Mechanism of Chloride Elimination from 3-Chloro- and 2,4-Dichloro-
cis,cis-Muconate: New Insight Obtained from Analysis of
Muconate Cycloisomerase Variant CatB-K169A†

URSULA KAULMANN,‡ STEFAN R. KASCHABEK,§ AND MICHAEL SCHLOÈMANN†*

Institut für Mikrobiologie, Universität Stuttgart, D-70569 Stuttgart,1 and Chemische Mikrobiologie,
Bergische Universität—Gesamthochschule Wuppertal, D-42097 Wuppertal,2 Germany

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Chloromuconate cycloisomerases of bacteria utilizing chloroaromatic compounds are known to convert 3-chloro-cis,cis-muconate to cis-dienelactone (cis-4-carboxymethylenebut-2-en-4-olide), while usual muconate cycloisomerases transform the same substrate to the bacteriotoxic protoanemonin. Formation of protoanemonin requires that the cycloisomerization of 3-chloro-cis,cis-muconate to 4-chloromuconolactone is completed by protonation of the exocyclic carbon of the presumed enol/enolate intermediate before chloride elimination and decarboxylation take place to yield the final product. The formation of cis-dienelactone, in contrast, could occur either by dehydrohalogenation of 4-chloromuconolactone or, more directly, by chloride elimination from the enol/enolate intermediate. To reach a better understanding of the mechanisms of chloride elimination, the proton-donating Lys169 of Pseudomonas putida muconate cycloisomerase was changed to alanine. As expected, substrates requiring protonation, such as cis,cis-muconate as well as 2- and 3-methyl-, 3-fluoro-, and 2-chloro-cis,cis-muconate, were not converted at a significant rate by the K169A variant. However, the variant was still active with 3-chloro- and 2,4-dichloro-cis,cis-muconate. Interestingly, cis-dienelactone and 2-chloro-cis-dienelactone were formed as products, whereas the wild-type enzyme forms protoanemonin and the not previously isolated 2-chloroprotolanemonin, respectively. Thus, the chloromuconate cycloisomerases may avoid (chloro-)protoanemonin formation by increasing the rate of chloride abstraction from the enol/enolate intermediate compared to that of proton addition to it.

Chloroaromatic compounds, in general, tend to be relatively persistent to microbial degradation (13, 21). Nevertheless, some of these compounds can be mineralized by specialized bacteria, in many cases via ortho cleavage of chlorocatechol intermediates. Cycloisomerization of the chloro-cis,cis-muconates resulting from ring cleavage is a key reaction, because in chlorocatechol assimilating bacteria it is accompanied by dehalogenation (Fig. 1) (11, 12, 38).

For a long time, muconate cycloisomerases (EC 5.5.1.1) of catechol catabolic pathways and chloromuconate cycloisomerases (EC 5.5.1.7) of chlorocatechol degradative pathways were assumed to catalyze just the cycloisomerization reaction of, for example, 2-chloro- and 3-chloro-cis,cis-muconate to 5-chloromuconolactone (4-carboxychloromethylbut-2-en-4-olide) and 4-chloromuconolactone (4-carboxymethyl-4-chlorobut-2-en-4-olide), respectively (34). Chloride elimination to trans-diene lactone and cis-dienelactone, respectively, was assumed to occur spontaneously in a secondary reaction. However, more recently Vollmer et al. (42) showed that proteobacterial muconate cycloisomerases form a pH-dependent equilibrium mixture of 2- and 5-chloromuconolactone from 2-chloro-cis,cis-

muconate (Fig. 1), proving that these enzymes, in contrast to proteobacterial chloromuconate cycloisomerases, cannot cause dehalogenation during conversion of 2-chloro-cis,cis-muconate. Moreover, Blasco et al. (3) have shown that muconate cycloisomerases convert 3-chloro-cis,cis-muconate predominantly to the antibiotic protoanemonin and not to cis-dienelactone as assumed before (34). Only for chloromuconate cycloisomerases has cis-dienelactone been shown to be the product (23, 34).

Blasso et al. (3) proposed that both muconate and chloromuconate cycloisomerases form 4-chloromuconolactone as an intermediate of 3-chloro-cis,cis-muconate conversion. This would be further transformed in different ways by the two classes of enzymes. However, since the muconate and chloromuconate cycloisomerases catalyze syn additions to a double bond (2, 7), the α,β elimination of HCl from a 4-chloromuconolactone intermediate to yield cis-dienelactone would imply that exactly the same proton as added in the first part of the reaction would be removed in the second (33). Consequently, chloride had been assumed to be abstracted before a proton could be added to what was then regarded as a carbanion intermediate (25). Recent comparative studies on the reaction mechanisms of muconate cycloisomerase and mandelate racemase suggested that the intermediate to which a proton is added in the reaction with cis,cis-muconate is an enol/enolate and not a carbanion (15). Thus, one might assume that in the reaction of chloromuconate cycloisomerases with 3-chloro-cis,cis-muconate, the corresponding enol/enolate intermediate is not protonated but rather loses the negative charge by chloride abstraction. The formation of protoanemonin in the reaction of muconate cycloisomerase with 3-chloro-cis,cis-muconate, in contrast, should
involve a protonation reaction, because two hydrogen atoms are present on the exocyclic carbon.

