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Formation of Protoanemonin from 2-Chloro-*cis*,*cis*-Muconate by the Combined Action of Muconate Cycloisomerase and Muconolactone Isomerase

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Muconate cycloisomerases are known to catalyze the reversible conversion of 2-chloro-*cis*,*cis*-muconate by 1,4- and 3,6-cycloisomerization into (4*S*)-(+)-2-chloro- and (4*R*/5*S*)-(+)-5-chloromuconolactone. 2-Chloromuconolactone is transformed by muconolactone isomerase with concomitant dechlorination and decarboxylation into the antibiotic protoanemonin. The low k_{cat} for this compound compared to that for 5-chloromuconolactone suggests that protoanemonin formation is of minor importance. However, since 2-chloromuconolactone is the initially predominant product of 2-chloromuconate cycloisomerization, significant amounts of protoanemonin were formed in reaction mixtures containing large amounts of muconolactone isomerase and small amounts of muconate cycloisomerase. Such enzyme ratios resemble those observed in cell extracts of benzoate-grown cells of *Ralstonia eutropha* JMP134. In contrast, *cis*-dienelactone was the predominant product formed by enzyme preparations, in which muconolactone isomerase was in vitro rate limiting. In reaction mixtures containing chloromuconate cycloisomerase and muconolactone isomerase, only minute amounts of protoanemonin were detected, indicating that only small amounts of 2-chloromuconolactone were formed by cycloisomerization and that chloromuconate cycloisomerase actually preferentially catalyzes a 3,6-cycloisomerization.

The aerobic degradation of aromatic compounds usually involves their successive activation and modification so that they are channeled toward a few dihydroxylated intermediates such as catechol, gentisate, or protocatechuate, which are then subject to aromatic ring cleavage. Some of the enzyme systems capable of activating aromatic compounds have broad substrate specificity and transform chlorinated substrate analogues, often resulting in the formation of chlorinated catechols (22, 23). However, only a small fraction of bacteria able to transform chloroaromatics into chlorocatechols are capable of mineralizing chlorocatechols (22), since this usually necessitates the presence of enzymes of the chlorocatechol pathway (7–9, 25). Thus, chlorocatechols can be regarded as environmentally important intermediates, and when they are processed by enzymes that are members of widespread pathways for the metabolism of catechol, their metabolic fate can be of environmental significance.

It has recently been shown that there are severe differences between reactions catalyzed by the chlorocatechol and the 3-oxoadipate pathway enzymes. In both cases, chlorocatechols were subject to intradiol cleavage, with the corresponding *cis*,*cis*-muconates as products (2, 7, 8). However, muconate and chloromuconate cycloisomerases perform distinct reactions. Whereas chloromuconate cycloisomerases catalyze a dehalogenation of 3-chloro-*cis*,*cis*-muconate to form *cis*-dienelactone, muconate cycloisomerases catalyze a dehalogenation and decarboxylation to form the antibiotic protoanemonin (2). Protoanemonin formation, in turn, was assumed to be the reason for the poor survival of polychlorinated-biphenyl-cometabolizing organisms in soil microcosms due to channeling of intermediary chlorobenzoate into the 3-oxoadipate pathway (1). Also, in the case of 2-chloromuconate turnover, muconate and chloromuconate cycloisomerases were shown to catalyze different reactions (Fig. 1). Whereas chloromuconate cycloisomerase catalyzes dehalogenation to form *trans*-dienelactone (28), muconate cycloisomerases catalyze cycloisomerization only, to form both 2-chloromuconolactone (2CML) and 5CML as stable products (29). Only chloromuconate cycloisomerase, but not muconate cycloisomerase, catalyzes the dehalogenation of 5CML (28, 29). Also, 2CML, in contradiction to previous assumptions, was biologically active and was converted to *trans*-dienelactone via 2-chloromuconate and 5CML by chloromuconate cycloisomerase. In addition to those enzymes purified from gram-negative bacteria, muconate cycloisomerases with distinct catabolic properties have been purified from *Rhodococcus* strains (27). None of the *Rhodococcus* enzymes could dehalogenate 2-chloromuconate, and 5CML was the only reaction product, showing that the enzymes discriminate between the different cycloisomerization possibilities. Based on kinetic data, Vollmer and Schlömann (28) postulated that chloromuconate cycloisomerases of gram-negative bacteria also preferentially catalyze a 3,6-cycloisomerization, resulting in the formation of 5CML, which is subsequently dehalogenated to form *trans*-dienelactone. It has recently been shown that 5CML is also a substrate of muconolactone isomerase (20). This enzyme functions in the 3-oxoadipate pathway, converting muconolactone, formed by cycloisomerization of muconate, into 3-oxoadipate enol-lactone (12). Muconolactone isomerase catalyzes the dehalogenation of 5CML to form *cis*- and *trans*-dienelactone in a 3:1 to 4:1 ratio. The mechanism was proposed via abstraction of the C-4 proton followed by

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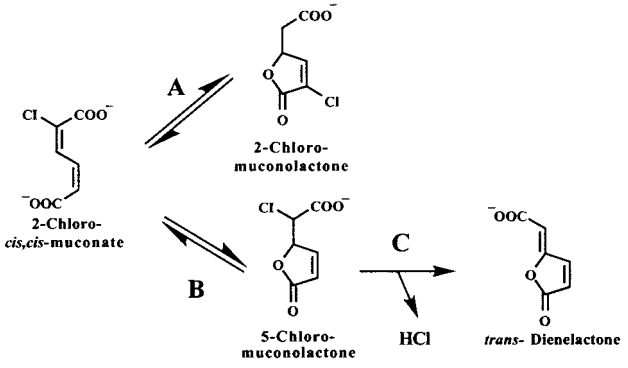

FIG. 1. Transformation of 2CM by muconate and chloromuconate cycloisomerases. Most muconate cycloisomerases described thus far catalyze a 1,4-cycloisomerization to form 2CML (reaction A) and a 1,6-cycloisomerization to form 5-CML (reaction B) (29). Muconate cycloisomerase isolated from *Rhodococcus erythropolis* 1CP catalyze reaction B only (27). Chloride elimination from 5CML to give *trans*-dienelactone is catalyzed exclusively by chloromuconate cycloisomerases (28).

spontaneous chloride elimination. The metabolism of 2-chloro-*cis*,*cis*-muconate via the 3-oxoadipate pathway should then, taking into the account the equilibrium between 2CML and 5CML and 2-chloro-*cis*,*cis*-muconate (2CM) catalyzed by muconate cycloisomerase, result in the formation of predominantly *cis*-dienelactone from 2-chloromuconate. However, like 5CML, 2CML harbors a proton at C-4, which theoretically can be abstracted by muconolactone isomerase. In the present report we show that 2CML is also transformed by muconolactone isomerase with protoanemonin as product and that significant amounts of protoanemonin can be formed when 3-chlorocatechol is misrouted into the 3-oxoadipate pathway.

