values were calculated on the basis of a subunit molecular mass of 40 kDa.
[c] —, in HPLC experiments, 2-fluoro-*cis*,*cis*-muconate at a concentration of 0.5 mM did not inhibit 3-chloro-*cis*,*cis*-muconate (0.2 mM) conversion.
[d] Detection limit for specific activity under the experimental conditions (e.g., at a substrate concentration of 0.1 mM).

TABLE 2. Substrate specificity of muconate cycloisomerase from *R. erythropolis* 1CP

Substrate	$K_{m,app}$ (μM)[a]	V_{max} (U/mg)[a]	k_{cat} (1/min)[b]	$k_{cat}/K_{m,app}$ ($\mu M^{-1} \cdot min^{-1}$)
cis,*cis*-Muconate	81.3 ± 14.0	36.9 ± 3.9	1,480	18.2
2-Chloro-*cis*,*cis*-muconate	55.9 ± 0.1	5.4 ± 0.1	216	3.9
3-Chloro-*cis*,*cis*-muconate	1,240 ± 694	13.2 ± 6.8	528	0.4
2-Methyl-*cis*,*cis*-muconate	66.7 ± 23.6	3.0 ± 0.5	120	1.8
3-Methyl-*cis*,*cis*-muconate	48.7 ± 10.7	4.8 ± 0.5	192	3.9
2,4-Dichloro-*cis*,*cis*-muconate		(<0.01)[c]		

[a] Calculated by nonlinear regression with the program Enzfitter. Standard deviations are given as calculated by this program.
[b] The k_{cat} values were calculated on the basis of subunit molecular mass of 40 kDa.
[c] Detection limit for specific activity under the experimental conditions for photometric measurements (e.g., at a substrate concentration of 0.1 mM). The lack of activity was verified by HPLC experiments (substrate concentration, 0.1 mM; detection limit, 0.06 U/mg).

3-chloro- and 2,4-dichloro-*cis*,*cis*-muconate as substrates but not with 2-chloro-*cis*,*cis*-muconate. Overlay UV spectra of 2-chloro-*cis*,*cis*-muconate turnover showed a decrease in A_{267} and the occurrence of a new absorption maximum at 205 nm but not at 277 nm (Fig. 3A). Two isosbestic points were observed, providing evidence that the product absorbing at 205 nm was formed from 2-chloro-*cis*,*cis*-muconate without accumulation of an intermediate. HPLC analyses of samples from such reaction mixtures showed disappearance of 2-chloro-*cis*,*cis*-muconate and the appearance of only one compound. This product had the same retention volume as authentic 5-chloromuconolactone (Fig. 4), and coinjection of both compounds gave one peak. 2-Chloromuconolactone could not be detected as a product. Only part of the 2-chloro-*cis*,*cis*-muconate was converted to 5-chloromuconolactone even after prolonged incubation (Fig. 3A [results corroborated by HPLC experiments at an initial substrate concentration of 0.5 mM]). When (+)-5-chloromuconolactone was used as a substrate, overlay UV spectra showed an appearance of a maximum at 267 nm, indicating formation of 2-chloromuconate (Fig. 3B). Thus, as observed previously with muconate cycloisomerases of gram-negative bacteria (33), the reaction is reversible and both compounds seem to enter an equilibrium in the course of the reaction. According to the changes in UV absorption, *trans*-dienelactone was not formed from (+)-5-chloromuconolactone. When (+)-2-chloromuconolactone (0.1 mM) was tested as a substrate, no spectral changes were observed even in the presence of an enzyme concentration fourfold that given in Fig. 3.

Muconate cycloisomerase, like chloromuconate cycloisomerase, during 2-chloro-*cis*,*cis*-muconate conversion, formed 5-chloromuconolactone (Fig. 4) as the only product. Changes in UV spectra were similar to those presented in Fig. 3, and the findings were backed up by HPLC investigations. Again, (+)-5-chloromuconolactone was used as a substrate, thus showing that, as in the case of chloromuconate cycloisomerase, the