To test these hypotheses on the enzymatic reaction mechanism, the Lys169 residue of *Pseudomonas putida* muconate cycloisomerase and the Lys165 residue of the chloromuconate cycloisomerase TfdD of pJP4, which are known to provide the proton for the protonation reaction (15, 32), were changed to alanine, and the catalytic properties of the resulting enzyme variants (CatB-K169A and TfdD-K165A) were investigated with various substrates.

(Some of the results have been published in a preliminary communication [U. Schell and M. Schlömann, Bioengineering (special ed.) abstr. PF220, 1998].)

### MATERIALS AND METHODS

Strains, plasmids, and cultivation conditions. *Escherichia coli* strain DH5α was purchased from GIBCO BRL. *E. coli* strain BL21(DE3,pLysS) (37) was used for gene expression under T7lac promoter control. Plasmids used in this study are listed in Table 1. Plasmid-containing strains were usually grown aerobically at 30 or 37°C with constant shaking in 2xYT medium (31) supplied with ampicillin (100 μg/ml). For growth on plates, Luria-Bertani (LB) medium (31) was supplemented with 1.5% (wt/vol) agar.

![FIG. 1. Reactions catalyzed by proteobacterial muconate cycloisomerases (MCI) and chloromuconate cycloisomerases (CMCI). Numbers on the arrows indicate whether the reaction is a 1,4- or a 3,6-cycloisomerization. “CMCI?” indicates that for chloromuconate cycloisomerases it is not clear whether cis-dienelactone is formed directly or via (+)-4-chloromuconolactone as intermediate. No attempt was made to differentiate between fast and slow turnover. The formation of chloromuconolactones involves the syn addition of a proton (bold italics) to the Ca atom.](image)

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relevant characteristics*</th>
<th>Source and/or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript II SK(+)</td>
<td>Phagemid derived from pUC19, lacZp, lacZ’, Ap’, f1(+) and ColE1 origin</td>
<td>Stratagen; 1</td>
</tr>
<tr>
<td>pBluescript II KS(+)</td>
<td>Same as pBluescript II SK(+) but with multiple cloning site in reverse orientation</td>
<td>Stratagen; 1</td>
</tr>
<tr>
<td>pDCA11</td>
<td>pUC28(+) (Ap+) with a 3.4-kb PstI-fragment from Ralstonia eutropha JMP134(pJP4) comprising tfdEFB</td>
<td>41</td>
</tr>
<tr>
<td>pTFDC1</td>
<td>pRSET6a (Ap+) with tfdC from R. eutropha JMP134(pJP4) flanked by NdeI and BamHI sites</td>
<td>44</td>
</tr>
<tr>
<td>pCATB1</td>
<td>pET-11a* (Ap+) with catB from <em>P. putida</em> PRS2000 flanked by NdeI and BamHI sites</td>
<td>43</td>
</tr>
<tr>
<td>pCATB7</td>
<td>pBluescript II KS(+) (Ap+) with 419-bp HindIII-SacII fragment of catB from pPX31</td>
<td>M. D. Vollmer, unpublished results</td>
</tr>
<tr>
<td>pCATB8</td>
<td>pCATB1 (Ap+) with mutated 309-bp NotI-SacII fragment of catB; encodes CatB-K169A</td>
<td>This study</td>
</tr>
<tr>
<td>pTFDD1</td>
<td>pRSET6a (Ap+) with tfdd from <em>R. eutropha</em> JMP134(pJP4) flanked by NdeI and BamHI sites</td>
<td>44</td>
</tr>
<tr>
<td>pDCA7-1</td>
<td>pBluescript II KS(+) (Ap+) with 1.4-kb DraII-SacII fragment from <em>R. eutropha</em> JMP134(pJP4) comprising tfdC’DE’</td>
<td>44</td>
</tr>
<tr>
<td>pTFDD2</td>
<td>pBluescript II SK(+) (Ap+) with 568-bp EagI-AccI fragment of tfdD from pDCA7-1</td>
<td>This study</td>
</tr>
<tr>
<td>pTFDD15</td>
<td>pTFDD1 (Ap+) with mutated 588-bp EagI-AccI fragment of tfdD, encodes TfdD-K165A</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Ap’, resistance to ampicillin.
DNA preparation and in vitro manipulation. Plasmid DNA was isolated by use of a Pharmacia FlexiPrep kit. Restriction endonuclease digests, ligations, and detection of colonies carrying an insert were performed according to standard procedures. DNA fragments were isolated from gels or purified in solution by use of a Geneclean II kit (Bio101, La Jolla, Calif.). Transformation of E. coli strains was achieved by the method of Inoue et al. (19).