MATERIALS AND METHODS

Chemicals and biochemicals. Chemicals were purchased from Mallinckrodt Baker (Griesheim, Germany), Sigma-Aldrich Chemie (Taufkirchen, Germany), and Merck AG (Darmstadt, Germany). 2-Chloro-*cis*,*cis*-muconate and (4*R*,5*R*/4*S*,5*S*)-5-chloro-3-methylmuconolactone were prepared as described by Pieper et al. (14, 17). (4*R*,5*S*)-(+)-5-Chloromuconolactone [(4*R*,5*S*)-(+)-5CML] and (4*S*)-2-chloromuconolactone [(4*S*)-2CML] were prepared as described by Prucha et al. (21). Larger amounts of (4*R*/4*S*)-2CML were prepared chemically by incubating 150 mg of 2-chloromuconate in 7.5 ml of H_2SO_4 (75%) overnight at room temperature. The reaction was stopped by dilution with 20 ml of H_2O and addition of NH_4OH to give a final pH of 3.5. The reaction mixture was extracted four times with equal volumes of ethyl acetate, dried over $MgSO_4$, evaporated to dryness, and redissolved in 20 ml of H_2O. High-performance liquid chromatography (HPLC) analysis showed that beside residual 2CM and 2-chloro-*cis*,*trans*-muconate, the solution contained mainly 2CML. The pH of the solution was adjusted to 5. Extraction with ethyl acetate (five times with equal volumes) led to the removal of 2CML only. This extract was again dried over $MgSO_4$ and evaporated to dryness.

Organism. The 2,4-dichlorophenoxyacetate-degrading organism *Ralstonia eutropha* JMP134 was isolated by Don and Pemberton (5).

Culture conditions and preparation of cell extracts. Growth in liquid culture was performed as previously described (18), with the mineral salts medium of Dorn et al. (6) modified such that the concentration of the buffer was twice that described and with benzoate concentrations of up to 15 mM or 2,4-dichlorophenoxyacetate concentrations of 5 mM. Cell extracts were prepared as described previously (18).

Enzyme assays. Muconolactone isomerase (EC 5.3.3.4) and methylmuconolactone isomerase were assayed by the method of Prucha et al. (20) in 50 mM potassium/sodium phosphate (pH 7.5), with 0.1 mM (4*S*,5*S*/4*R*,5*R*)-5-chloro-3-methylmuconolactone as substrate. Product accumulation was analyzed spectrophotometrically at 270 nm ($\varepsilon_{3\text{-methyl-}trans\text{-dienelactone}}$ = 15,200 M^{-1} cm^{-1}). Transformation of 2CML was monitored spectrophotometrically at 260 nm ($\varepsilon_{protoanemonin}$ = 15,000 M^{-1} cm^{-1}). Substrate concentrations of 0.05 to 2 mM were used for determination of K_m and k_{cat} values.

Chlorocatechol 1,2-dioxygenase, muconate cycloisomerase, chloromuconate cycloisomerase, and dienelactone hydrolase were measured as previously described (7, 24, 25). 3-Chloromuconate as a substrate for chloromuconate cycloisomerase was prepared in situ from 4-chlorocatechol by using partially purified chlorocatechol 1,2-dioxygenase (11).

Specific activities are expressed as micromoles of substrate converted or product formed per minute per gram of protein at 25°C. Protein concentrations were determined by the Bradford procedure (3).

Enzyme purification. For analysis of kinetic properties, muconolactone isomerase and methylmuconolactone isomerase were purified to homogeneity from benzoate- or 4-methylmuconolactone-grown cells as previously described (19, 20). For all other experiments, partially purified enzymes from benzoate-grown cells (muconate cycloisomerase and muconolactone isomerase) or from 2,4-dichlorophenoxyacetate-grown cells (chlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase, and dienelactone hydrolase) were used. Cells from 1 to 2 liters of culture fluid were harvested during late exponential growth, and the cell extract (usually 0.8 to 1.5 ml containing 50 to 200 mg of protein) was directly applied to a MonoQ HR10/10 column. Proteins were eluted with a linear gradient of NaCl (0 to 0.5 M) in 50 mM Tris-HCl (pH 7.5) supplemented with 2 mM $MnCl_2$, in a total volume of 200 ml (flow rate, 2 ml/min; fraction volume, 4 ml). Muconolactone isomerase of benzoate-grown cells under these conditions did not bind to the column. Fractions containing this enzyme were incubated for 10 min at 70°C. The supernatant after centrifugation contained partially purified muconolactone isomerase. Other enzymes used in transformation experiments eluted at 0.16 M (dienelactone hydrolase), 0.22 M (muconate cycloisomerase), 0.29 M (chlorocatechol dioxygenase), or 0.43 M NaCl (chloromuconate cycloisomerase) and were free of any other enzyme possibly interfering with the reaction analyzed.