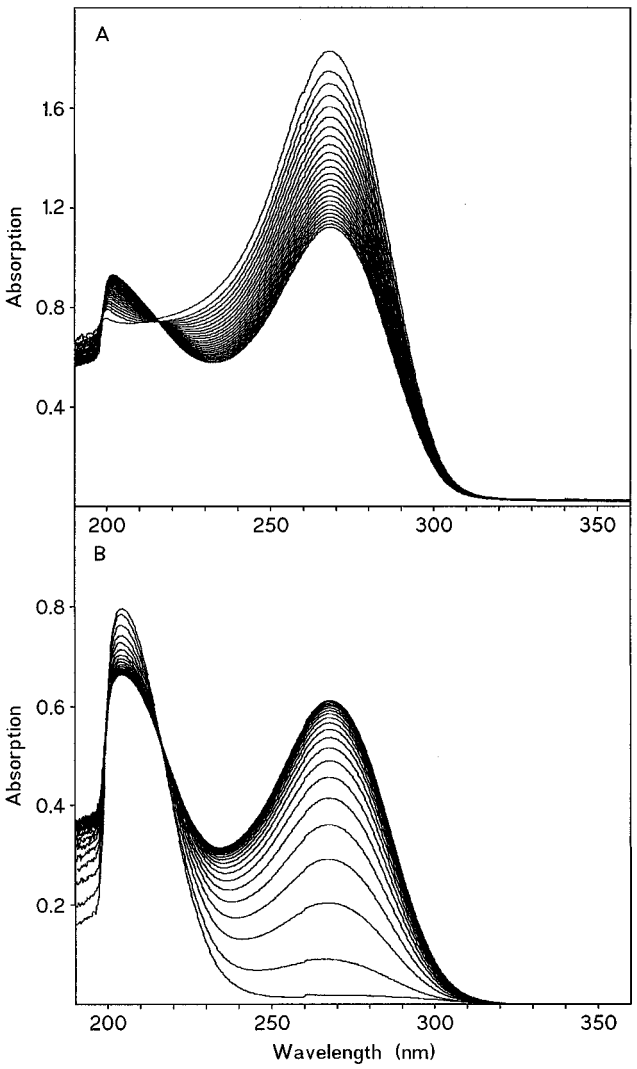

FIG. 3. (A) Spectral changes during conversion of 2-chloro-*cis*,*cis*-muconate by chloromuconate cycloisomerase of *R. erythropolis* 1CP. The reaction mixture (1 ml) contained 30 mM Tris-HCl (pH 7.0), 2 mM $MnCl_2$, 0.1 mM 2-chloro-*cis*,*cis*-muconate, and 1 μg of purified chloromuconate cycloisomerase. UV spectra between 190 and 360 nm were run every 2 min. (B) Conversion of (+)-5-chloromuconolactone (initial concentration not precisely quantitated) under the same conditions described for panel A.

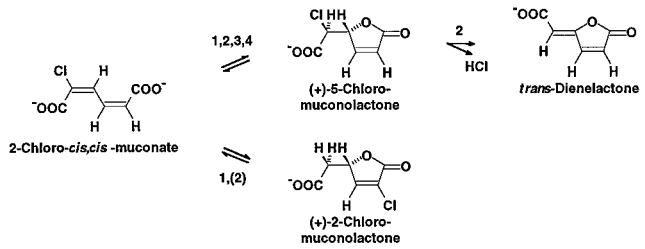

FIG. 4. Reactions catalyzed by various bacterial muconate and chloromuconate cycloisomerases with 2-chloro-*cis*,*cis*-muconate as a substrate (references 33 and 34 and this paper). The numbers on the arrows indicate the groups of cycloisomerases, as defined in Table 3 and the text, which catalyze the respective reactions. The 2 in parentheses is meant to summarize the fact that chloromuconate cycloisomerases of gram-negative bacteria catalyze conversion of (+)-2-chloromuconolactone but that at least in the case of the pJP4-encoded enzyme, the opposite direction of this reaction, formation of 2-chloromuconolactone from 2-chloro-*cis*,*cis*-muconate, appears to be considerably slower than the formation of 5-chloromuconolactone.

TABLE 3. Overview of the properties of different groups of cycloisomerases[a]

Property	Muconate cycloisomerase from gram-negative strains (group 1)[b]	Chloromuconate cycloisomerase from pAC27 and pJP4 (group 2)	Muconate cycloisomerase from *R. erythropolis* 1CP (group 3)	Chloromuconate cycloisomerase from *R. erythropolis* 1CP (group 4)
$k_{cat}/K_{m,app}$ for 3CM[c] and 2,4DCM[c]	Low	High	Low	High
Conversion of (+)-2-chloromuconolactone	Yes	Yes	No	No
Discrimination between directions during 2CM[c] cycloisomerization	No	Yes?[d]	Yes	Yes
Dehalogenation during 2CM cycloisomerization	No	Yes	No	No

[a] Information was compiled from this paper and references 12, 25, 33, and 34.
[b] Investigated until now in *Pseudomonas* sp. strain B13, *P. putida* PRS2000, and *A. calcoaceticus* ADP1.
[c] 2CM, 3CM, and 2,4DCM stand for 2-chloro-, 3-chloro-, and 2,4-dichloro-*cis*,*cis*-muconate, respectively.
[d] In the course of 2-chloro-*cis*,*cis*-muconate turnover, small amounts of 2-chloro- and 5-chloromuconolactone occurred temporarily as intermediates, indicating that the pAC27- and pJP4-encoded chloromuconate cycloisomerases would not discriminate between 1,4-cycloisomerization, yielding 2-chloromuconolactone, and 3,6-cycloisomerization, yielding 5-chloromuconolactone (Fig. 4). However, 5-chloromuconolactone appeared to be converted faster than 2-chloromuconolactone (at least by the pJP4-encoded enzyme). Thus, despite similar concentrations detected by HPLC, 5-chloromuconolactone is probably formed in larger amounts than 2-chloromuconolactone, and consequently there seems to be discrimination between the two directions of cycloisomerization (34).

cycloisomerization is reversible. Activity towards (+)-2-chloromuconolactone was at the detection limit with overlay UV spectra as well as HPLC analyses (substrate concentration, 0.1 mM; detection limit, ca. 0.03 U/mg).