PCR mutagenesis. The CatB-K169A variant was generated by PCR-based site-directed mutagenesis as described by Michael (24), using two outer amplification primers and one mutagenic phosphorylated oligonucleotide in one PCR. To avoid mutations other than the one desired, mutagenesis was carried out with pCATB7, which had been constructed earlier by cloning the 419-bp SacII-HindIII fragment of mutagenic catB (M. D. Vollmer, unpublished results). The sequences of the two outer amplification primers were 5′-CTGAAATATCCCTCACTAAAG-3′ (BS-T3, directed toward the T3 promoter region of the vector) and 5′-AATTTGAATACGACTCATATAG-3′ (BS-T7), reverse primer directed toward the T7 promoter region of the vector). Since these primers were originally designed for use with pBluescript and not pBluescript II, some 5′-terminal residues of the primers (bold) were not complementary to the template. To create the Lys (AAG) 169-to-Ala (GCC) mutation, a mutant oligonucleotide was derived for positions 491 to 515 of catB (BS_T7, reverse primer directed toward the T7 promoter region of the vector) and 5′-CATGTCGACTCACTATAG-3′ (BS-T3, directed toward the T3 promoter region of the vector) and designed to create the same substitution on DNA and protein in pBluescript II SK(+) and JMP134(pJP4). The site-directed TfdD-K165A fragment of 5,800 M -1 cm -1, if the reaction proceeded via 2-chloro-cis-diene lactone to 2-chloro maleylactate (23), or 4,300 M -1 cm -1 if the conversion proceeded to 2-chloropropanoammonium (see Results). Correspondingly, a value of 12,400 M -1 cm -1 was used when 3-chloro-cis,cis-muconate was converted via cis-diene lactone to maleylactate, and a value of 4,000 M -1 cm -1 (43) was used when protoanemonin was formed. Protein concentrations were calculated by the Bradford method (4), with bovine serum albumin as the standard.

Enzyme expression and purification. E. coli BL21(DE3, pLysS) was used as the host strain to express wild-type CatB from pCATB1 (43), CatB-K169A from pCATB8, and TfdD-K165A from pTFDD15. Growth and induction conditions were as described for overexpression of wild-type CatB (43) and wild-type TfdD (44), respectively. After induction of expression, the preparation of extracts, and the enzyme purifications, in general, were performed as described by Vollmer et al. (43). The purification comprised an initial anion-exchange chromatography (Q Sepharose high-performance HR16/10) which was followed by hydrophobic interaction chromatography (Phenyl-Superose HR10/10). In the case of wild-type CatB, fractions with the highest specific activity with cis,cis-muconate eluted from the first column at ca. 0.16 M NaCl and from the second column at ca. 0.07 M (NH₄)₂SO₄. In the case of CatB-K169A, activities with cis,cis-muconate, due to the mutation, were so low that assays would have required too much enzyme. Thus, those fractions which, as judged by reference to wild-type CatB, were expected to contain the variant CatB were checked for the presence of a 40-kDa band by sodium dodecyl sulfate (SDS)-gel electrophoresis (42) with subsequent Coomassie brilliant blue R-250 staining. Fractions with strongest 40-kDa bands eluted from the first column at ca. 0.18 M NaCl and from the second column at ca. 0.11 M (NH₄)₂SO₄.

TfdD-K165A was purified from a 2-liter culture because expression of an R. eutropha gene from the pSETSa vector proved to be not as effective as expression of a P. putida gene from pET11a* (43, 44). As with the CatB-K169A purification, the presence of overexpressed protein was checked only by SDS-gel electrophoresis since activities were very low. Fractions with the strongest 40-kDa bands eluted from the first column at ca. 0.37 M NaCl and from the second column at ca. 0.20 M (NH₄)₂SO₄ similar to the wild-type enzyme (45).

The purity of the (chloro)muconate cycloisomerase preparations was analyzed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie brilliant blue (see above). In the case of CatB, 21 mg of pure enzyme was obtained. The resulting preparations of CatB-K169A and TfdD-K165A contained 12 and 8.2 mg, respectively, of pure enzyme.

HPLC. Substrates and products were quantified by reversed-phase high-pressure liquid chromatography (HPLC) with a SII 100 C8 reversed-phase column (length, 250 mm; internal diameter, 4.6 mm; Grom, Herrenberg, Germany) protected by a LiChrospher RP8 precolumn (20 by 4.6 mm; Grom). Usually, samples of 10 μl were analyzed. The column effluent was monitored simultaneously at 210 and 260 nm by use of a variable-wavelength detector (Waters 490 programmable wavelength detector; Waters, Milford, Mass.). For analysis of products from cis,cis-muconate and 2,4-dichloro-cis,cis-muconate, 40% (wt/vol) methanol containing 0.1% (wt/vol) H₃PO₄ was used as the solvent at a flow rate of 0.7 ml min⁻¹. Typical retention volumes were as follows: compound X (presumed reaction product of 2-chloro-cis-diene lactone and Tris), 3.0 ml; muconolactone, 3.2 ml; 2-chloromaleylacetic acid, 3.3 ml; 2-chloro-cis-acetylacrylic acid, 4.0 ml; 2,4-dihalo-cis,cis-muconic acid, 4.6 ml; 3-chloro-cis,cis-muconic acid and protoanemonin, 4.8 ml; 2-chloro-cis-diene lactone, 5.9 ml; and 2-chloropropanoammonium, 6.3 ml. Since protoanemonin could not be separated well from 3-chloro-cis,cis-muconic acid under these conditions, 25% (wt/vol) methanol containing 0.1% (wt/vol) H₃PO₄ was used as the solvent for analysis of products from 3-chloro-cis,cis-muconate turnover (flow rate, 0.9 ml min⁻¹). Typical retention volumes were as follows: maleylactate, 3.3 ml; cis,cis-acetylacrylic acid, 3.8 ml; trans-diene lactone, 4.4 ml; cis-diene lactone, 5.9 ml; protoanemonin, 6.4 ml; and 3-chloro-cis,cis-muconic acid, 7.4 ml.