Analytical methods. HPLC of low-molecular-weight compounds was performed with a Lichrospher SC 100 RP8 reverse-phase column (125 by 4.6 mm) (Bishoff, Leonberg, Germany). Methanol-H_2O (25:75) containing 0.1% (vol/vol) H_3PO_4 was used as eluent at a flow rate of 1 ml/min. The column effluent was monitored simultaneously at 210, 260, and 270 nm by a diode array detector (Shimadzu Corp., Kyoto, Japan). Typical retention volumes were as follows: 2-chloro-*cis*,*cis*-muconate, 7.7 ml; 2CML, 2.5 ml; 5CML, 1.1 ml; *cis*-dienelactone, 4.1 ml; *trans*-dienelactone, 1.8 ml; and protoanemonin, 3.4 ml. Kinetic measurements were recorded on a UV 2100 spectrophotometer (Shimadzu Corp.). ^1H nuclear magnetic resonance spectra were recorded on a CXP 300 spectrometer (Bruker) with tetramethylsilane as the internal standard and deuterated acetone as the solvent.

Transformation of 2-chloromuconate by enzyme mixtures. Transformation of 2-chloromuconate was usually performed in 50 mM Tris-HCl (pH 7.5). The reaction mixtures contained 100 μM 2-chloromuconate, 1 to 50 mU of muconolactone isomerase, and 5 to 200 mU of muconate cycloisomerase in a total volume of 0.1 ml. For analyzing the cycloisomerization direction preformed by chloromuconate cycloisomerase, the reaction mixture contained 100 μM 2-chloromuconate, 2 to 5 mU of chloromuconate cycloisomerase, and 2 to 50 mU of muconolactone isomerase in a total volume of 0.1 ml. The product ratios produced in the presence of characteristic enzyme ratios were determined in triplicate. A milliunit is defined as the amount of enzyme transforming 1 nmol of 3-methyl-5CML (muconolactone isomerase), *cis*,*cis*-muconate (muconate cycloisomerase), or 3-chloromuconate (chloromuconate cycloisomerase) per min when the substrate is supplied at 100 μM.

Mathematical calculations. Sets of linear equations were solved using MAPLE V (Waterloo, Ontario, Canada). Numerical calculations were done with MATLAB 5 (The Math Works, Natik, Mass.).

RESULTS

Metabolism of 2CML by muconolactone isomerase and methylmuconolactone isomerase of R. eutropha JMP134. (4*S*)-2CML and a racemic mixture of (4*S*/4 *R*)-2CML were subject to biotransformation by purified muconolactone isomerase and methylmuconolactone isomerase. Biologically synthesized (4*S*)-2CML was quantitatively transformed by both enzymes, whereas only 50% ± 5% of chemically synthesized (4*R*/4*S*)-2CML was transformed in each case. Repeated UV

TABLE 1. Catalytic properties of muconolactone isomerase and methylmuconolactone isomerase from *R. eutropha* JMP134[a]

Substrate	Enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1} \cdot min^{-1}$)
(4*S*)-Muconolactone	MLI	6.0 ± 1.2	41,000 ± 6,900	6,830
	MMLI	8.9 ± 0.7	22,000 ± 1,600	2,500
(4*R*,5*R*/4*S*,5*S*)-5-Chloro-3-methylmuconolactone	MLI	0.66 ± 0.02	2,550 ± 80	3,870
	MMLI	9.8 ± 1.6	16,000 ± 1,600	1,600
(4*R*,5*S*)-5CML	MLI	10.3 ± 1.8	357,000 ± 46,000	34,700
	MMLI	5.2 ± 0.8	19,000 ± 2,100	3,600
(4*S*)-2CML	MLI	3.6 ± 0.5	430 ± 45	120
	MMLI	10.2 ± 1.3	490 ± 55	50

[a] Catalytic properties of the enzymes with (4*S*)-muconolactone, (4*R*,5*R*/4*S*,5*S*)-5-chloro-3-methylmuconolactone, and (4*R*,5*S*)-5CML have been reported by Prucha et al. (19, 20) and include the previously determined standard deviations.

spectra during turnover of (4*S*)-2CML and (4*R*/4*S*)-2CML by muconolactone isomerase or methylmuconolactone isomerase showed the formation of a new absorption maximum at 260 nm. This absorption maximum is significantly different from the absorption maxima at 274 to 277 nm reported for *cis*- or *trans*-dienelactone (26) but similar to the one observed for protoanemonin (2). Actually, HPLC analysis, including analysis of in situ UV absorption spectra of the metabolite formed from (4*S*)-2CML and from (4*S*/4*R*)-2CML by muconolactone isomerase or methylmuconolactone isomerase and of authentic protoanemonin showed identical retention volumes and identical spectra. To further reveal the identity of the product to protoanemonin, (4*R*/4*S*)-2CML was transformed by partially purified muconolactone isomerase. A total amount of 15 mg of (4*R*/4*S*)-2CML in a total volume of 20 ml of 50 mM phosphate buffer (pH 7) was incubated overnight with 2 U of partially purified muconolactone isomerase. Extraction with diethyl ether under neutral conditions resulted in extraction from the incubation mixture of the reaction product only, whereas residual 2CML remained in the aqueous phase. ^{1}H nuclear magnetic resonance analysis of the purified product showed signals, which proved the identity of the isolated product to protoanemonin (four olefinic protons with chemical shifts of 6.38, 7.76, 5.07 and 5.21 ppm, exhibiting a geminal coupling of 2.8 Hz between H-3 and H-4, a vicinal coupling of 5.6 ppm between H-1 and H-2, and long-range couplings between H-1 and H-4 as well as H-1 and H-3). Based on the identification, accumulation of protoanemonin from (4*S*)-2CML was quantified by use of an authentic standard and summed up to more than 90% of substrate added.

Catalytic efficiencies of muconolactone isomerase and methylmuconolactone isomerase with 2CML. The catalytic properties of purified muconolactone isomerase and methylmuconolactone isomerase were measured with (4*S*)-2CML (Table 1) and compared to those previously reported with muconolactone, 5CML and 5-chloro-3-methylmuconolactone (19, 20). As for the other substrates, both enzymes exhibit relatively high K_m values for (4*S*)-2CML. k_{cat} values were in the range of 430 to 490 min^{-1}, much lower than with other substrates tested. Taking into consideration the k_{cat}/K_m value as an indication of specificity, 2CML is evidently the least preferred substrate of the range of substrates tested.