Comparison of the catalytic properties with those of cycloisomerases of other bacteria. The identification of 5-chloromuconolactone as the product of 2-chloro-*cis*,*cis*-muconate turnover by chloromuconate cycloisomerase of *R. erythropolis* 1CP shows that this enzyme is unable to catalyze a dehalogenation of the lactone. In this property, it differs significantly from its counterparts in gram-negative bacteria, which for some time have been known to convert 2-chloro-*cis*,*cis*-muconate to *trans*-dienelactone, a reaction which includes a chloride elimination (12, 25) (Fig. 4). The observation also shows that the capability to catalyze a chloride elimination during 2-chloro-*cis*,*cis*-muconate conversion is not necessarily coupled to the ability to convert and dehalogenate 3-chloro- and 2,4-dichloro-*cis*,*cis*-muconate.

Another implication of 5-chloromuconolactone being the only product formed from 2-chloro-*cis*,*cis*-muconate is that both the muconate and the chloromuconate cycloisomerase of *R. erythropolis* 1CP discriminate between the two possible directions of 2-chloro-*cis*,*cis*-muconate cycloisomerization. This contrasts with the behavior of the muconate cycloisomerases of the gram-negative strains *Pseudomonas* sp. strain B13, *P. putida* PRS2000, and *Acinetobacter calcoaceticus* ADP1, which have recently been shown to convert 2-chloro-*cis*,*cis*-muconate to a mixture of (+)-2-chloro- and (+)-5-chloromuconolactone (33) (Fig. 4). The chloromuconate cycloisomerase encoded by pJP4, on the other hand, appears to resemble the *Rhodococcus* enzymes in also having a preference for 5-chloromuconolactone formation (34).

A third aspect relevant to the comparison of the *R. erythropolis* 1CP cycloisomerases with those of gram-negative bacteria is the ability or inability to convert (+)-2-chloromuconolactone. While the muconate and chloromuconate cycloisomerases of gram-negative bacteria form the same products from this compound as from 2-chloro-*cis*,*cis*-muconate or (+)-5-chloromuconolactone (33, 34), neither *Rhodococcus* enzyme showed significant activity with this substrate.

If substrate specificity, especially with respect to 3-chloro- and 2,4-dichloro-*cis*,*cis*-muconate, is taken into consideration as an additional catalytic property, four groups of bacterial muconate and chloromuconate cycloisomerases need to be differentiated (Table 3): (i) muconate cycloisomerases, as found in gram-negative bacteria such as *P. putida* or *A. calcoaceticus*; (ii) chloromuconate cycloisomerases, as encoded by plasmids of gram-negative bacteria such as pJP4 or pAC27; (iii) muconate cycloisomerases, as in *R. erythropolis* 1CP; and (iv) chloromuconate cycloisomerases, as in *R. erythropolis* 1CP. For phenotypic groups 1 and 2, it has already been shown by DNA sequencing that they correspond to similarities at the genetic level and that the enzymes of both groups are homologous to each other (for a review, see reference 22). Comparison of the N-terminal sequences shown above (Fig. 2) suggests that the *R. erythropolis* 1CP cycloisomerases (groups 3 and 4) also have been derived from the same precursor as the cycloisomerases of gram-negative bacteria.

Among the criteria listed in Table 3, the muconate and the chloromuconate cycloisomerases of *R. erythropolis* 1CP differ only in one—the magnitude of the $k_{cat}/K_{m,app}$ values for 3-chloro- and 2,4-dichloro-*cis*,*cis*-muconate. In contrast, comparisons with the respective enzymes of gram-negative strains showed correspondence in only two criteria and differences in the other (Table 3). Thus, the catalytic properties of the cycloisomerases suggest that the two enzymes from *R. erythropolis* 1CP might be more closely related to each other than to the corresponding enzymes from gram-negative bacteria. Such an interpretation is consistent with the pattern of N-terminal sequence similarities described above. If one assumes that muconate turnover is older than chloromuconate conversion, a relatively close relatedness of the *Rhodococcus* enzymes would imply that the capability to convert 3-chloro- and 2,4-dichloro-*cis*,*cis*-muconate effectively arose at least twice independently by functionally convergent evolution (5)—at least once among the proteobacteria and at least once among the gram-positive bacteria. The results with the cycloisomerases of *R. erythropolis* 1CP, which are reported here, are in concordance with those with chlorocatechol 1,2-dioxygenase and dienelactone hydrolase, which have been shown to differ considerably from their counterparts in gram-negative bacteria (15, 16). Genetic work to provide clear-cut proof for the assumed convergence is in progress.

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