For the preparation of 2-chloropropanoammonium after conversion of 3,5-dichlo-
rotacone, a Grom SIL 100 C8 reversed-phase column of 125-mm length and 4.6-mm internal diameter was used with 40% (v/v) methanol without H3PO4 as the solvent and a flow rate of 1 ml min⁻¹. Retention volumes were as follows: 2,4-dichloro-cis,cis-muconate, 1.2 ml; 2-chloro-cis-diene-lactone, 1.6 ml; 2-chloro-rotopprotoanemonin, 3.2 ml; and 3,5-dichlorocatechol, 7.9 ml.

Preparation and identification of 2-chloroprotoanemonin. The reaction mixture contained, in a final volume of 100 ml, 5 mM of BisTris-HCl [bis-(2-hydroxyethyl)iminotris(hydroxymethyl)methane-HCl] (pH 6.5), 0.2 mM of MnSO4, 300 μM of 3,5-dichlorocatechol, 24 U (measured with 3,5-dichlorocatechol) of chlorocatechol 1,2-dioxygenase, provided as a cell extract of E. coli BL21(DE3)pLysS[pTFDC1] (44), and 4,100 U (measured with cis,cis-muconate as partial purified P. putida muconate cycloisomerase. 3,5-Dichlorocatechol (used as 20 mM stock solution) as well as muconate cycloisomerase were added stepwise during the conversion. The mixture was incubated at 25°C for 6 h and stirred slightly. The progress of the reaction was followed by HPLC analyses (for conditions, see above). After 6 h, a compound later identified as 2-chloroprotoanemonin was the major metabolite detected. Protein was removed at 4°C by ultrafiltration with an Amicon 8050 cell using a Diaflo ultrafiltration membrane (type PM10; Amicon). The preparation was extracted twice with 25 and 10 ml of diethyl ether. The combined organic phases were dried over Na2SO4, vaporized using a rotation evaporator (V2000; Heidolph, Kelheim, Germany), and finally completely dried with an Alpha 1-5 freeze-dryer (Christ, Osterode, Germany). Small amounts (5 to 10 mg) of a white substance which appeared to have a melting temperature between 0 and 10°C were obtained. High-resolution nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AC 250 spectrometer with the Pulse-Fourier transform technique and with nominal frequencies of 500.133 MHz for 1H NMR and 125.774 MHz for 13C NMR. The samples were dissolved in deuterated methanol, and tetramethylsilane was used as the internal standard. To estimate the extinction coefficient of 2-chloroprotoanemonin, the spectrum of a 0.1 mM solution was recorded between 200 and 400 nm on a double-beam spectrophotometer (Kontron Unikov 941 Plus) against water as the background.

Preparation of a solution containing a 2-chloro-cis-diene-lactone–Tris reaction product (compound X). To check the absorption spectra of compound X under acidic and neutral conditions, the compound was prepared in a small scale by incubating a 0.5 mM 2-chloro-cis-diene-lactone solution in 50 mM Tris-HCl (pH 7.5) at room temperature for 12 h. Yielding X as the main product (at maximum, 82%). Upon acidification of 1 ml of this solution to pH 3.0, most of the by-product 2-chloromaleylacetate (16%), but not compound X, could be removed by extraction with the same volume of ethylacetate from the water phase. Compound X was therefore isolated from the latter by evaporation of the water under reduced pressure. Of the remaining pellet, 0.5 mg was dissolved in 2 ml of water, and the spectrum was recorded between 200 and 400 nm (see above). After acidification to pH 2.0 by addition of 1 μl of 85% H3PO4, a second spectrum was recorded.

Preparation of a solution containing the presumed 2-chloro-trans-diene-lactone. To provide an HPLC standard for experiments on 2,4-dichloro-cis,cis-muconate conversion, preparation of the presumed 2-chloro-trans-diene-lactone from 2-chloro-cis-diene-lactone (20) was attempted by UV irradiation at 254 nm in an analogous manner to the preparation of trans-diene-lactone from cis-diene-lactone (33) (Fig. 2). Irradiation was carried out at 4°C. A clear identification of this compound was supported by an additional spectrum obtained after irradiation at 4°C. The spectrum of the putative 2-chloro-trans-diene-lactone, performed under stopped-flow conditions by HPLC, showed an absorption maximum at 282 nm similar to that of 2-chloro-cis-diene-lactone (λmax = 283 nm [20]).