Transformation of 2CM by the combined action of muconate cycloisomerase and muconolactone isomerase. Muconate cycloisomerases have been reported to catalyze the formation of an equilibrium between 2CM, 2CML, and 5CML (29). We could confirm that at equilibrium at a pH of 7.5, those compounds, as indicated previously (29), were present in a 4:1:2 mixture. Considering the poor activity of muconolactone isomerase with 2CML compared to that with 5CML, one might assume at first glance that formation of protoanemonin from 2CM is not of major importance. However, it is evident that the actual rate of formation of protoanemonin depends on the kinetics of both muconate cycloisomerase and muconolactone isomerase. In the presence of an excess of muconate cycloisomerase, it can be assumed that this enzyme catalyzes the formation of the respective equilibrium and muconolactone isomerase acts on a 1:2 mixture of 2CML and 5CML. Consequently, under those conditions, *cis*-dienelactone is expected to be the dominant reaction product. Actually, when 2CM was converted by muconate cycloisomerase (1.5 U/ml) and the resulting product mixture was supplemented with muconolactone isomerase (50 mU/ml), only minor amounts of protoanemonin, summing to 4% ± 1% of the applied 2CM, were produced (Fig. 2A). *cis*- and *trans*-dienelactone were formed in a 4:1 ratio as reported for transformation of 5-CML by purified muconolactone isomerase (20), and they dominated the final product mixture. Similar results were obtained when 2CM was transformed by a mixture of an excess muconate cycloisomerase and limiting amounts of muconolactone isomerase (Fig. 2B) (enzyme ratio, 83:1).

The extent of production of protoanemonin was higher than that expected from a comparison of the respective k_{cat} and K_m values (about 290:1 for the ratio of 5CML to 2CML). However, it should be noted that the k_{cat} for 5CML was calculated based on substrate concentrations of up to 2 mM only (20), and thus, due to the high K_m value, kinetic data represent only approximate values. Obviously the k_{cat} for 5CML is significantly lower than was previously determined.

As reported by Vollmer et al. (29) for the *Pseudomonas putida* muconolactone isomerase, during the initial reaction 2CM was converted predominantly into 2CML, indicating that this conversion is favored kinetically. In contrast, 5CML is the thermodynamically preferred product, and after the reaction reached equilibrium, this compound dominated over 2CML. Similar results were observed with the JMP134 enzyme (data not shown). We therefore assumed that in the presence of rate-limiting amounts of muconate cycloisomerase, 2CML will be the dominating substrate for muconolactone isomerase and that therefore larger amounts of protoanemonin should be

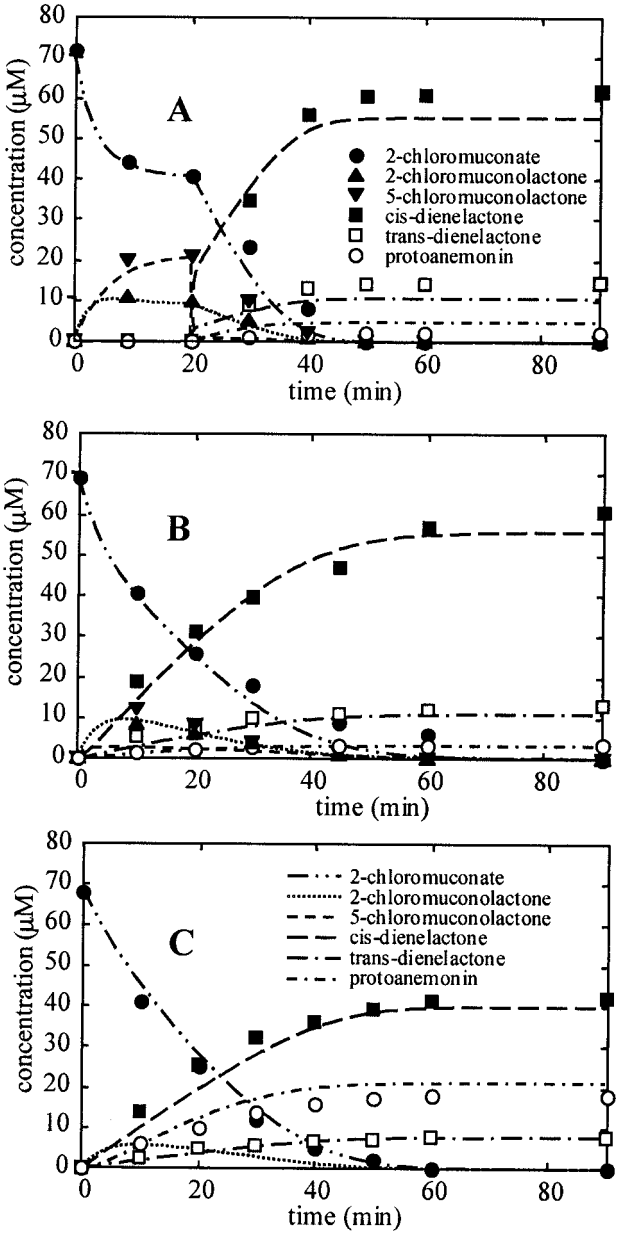

FIG. 2. HPLC analyses of the conversion of 2CM by mixtures of muconate cycloisomerase and muconolactone isomerase of *R. eutropha* JMP134. Reaction mixtures (0.1 ml) contained 30 mM Tris-HCl (pH 7.5) supplemented with 1 mM $MnCl_2$, 0.07 mM 2CM, and 150 mU of muconate cycloisomerase plus 5 mU of muconolactone isomerase (added after 20 min of reaction) (A), 100 mU of muconate cycloisomerase plus 1.2 mU of muconolactone isomerase (B), or 60 mU of muconate cycloisomerase plus 12 mU of muconolactone isomerase (C). Symbols represent experimental data; broken lines represent the simulations by the model.

formed under those conditions. When 2CM was transformed by an enzyme mixture of muconate cycloisomerase corresponding to an activity of 600 mU/ml and muconolactone isomerase corresponding to an activity of 120 mU/ml (enzyme ratio, 5:1), 25% ± 5% of applied 2CM was transformed into protoanemonin (Fig. 2C). The ratio of *cis*-dienelactone to protoanemonin formed under these conditions was 2.1:1. In cell