Chemicals. Catechols and cis,cis-muconates, in general, were available from the same sources as described before (43). All other substituted cis,cis-muconic acids were synthesized enzymatically from the corresponding catechols, using cell extract derived from E. coli BL21(DE3)pLysS[pTFDC1] (44). Protoanemonin was freshly prepared by converting 3-chloro-cis,cis-muconate by large amounts of purified P. putida muconate cycloisomerase (3). trans-Diene-lactone was obtained from cis-diene-lactone and 2-chloro-cis-diene-lactone were available from previous syntheses (17, 20, 30). Maleylactate and 2-chloromaleylactate were prepared from cis-diene-lactone and 2-chloro-cis-diene-lactone, respectively, by alkaline hydrolysis (11). trans-Acetylacrylic acid was purchased from Lancaster, cis-Acetylacrylic acid acylate was synthesized from maleylactic acid under acidic conditions (33). In an analogous manner, 2-chloro-cis-acetylacrylic acid acylate was prepared from 0.25 mM 2-chloromaleylactic acid by incubation for 120 min in 10 mM citrate buffer (pH 3.0) (25°C). A product with the same retention volume was detected by heating an acidified solution of 2-chloromaleylactic acid (ca. pH 4) at 95°C for 2.5 h. A product suggested to have been formed from 2-chloromaleylactic acid by thermal decarboxylation and acidification was identified by Tiedje et al. (38) as 2-chloro-cis-acetylacrylic acid acylate.

RESULTS

Drastically reduced catalytic efficiency of muconate cycloisomerase variant K169A with most cis,cis-muconates. Wild-type muconate cycloisomerase and the CatB-K169A variant were both purified to homogeneity as judged by SDS-polyacrylamide gel electrophoresis (single bands at ca. 40 kDa). CatB, purified 6.6-fold, gave a preparation with a specific activity of 224 U mg⁻¹ measured with 0.1 mM cis,cis-muconate (Table 2). The purification of CatB-K169A was performed twice and yielded preparations with a specific activity of 0.003 U mg⁻¹ or less (Table 2). The specific activity could be measured only by using 400 to 500 μg of enzyme in the assay and represented only 0.0015% or less of that of CatB, in accord with Lys169 being essential for the enzyme mechanism. By HPLC, muconolactone was shown to be formed by both CatB and CatB-K169A. CatB-K169A showed a residual activity with 0.1 mM 3-fluoro-cis,cis-muconate in the same order of magnitude as with cis,cis-muconate (0.001 U mg⁻¹). When CatB-K169A was tested for conversion of 2-chloro-, 2-methyl-, and 3-methyl-cis,cis-muconate, specific activities were below the detection limit of 0.0005 U mg⁻¹.
Conversion of 3-chloromuconate by *P. putida* muconate cycloisomerase and CatB-K169A. When 3-chloro-cis,cis-muconate conversion was followed by monitoring the extinction at 260 nm over at least 2 min in assays without auxiliary dienelactone hydrolase, a difference in product formation by CatB and CatB-K169A became obvious. An enzyme assay with CatB (0.6 U ml\(^{-1}\)) measured with cis,cis-muconate showed a decrease of \(E_{260}\) within the first ca. 20 s of measurement and then again an increase, indicating the formation of an intermediate with low absorption at 260 nm, presumably 4-chloromuconolactone, and a subsequent spontaneous or enzyme-catalyzed conversion of the latter to protoanemonin (\(\lambda_{\text{max}} = 260\) nm) (3). In contrast, an amount of CatB-K169A which corresponded to this experiment with respect to 3-chloro-cis,cis-muconate conversion (only 0.002 U ml\(^{-1}\)) measured with cis,cis-muconate) showed a continuous slight decrease but no increase of \(E_{260}\).

Conversion of 3-chloro-cis,cis-muconate by CatB-K169A and CatB was subsequently investigated by running overlay UV spectra of enzyme assays and by analyses of products by HPLC (Fig. 3). When a 3-chloro-cis,cis-muconate-containing reaction mixture was incubated with CatB, due to the relatively long cycle times between the spectra and because of similar absorbances of the substrate and the product protoanemonin, practically no absorbance changes occurred. With CatB-K169A, however, a strong shift toward 280 nm was observed. Monitoring of the CatB-K169A-catalyzed turnover by HPLC clearly showed the formation of a compound which cochromatographed with authentic cis-dienelactone.

Based on this knowledge, the specific activity of CatB with 3-chloro-cis,cis-muconate was determined to be 3.4 U mg\(^{-1}\), using a \(D_m = 4,000\ M^{-1}\ cm^{-1}\) for the first 60 s

### TABLE 2. Activity of muconate cycloisomerase variant CatB-K169A with various substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sp act with 0.1 mM substrate (U mg(^{-1}))(^a)</th>
<th>Reduction of activity(^c) (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cis,cis-Muconate</td>
<td>224</td>
<td>(&lt;0.0005–0.003) 7 \times 10^4</td>
</tr>
<tr>
<td>3-Fluoro-cis,cis-muconate</td>
<td>110(^b)</td>
<td>0.001 11 \times 10^4</td>
</tr>
<tr>
<td>3-Chloro-cis,cis-muconate</td>
<td>3.4(^e)</td>
<td>0.010–0.011 310</td>
</tr>
<tr>
<td>2,4-Dichloro-cis,cis-muconate</td>
<td>0.052(^e)</td>
<td>0.017–0.020(^f) 2.6</td>
</tr>
</tbody>
</table>

\(^a\) Assays with chloro-substituted substrates were performed in the presence of dienelactone hydrolase as auxiliary enzyme. Unless mentioned otherwise, the extinction coefficients of Dorn and Knackmuss (9) were used.

\(^b\) Where a range is given, the two activities were determined with two independently purified variant preparations.

\(^c\) The number given reflects the minimal value, i.e., the activity of the wild-type enzyme divided by the higher one of the values determined for the variant.

\(^d\) The specific activity with 3-fluoro-cis,cis-muconate was not measured directly but calculated from the activity with cis,cis-muconate by using kinetic data for the enzyme as determined by Vollmer et al. (43).

\(^e\) \(\Delta E = 4,300\ M^{-1}\ cm^{-1}\) for the first 60 s (43).

\(^f\) \(\Delta E = 4,000\ M^{-1}\ cm^{-1}\), determined as described in the text.

\(^g\) \(\Delta E = 5,800\ M^{-1}\ cm^{-1}\) (23).

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**Fig. 3.** Conversion of 3-chloro-cis,cis-muconate by wild-type CatB and CatB-K169A at pH 7.5. For overlay spectra, reaction mixtures (1 ml) contained 30 mM Tris-HCl (pH 7.5), 1 mM MnSO\(_4\), 0.1 mM 3-chloro-cis,cis-muconate, and 0.001 to 0.002 U enzyme (measured with cis,cis-muconate). Reference cuvettes contained the same mixtures without substrate. Ten spectra were recorded at 25°C between 200 and 400 nm every 10 min, and at least 90% of the substrate was converted after the last cycle. Arrows indicate the shift of the absorption maximum. Product formation from 3-chloro-cis,cis-muconate was additionally examined by HPLC analyses. Reaction conditions were the same as described above, except that 0.5 mM substrate was supplied and more enzyme was added (0.002 U of CatB and 0.007 U of CatB-K169A). 3-Chloro-cis,cis-muconate (■) was converted to cis-dienelactone (▼), maleylacetate (○), cis-acetylacrylate (▲), and protoanemonin (△). trans-Dienelactone was formed by CatB-K169A in concentrations no more than 0.014 mM (not shown).
The specific activity of CatB-K169A with 3-chloro-
cis,cis-muconate was estimated to be ca. 0.011 U mg
-1 (Table 2). Thus, while the K169A mutation resulted in at least a 7 \times 10^3-fold reduction of the reaction rate with cis,cis-muconate and in a ca.
11 \times 10^3-fold reduction of the reaction rate with 3-fluoro-
cis,cis-muconate, turnover of 3-chloro-
cis,cis-muconate was reduced only by a factor of 310 (Table 2).

Conversion of 2,4-dichloromuconate by CatB-K169A. The conversion of 2,4-dichloro-
cis,cis-muconate by CatB-K169A was likewise investigated by overlay UV spectra and HPLC
measurements (Fig. 4, right). The peak maximum at 267 nm initially shifted toward 280 nm, and a new maximum subsequently appeared at about 254 nm. Monitoring of the CatB-K169A-
catalyzed turnover by HPLC clearly showed the immediate formation of a compound which cochromatographed with authentic, i.e., chemically synthesized, 2-chloro-
cis,cis-dienelactone (\(\lambda_{\text{max}} = 283 \text{ nm}\) [20]). 2-Chloro-cis-dienelactone, however, proved to be relatively unstable at pH 7.5. Its concentrations never exceeded 30% of provided substrate.

The accumulation of a new compound X as the main product of 2,4-dichloro-
cis,cis-muconate conversion could be shown to result from a nonenzymatic reaction of 2-chloro-
cis-diene-
lactone. Stability experiments with 0.5 mM 2-chloro-
cis-diene-
lactone in Tris-HCl (pH 7.5) (in the absence of enzyme) gave a product with the same UV spectrum and chromatographic behavior. Thus, a solution of X prepared from chemically
synthesized 2-chloro-cis-dienelactone revealed different absorption spectra of X at neutral (\(\lambda_{\text{max}} = 252 \text{ nm}\)) and acidic (\(\lambda_{\text{max}} = 229 \text{ nm}\)) conditions. This corresponds well to the different absorption maxima of X from enzymatic 2,4-dichloro-
cis,cis-muconate conversion as determined by HPLC under acidic, stopped-flow conditions (230 nm) or by overlay UV spectra at pH 7.5 (shift toward 254 nm [Fig. 4, right]). 2-Chloromaleylacetate and the presumed 2-chloro-trans-dienelactone appeared as minor by-products from nonenzymatic 2-chloro-
cis-dienelactone conversion at pH 7.5.