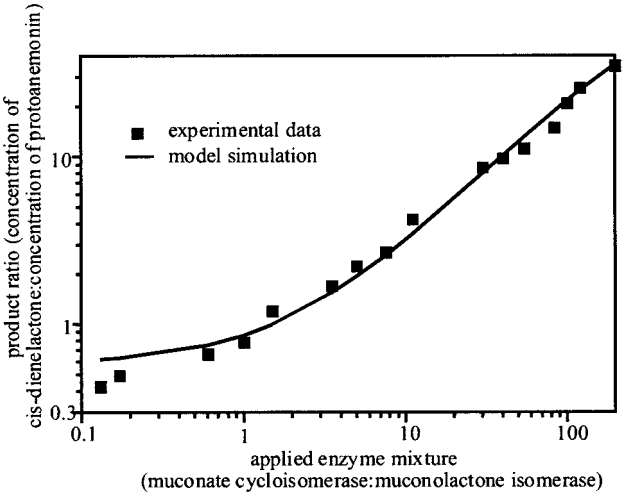

FIG. 3. Products formed after complete conversion of 2CM by different mixtures of muconate cycloisomerase and muconolactone isomerase of *R. eutropha* JMP134. Shown is the ratio at which *cis*-dienelactone and protoanemonin are formed. *trans*-Dienelactone was always formed in amounts corresponding to 20 to 25% that of *cis*-dienelactone. Activity ratios were calculated based on the activity of muconate cycloisomerase with 0.1 mM 2CM and of the activity of muconolactone isomerase with 0.1 mM 5-chloro-3-methylmuconolactone.

extracts of benzoate-grown cells of JMP134, typical muconate cycloisomerase activities were 370 ± 80 U/g of protein and typical muconolactone isomerase activities were 180 ± 50 U/g of protein, with activity ratios of muconate cycloisomerase to muconolactone isomerase of 2:1 to 3:1. Even though no intermediate formation of 2CML nor 5CML was observed when applying muconate cycloisomerase and muconolactone isomerase in a ratio of 5:1, the reaction could be shifted more drastically toward the formation of protoanemonin by further increasing the relative amount of muconolactone isomerase in the reaction mixture (Fig. 3). In the presence of an excess of muconolactone isomerase, protoanemonin was the dominating reaction product (*cis*-dienelactone and protoanemonin were formed in a ratio of 0.4:1). In the presence of an excess of muconate cycloisomerase, *cis*-dienelactone strongly dominated the product mixture (ratios of up to 35:1).

Modeling of protoanemonin formation. The experimental results so far indicate that protoanemonin formation from 2CM is based on the action of two enzymes (Fig. 4). Muconate cycloisomerase catalyzes the formation of an equilibrium between 2CM, 2CML, and 5CML. Thus, the breakdown of the enzyme-substrate complex can be considered reversible in all directions. Muconolactone isomerase converts 2CML to protoanemonin and 5CML to a mixture of *cis*- and *trans*-dienelactone, and thus irreversible breakdown of the enzyme-substrate complexes can be assumed in this case. Because both CMLs are substrate to the same enzyme, the mechanism is considered similar to a competitive inhibition, where each CML inhibits the reaction rate of the other. To support this assumption, a kinetic model was established.

Application of the steady-state method to the reaction scheme leads to the equations below. The following abbrevia-

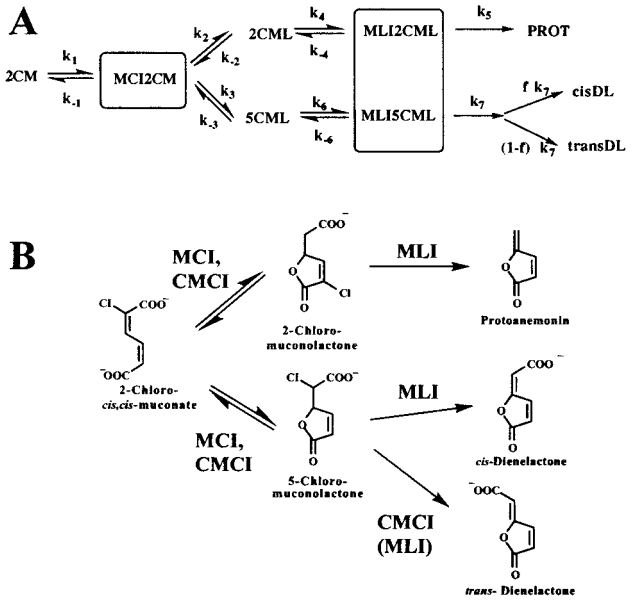

FIG. 4. Reaction scheme for the transformation of 2CM by muconate cycloisomerase and muconolactone isomerase (A) and the transformation of 2CM muconate by mixtures of muconate or chloromuconate cycloisomerase (MCI and CMCI) and muconolactone isomerase (MLI) (B).

tions are used: 2CM, 2-chloromuconate; 2CML, 2-chloromuconolactone; 5CML, 5-chloromuconolactone; Prot, protoanemonin; *cis*DL, *cis*-dienelactone; *trans*DL, *trans*-dienelactone; DL, dienelactone; MCI, muconate cycloisomerase; MLI, muconolactone isomerase.