Compound X is most probably a reaction product of 2-chloro-cis-dienelactone with Tris buffer. As illustrated by the UV spectra (Fig. 5), alkaline hydrolysis as well as hydrolysis of 2-chloro-cis-dienelactone in the presence of imidazole-HCl yielded 2-chloromaleylacetate (\(\lambda_{\text{max}} = 250 \text{ nm}, \epsilon = 8,400 \text{ M}^{-1} \text{ cm}^{-1}\)) as the product. In contrast, the reaction of 2-chloro-cis-
dienelactone in the presence of Tris-HCl gave a product with a similar \(\lambda_{\text{max}}\) but with a significantly higher molecular absorption coefficient (\(\epsilon = 15,000 \text{ M}^{-1} \text{ cm}^{-1}\)). In contrast to the products of alkaline- as well as imidazole-HCl-catalyzed hydrolysis, the product obtained with Tris showed no biological activity with maleylacetate reductase (data not shown). The fact that compound X occurred with Tris-HCl (pH 7.5) but not with imidazole-HCl (pH 7.5) as the buffer suggests that hydroxyl groups of the Tris buffer might be the reactive groups.

Since with respect to product formation from 2,4-dichloro-
cis,cis-muconate, CatB-K169A resembled the chloromuconate

FIG. 4. Conversion of 2,4-dichloro-
cis,cis-muconate by wild-type CatB and CatB-K169A at pH 7.5. For overlay spectra, reaction mixtures (1 ml) contained 30 mM Tris-HCl (pH 7.5), 1 mM MnSO₄, 0.1 mM 2,4-dichloro-
cis,cis-muconate, and 0.001–0.002 U enzyme (measured with 3-chloro-
cis,cis-muconate). Reference cuvettes contained the same mixtures without substrate. Ten spectra were recorded as described in the legend to Fig. 3, and with both enzymes the substrate was completely converted after the last cycle. Arrows indicate the shift of the absorption maximum. For analysis of 2,4-dichloro-
cis,cis-muconate turnover by HPLC, reaction conditions were the same as described above, except that 0.5 mM substrate was supplied and more enzyme was added (approximately 0.003 U). 2,4-Dichloro-
cis,cis-muconate (\(\square\)) was converted to 2-chloro-
cis-cis-dienelactone (\(\triangle\)), 2-chloromaleylacetate (\(\Delta\)), 2-chloro-
cis-acetylcylactate (\(\bullet\)), 2-chloroprotanemonin (\(\odot\)), 2-chloro-trans-dienelactone (\(\heartsuit\)), a reaction product of 2-chloro-cis-dienelactone and Tris, i.e., compound X (\(\clubsuit\)), and the unknown compound Y (\(\lozenge\)). For compounds X and Y, peak areas at 210 nm are given in relative units. From a preparation of compound X as described in Material and Methods, a peak area of 660 relative units was assumed to be equivalent to 0.1 mM.
cycloisomerasers, the difference of extinction coefficients of substrate and product was assumed to be 5,800 M⁻¹ cm⁻¹ (23). With this Δɛ, values of up to 0.020 U mg⁻¹ were determined for the specific activity of CatB-K169A with 2,4-dichloro-cis,cis-muconate (Table 2).

Conversion of 2,4-dichloromuconate by wild-type muconate cycloisomerase to 2-chloroprotoanemonin. When a 2,4-dichloro-cis,cis-muconate-containing reaction mixture with CatB was followed by overlay UV spectra, the peak maximum first mainly decreased and then showed a gradual shift from 267 nm toward 250 nm (Fig. 4). This shift even continued for some time after the original substrate was completely converted. When following the CatB-catalyzed turnover of 2,4-dichloro-cis,cis-muconate by HPLC, we observed a product which had a retention volume only slightly different from that of 2-chloro-cis-dienelactone but differed significantly from the latter in having a higher relative absorption at 260 nm, compared to 210 nm. This compound could be easily extracted with diethyl ether under approximately neutral conditions and was identified as 2-chloroprotoanemonin (4-methylene-2-chlorobut-2-en-4-olide) (see below).

2-Chloroprotoanemonin proved to be considerably more stable at pH 6.5 (half-life of 11 h) than at pH 7.5 (half-life of <2 h). The by-products observed during 2,4-dichloro-cis,cis-muconate conversion at pH 7.5 (Fig. 4, left) included (i) the presumed 2-chloro-cis-acetylactylate, (ii) a compound with the same retention volume and the same relative absorption at 210 and 260 nm as the presumed reaction product of 2-chloro-cis-dienelactone and Tris (compound X; see above), and (iii) a third, as yet unidentified compound Y eluting from the reversed-phase column between the latter two compounds. It thus had a retention volume similar to that of 2-chloromaleyl-acetic acid but differed from the latter in having a higher relative absorption at 260 nm, compared to 210 nm.

The Δɛ at 260 nm for formation of 2-chloroprotoanemonin from 2,4-dichloro-cis,cis-muconate was determined to be 4,300 M⁻¹ cm⁻¹. This value was determined by monitoring E₂₆₀ during 2,4-dichloro-cis,cis-muconate conversion by 18 U of CatB (measured with cis,cis-muconate) in a 1-ml reaction mixture with dienelactone hydrolase and by correlating the ΔE₂₆₀ to the difference in substrate concentrations as analyzed by HPLC prior to CatB addition and after 1.5 min. A specific activity of 0.052 U mg⁻¹ was calculated for CatB with 2,4-dichloro-cis,cis-muconate by a factor of only 2.6, i.e., less than that of 3-chloro-cis,cis-muconate and much less than that of cis,cis-muconate and other substituted muconates (Table 2).