(i) Muconate cycloisomerase:

$$\frac{d[\text{MCI2CM}]}{dt} = 0 = k_1[\text{MCI}][\text{2CM}] - k_{-1}[\text{MCI}][\text{MCI2CM}] - k_2[\text{MCI2CM}] + k_{-2}[\text{2CML}][\text{MCI}] - k_3[\text{MCI2CM}] + k_{-3}[\text{MCI}][\text{5CML}] \quad (1)$$

$$[\text{MCI}]_{\text{total}} = [\text{MCI}] + [\text{MCI2CM}] \quad (2)$$

$$v_{\text{2CM}} = \frac{d[\text{2CM}]}{dt} = k_{-1}[\text{MCI2CM}] - k_1[\text{MCI}][\text{2CM}] \quad (3)$$

$$v_{\text{2CML}} = \frac{d[\text{2CML}]}{dt} = k_2[\text{MCI2CM}] - k_{-2}[\text{2CML}][\text{MCI}] \quad (4)$$

$$v_{\text{5CML}} = \frac{d[\text{5CML}]}{dt} = k_3[\text{MCI2CM}] - k_{-3}[\text{5CML}][\text{MCI}] \quad (5)$$

At equilibrium

$$k_2[\text{MCI2CM}] = k_{-2}[\text{2CML}][\text{MCI}] \quad (6)$$

$$k_3[\text{MCI2CM}] = k_{-3}[\text{5CML}][\text{MCI}] \quad (7)$$

$$\frac{[\text{2CML}]}{[\text{2CM}]} = a, \quad \frac{[\text{2CML}]}{[\text{2CM}]} = b \quad \text{(ratio of equilibrium concentrations)} \quad (8 \text{ and } 9)$$

(ii) Muconolactone isomerase:

$$\frac{d[\text{MLI2CML}]}{dt} = 0 = k_4[\text{MLI}][\text{2CML}] - k_{-4}[\text{MLI2CML}] - k_5[\text{MLI2CML}] \quad (10)$$

$$\frac{d[\text{MLI5CML}]}{dt} = 0 = k_6[\text{MLI}][\text{5CML}] - k_{-6}[\text{MLI5CML}] - k_7[\text{MLI5CML}] \quad (11)$$

$$[\text{MLI}]_{\text{total}} = [\text{MLI}] + [\text{MLI2CML}] + [\text{MLI5CML}] \quad (12)$$

$$v_{\text{Prot}} \frac{d[\text{Prot}]}{dt} = k_5[\text{MLI2CML}] \quad (13)$$

$$v_{\text{DL}} = \frac{d[\text{DL}]}{dt} = k_5[\text{MLI5CML}] \quad (14)$$

cis- and *trans*-dienelactone were considered to be formed at a fixed ratio:

$$v_{cis\text{DL}} = f v_{\text{DL}}, \; v_{trans\text{DL}} = (1 - f)v_{\text{DL}}, \quad (15)$$

(iii) Combination of the two enzyme reactions:

$$v_{\text{2CML}} = \frac{d[\text{2CML}]}{dt} = k_2[\text{MCI2CM}] - k_{-2}[\text{2CML}][\text{MCI}] - k_4[\text{MLI}][\text{2CML}] + k_{-4}[\text{MLI2CML}] \quad (16)$$

$$v_{\text{5CML}} = \frac{d[\text{5CML}]}{dt} = k_3[\text{MCI2CM}] - k_{-3}[\text{5CML}][\text{MCI}] - k_6[\text{MLI}][\text{5CML}] + k_{-6}[\text{MLI5CML}] \quad (17)$$

This set of linear equations was solved using Maple V, resulting in the following set of kinetic equations for the six compounds:

$$v_{\text{2CM}} = \frac{d[\text{2CM}]}{dt} = -[\text{MCI}]_{\text{total}} \frac{k_2\left([\text{2CM}] - \frac{[\text{2CML}]}{a}\right) + k_3\left([\text{2CM}] - \frac{[\text{5CML}]}{b}\right)}{\left[\text{2CM}] + \frac{K_a}{a}[\text{2CML}] + \frac{K_b}{b}[\text{5CML}] + K_c\right.} \quad (18)$$

$$v_{\text{2CML}} = \frac{d[\text{2CML}]}{dt} = k_2[\text{MCI}]_{\text{total}} \frac{[\text{2CM}] - \frac{1 + K_b}{a}[\text{2CML}] + \frac{K_b}{b}[\text{5CML}]}{[\text{2CM}] + \frac{K_a}{a}[\text{2CML}] + \frac{K_b}{b}[\text{5CML}] + K_c} - k_5[\text{MLI}]_{\text{total}} \frac{[\text{2CML}]}{[\text{2CML}] + K_{m1}\left(1 + \frac{[\text{5CML}]}{K_{m2}}\right)} \quad (19)$$

$$v_{\text{5CML}} = \frac{d[\text{5CML}]}{dt} = k_3[\text{MCI}]_{\text{total}} \frac{[\text{2CM}] - \frac{1 + K_a}{b}[\text{5CML}] + \frac{K_a}{a}[\text{2CML}]}{[\text{2CM}] + \frac{K_a}{a}[\text{2CML}] + \frac{K_b}{b}[\text{5CML}] + K_c}$$

$$- k_7[\mathrm{MLI}]_{\mathrm{total}} \frac{[5\mathrm{CML}]}{[5\mathrm{CML}] + K_{m2}\left(1 + \frac{[2\mathrm{CML}]}{K_{m1}}\right)} \quad (20)$$

$$\nu_{\mathrm{Prot}} \frac{d[\mathrm{Prot}]}{dt} = k_5[\mathrm{MLI}]_{\mathrm{total}} \frac{[2\mathrm{CML}]}{[2\mathrm{CML}] + K_{m1}\left(1 + \frac{[5\mathrm{CML}]}{K_{m2}}\right)} \quad (21)$$

$$\nu_{cis\mathrm{DL}} = \frac{d[cis\mathrm{DL}]}{dt} = fk_7[\mathrm{MLI}]_{\mathrm{total}} \frac{[5\mathrm{CML}]}{[5\mathrm{CML}] + K_{m2}\left(1 + \frac{[2\mathrm{CML}]}{K_{m1}}\right)} \quad (22)$$

$$\nu_{trans\mathrm{DL}} = \frac{d[trans\mathrm{DL}]}{dt} = (1 - f)k_7[\mathrm{MLI}]_{\mathrm{total}} \frac{[5\mathrm{CML}]}{[5\mathrm{CML}] + K_{m2}\left(1 + \frac{[2\mathrm{CML}]}{K_{m1}}\right)} \quad (23)$$

with

$$K_{\mathrm{a}} = \frac{k_2}{k_{-1}}, K_{\mathrm{b}} = \frac{k_3}{k_{-1}}, K_c = \frac{k_{-1} + k_2 + k_3}{k_1}, \quad K_{m1} = \frac{k_{-4} + k_5}{k_4}, K_{m2} = \frac{k_{-6} + k_7}{k_6} \quad (24\text{–}28)$$

To identify the model parameters and to simulate the dynamics and equilibrium states, equations 18 to 23 were integrated numerically using the MATLAB software platform. Parameter identification was done by comparing the model with the experimental data using a least-squares method (simplex algorithm). During the fitting process, the parameters K_a and K_b reached the lower set point (10^{-7}) of the fitting algorithm and were set to zero. A parameter sensitivity analysis revealed linear dependencies between the parameters k_5 and K_{m1} and between k_7 and K_{m2}. This means that, as determined experimentally (Table 1), the values of K_{m1} and K_{m2} (K_m values of the reactions) are much higher than the concentrations of 2CML and 5CML. Thus, these parameters could not be estimated separately and were therefore combined. The terms $k_5[\mathrm{MLI}]_{\mathrm{total}} \frac{[2\mathrm{CML}]}{[2\mathrm{CML}] + K_{m1}\left(1 + \frac{[5\mathrm{CML}]}{K_{m2}}\right)}$ and $k_7[\mathrm{MLI}]_{\mathrm{total}} \frac{[5\mathrm{CML}]}{[5\mathrm{CML}] + K_{m2}\left(1 + \frac{[2\mathrm{CML}]}{K_{m1}}\right)}$ in equations 19 to 23 can therefore be simplified to $\frac{k_5}{K_{m1}}[\mathrm{MLI}]_{\mathrm{total}}[2\mathrm{CML}]$ and $\frac{k_7}{K_{m2}}[\mathrm{MLI}]_{\mathrm{total}}[5\mathrm{CML}]$, respectively. The competitive inhibition between the two CMLs is of no significance in the concentration range used.

Good agreement between experimental data and model was obtained for the equilibrium states of the *cis*-dienelactone-to-protoanemonin concentration ratio as a function of the concentration ratio of the two enzymes, showing that the simple mechanism on which the model is based can describe the equilibrium states of protoanemonin formation by the two enzymes quite well (Fig. 3). Also, the time courses for CML conversion and formation of the end products protoanemonin, *cis*-dienelactone, and *trans*-dienelactone are well simulated by the model for all three experiments shown in Fig. 2. The identified velocity constant for 2CML formation, k_2, is approximately 50% higher than the velocity constant for 5CML formation, k_3. This leads to a more rapid formation of 2CML in the first 5 min, but eventually the equilibrium reaction takes over and leads to a higher accumulation of 5CML after 20 min. Thus, the assumption that 2CML is the kinetically favored product and 5CML the thermodynamically more stable product is also supported by the model (Fig. 2A). After addition of muconolactone isomerase, the model predicts a much faster conversion of 5CML to *cis*-dienelactone (and *trans*-dienelactone) compared with the 2CML conversion to protoanemonin (Fig. 2A). If muconolactone isomerase is present in the reaction mixture from the beginning, 5CML is rapidly converted to *cis*-dienelactone (and *trans*-dienelactone), resulting in a lower accumulation of this compound and in *cis*-dienelactone being the prominent product (Fig. 2B). A larger amount of muconolactone isomerase in the reaction mixture increases the velocity of the conversion of both CMLs proportionally, which reduces the accumulation of both compounds. In this way, the produced CMLs are rapidly converted and the higher formation rate of 2CML in combination with the reduced equilibrium reaction leads to an increased amount of protoanemonin in the reaction mixture (Fig. 2C). A comparison of the catalytic properties of the MLI reactions, estimated from the model (k_5/K_{m1} and k_7/K_{m2} [Table 2]) with experimentally derived data (k_{cat}/K_m [Table 1]), also shows good to moderate agreement. Some discrepancies between the model and experimental data, however, are observed for the time courses of the two intermediates, 2CML and 5CML. The model predicts accumulation of 2CML for the experiment in Fig. 2C, whereas no accumulation was detected experimentally. For 5CML, the conversion to *cis*- and *trans*-dienelactone is slower than predicted by the model (Fig. 2A and B).

TABLE 2. Identified model parameters by fitting the model to the experimental data[a]

Parameter	Value
k_2	3.45×10^{-6} μmol min^{-1} mU^{-1}[b]
k_3	2.38×10^{-6} μmol min^{-1} mU^{-1}[b]
K_c	6.92 μM
a	0.228
b	0.525
f	0.837
k_5/K_{m1}	0.354 min^{-1} $\mu\mathrm{M}^{-1}$[c]
k_7/K_{m2}	19.8 min^{-1} $\mu\mathrm{M}^{-1}$[c]

[a] For definition of the parameters, see Materials and Methods.
[b] For muconate cycloisomerase, the unknown conversion factor is included in the velocity constant.
[c] For muconolactone isomerase, an enzyme concentration of 1 μmol liter^{-1} corresponds to 335 mU ml^{-1} (measured with 0.1 mM 5-chloro-3-methylmuconolactone as substrate).

Transformation of 2CM by the combined action of chloromuconate cycloisomerase and muconolactone isomerase. Whereas muconate cycloisomerases of gram-negative bacteria catalyze the formation of an equilibrium between 2CM,

2CML, and 5CML, chloromuconate cycloisomerases described from those organisms can catalyze a dehalogenation. It was proposed (28) that chloromuconate cycloisomerases discriminate between the two possible modes of cycloisomerization and form predominantly 5CML, which is dehalogenated by the same enzyme to form *trans*-dienelactone. During 2CM turnover, small amounts of 2CML and 5CML (corresponding to 1.5 and 0.9%, respectively, of the applied substrate) were observed to accumulate, and 2CML was shown to be transformed into *trans*-dienelactone via 2CM and 5CML.

The ability of muconolactone isomerase to extract from the equilibrium any 2CML released into the medium during cycloisomerization of 2CM and to transform it into protoanemonin allows us to characterize the mode of cycloisomerization of chloromuconate cycloisomerases. The transformation of 2CM by chloromuconate cycloisomerase (10 to 200 mU/ml) was analyzed in the presence of various amounts of muconolactone isomerase (20 to 500 mU/ml), which should further transform intermediary 2CML. In the absence of muconolactone isomerase, *trans*-dienelactone was the only product observed after completion of the reaction. When chloromuconate cycloisomerase and muconolactone isomerase were added at activities corresponding to 200 U and 20 mU, respectively, both *cis*-dienelactone (3% ± 0.5%) and protoanemonin (2.5% ± 0.5%) were observed as additional products. Increasing the relative amount of muconolactone isomerase respective to chloromuconate cycloisomerase (500 and 10 mU, respectively) led to an increase in the formation of *cis*-dienelactone (4% ± 0.5%) and especially of protoanemonin (5% ± 0.5%).

DISCUSSION

The capabilities of enzymes of the 3-oxoadipate pathway to transform chlorinated catechols have only recently been investigated in detail. Various reports focused on the comparison of muconate and chloromuconate cycloisomerases, since those enzymes were observed to carry out very different reactions, i.e., the formation of protoanemonin and chloroprotoanemonin (2, 10) versus the formation of *cis*-dienelactone and 2-chloro-*cis*-dienelactone (15, 25) from 3-chloromuconate and 2,4-dichloromuconate, respectively, as well as the formation of 2CML and 5CML (29) versus the formation of *trans*-dienelactone (25, 28) from 2-chloromuconate. A second class of enzymes coming into focus are the muconolactone isomerases. Prucha et al. (19–21) showed that muconolactone isomerases are highly nonspecific and can transform differently substituted muconolactones. Chari et al. (4) have shown that transformation of muconolactone is initiated by the abstraction of the C-4 proton, which is subsequently transferred to the C-2 atom and that only the 4*S* enantiomer is used as a substrate. Similarly, it was evidenced that only the stereochemically identical 4*R* enantiomers of 5-chlorosubstituted muconolactones were transformed by this enzyme (21). Since only 50% of chemically prepared 2CML was shown to be transformed, in contrast to a complete transformation of 2CML resulting from muconate cycloisomerase-catalyzed transformation of 2-chloromuconate, it can again be assumed that only the 4*S* configuration is active.

It was postulated (20, 21) that the intermediate anions formed after abstraction of the 4*S* proton of 5-chlorosubstituted muconolactones are stabilized by elimination of chloride

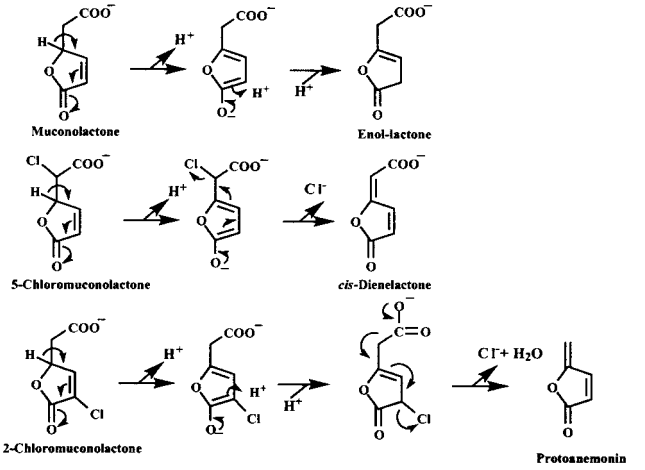

FIG. 5. Proposed reaction mechanism for dehalogenation of 2CML. The isomerization mechanism of muconolactone has been described by Ornston and Stanier (12) and Chari et al. (4), and dehalogenation of 5-chloromuconolactone has been described by Prucha et al. (20).

rather than by the addition of a proton, thereby resulting in the formation of a dienelactone. However, such a mechanism cannot explain the formation of protoanemonin from 2CML. We speculate that the formation of protoanemonin occurs by elimination of CO_2 and chloride from chlorosubstituted 3-oxoadipate enol-lactone, as shown in Fig. 5.

In general, the simple mechanism used for our model (Fig. 4) is quite well suited to describe protoanemonin formation as a function of the concentration ratio of the two enzymes, especially the equilibrium states. The discrepancies between model and experimental data during dynamic states, however, indicate that the proposed mechanism is an oversimplification. The formation of the two CMLs and the equilibrium reaction between them were assumed to be a simple reversible reaction. It was indicated by Vollmer and Schlömann (28) that part of muconolactones do not dissociate from cycloisomerases but remain bound to the enzyme. This could explain the failure to detect the accumulation of small amounts of CMLs predicted by the model in the presence of excess muconolactone isomerase.

Whereas 4-chlorocatechol is transformed by enzymes of the 3-oxoadipate pathway at a rate approximately 10% that of catechol, resulting in the quantitative production of protoanemonin (2, 8, 16), 3-chlorocatechol transformation is far more inefficient, with 3-chlorocatechol and 2-chloromuconate being transformed at about 1% the rate observed with the unsubstituted substrates (8, 16, 29). However, this low rate cannot be neglected. The activity of muconate cycloisomerase with 2-chloromuconate, compared to the activity of muconolactone isomerase with 5CML and even with 2CML, is limiting, at least in the JMP134 system analyzed, and such enzyme specificities seem not to be specific for the JMP134 enzymes but typical for enzymes of the 3-oxoadipate pathway (19, 29). Thus, it can be proposed that when induced for the degradation of aromatic compounds and confronted with 3-chlorocatechol, a significant portion of bacterial strains will generate muconate cycloisom-

erase in rate-limiting amounts and thus form protoanemonin as a dominant product.

Moreover, even when induced for the degradation of chloroaromatics and harboring enzymes of the chlorocatechol pathway, 2-chloromuconate cycloisomerization is a pathway bottleneck in JMP134. When growing on 3-chlorobenzoate, the wild-type strain excretes significant amounts of 2-chloromuconate (14) since both chloromuconate cycloisomerases of this strain are poorly effective with this substrate (11, 13). Thus, 2-chloromuconate is available to be transformed by muconate cycloisomerase, which is simultaneously induced, when JMP134 grows on 3-chlorobenzoate (14).

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