The product formed from 2,4-dichloro-cis,cis-muconate by muconate cycloisomerase was isolated as described in Materials and Methods. The ¹H NMR spectrum (Fig. 6) showed three olefinic protons, centered at two different carbon atoms. One of the protons had a chemical shift value of 7.75 ppm, which is typical for the β proton in an α,β-unsaturated carbonyl system. This proton did not show any long-range coupling to 5-H₅ or 5-H₆ and was identified as 3-H. A geminal coupling of 2.9 Hz was observed between 5-H₅ and 5-H₆. For the interpretation of ¹³C NMR spectra, it was useful to compare data with recently published data for 3-bromoprotoanemonin and protoanemonin (8). The most significant difference in chemical shifts of the carbon atoms is observed for C-2. The chlorine of 2-chloroprotoanemonin shifts the signal of C-2 around 5.5 ppm downfield compared to C-2 signals of protoanemonin and 3-bromoprotoanemonin. The signals of C-3 of both 2-chloro- and 3-bromoprotoanemonin were shifted upfield in comparison to protoanemonin by 5 and 6.7 ppm, respectively. The λ₅₅₅ of an aqueous solution of 2-chloroprotoanemonin was determined to be 268 nm, and ε was 15,780 M⁻¹ cm⁻¹. These values were quite similar to those reported for protoanemonin (λ₅₅₅ = 260 nm; ε = 15,100 M⁻¹ cm⁻¹) (3).

When 0.1 mM 2-chloroprotoanemonin dissolved in water was dropped on an LB plate which had been streaked with E. coli BL21(DE3,pLysS), overnight cell growth did not occur at the site of application. This preliminary experiment indicates that 2-chloroprotoanemonin, like protoanemonin, might be toxic for bacteria (3, 5, 35).
Inefficiency of TfdD-K165A with all tested cis,cis-muconates. TfdD-K165A which was also purified to homogeneity gave specific activities below 0.001 U mg\(^{-1}\) with most cis,cis-muconates tested. 2,4-Dichloro-cis,cis-muconate was the only substrate still converted. A specific activity of 0.005 U mg\(^{-1}\) was measured with 0.1 mM substrate. From 2,4-dichloro-cis,cis-muconate, TfdD-K165A formed the same product as CatB-K169A, i.e., 2-chloro-cis-dienelactone.

DISCUSSION

As outlined in the introduction, we wanted to test the hypothesis that the formation of protoanemonin from 3-chloro-cis,cis-muconate by muconate cycloisomerases requires enzymatic protonation of the respective enol/enolate intermediate, while the formation of cis-dienelactone from 3-chloro-cis,cis-muconate by chloromuconate cycloisomerases should not necessitate a protonation, but instead chloride abstraction could take place. We thus changed the lysine that has been suggested to be responsible for protonation (15) to an alanine in both \(P.\ putida\) muconate cycloisomerase CatB and in the \(pJP4\)-encoded chloromuconate cycloisomerase TfdD.

We expected to find activity of the TfdD variant with those substrates which should not require protonation, i.e., 3-chloro- and 2,4-dichloro-cis,cis-muconate (29, 34). We also expected the CatB and the TfdD variants to be inactive toward substrates necessarily requiring protonation, like cis,cis-muconate (36), 2-chloro-cis,cis-muconate (42), 3-fluoro-cis,cis-muconate (33), and 2-methyl- and 3-methyl-cis,cis-muconate (6, 22). We were curious to see what the CatB variant would do with substrates which are obviously protonated by the wild-type enzyme, specifically 3-chloro-cis,cis-muconate, and finally we wanted to investigate the product formation from 2,4-dichloro-cis,cis-muconate.

In contrast to our expectations, TfdD-K165A was completely inactive with 3-chloro-cis,cis-muconate, and 2,4-dichloro-cis,cis-muconate conversion was also severely affected. The lack of any activity of TfdD-K165A with cis,cis-muconate or methylmuconates, on the other hand, was in accord with our expectations, as was the fact that the product formed from 2,4-dichloro-cis,cis-muconate was 2-chloro-cis-dienelactone. One might explain the inefficiency of this enzyme variant by the change of the active site. In analogy to CatB (16), the binding pocket of TfdD is probably positively charged and should be created by \(\text{Mn}^{2+}\) and the amino groups of Lys163 and Lys165. Upon replacement of Lys165 by a nonpolar alanine, the substrate which is negatively charged at pH 7 might bind less effectively. The K165A replacement could also have resulted in other unintended, structural changes which might negatively affect not only the protonation of the enol/enolate intermediate but also other steps of the cycloisomerization reaction.
In concordance with our expectations, CatB-K169A converted cis,cis-muconate and 3-fluoro-cis,cis-muconate about 10^5-fold slower than the wild-type enzyme (Table 2), while the activities with other substrates requiring protonation (methylmuconates and 2-chloro-cis,cis-muconate) were below the detection limit. At the same time, 3-chloro- and 2,4-dichloro-cis,cis-muconate were still converted at a considerable rate, thus proving that the almost complete inactivation of CatB observed with the other substrates was not due to some non-specific effect as discussed above for TfdD-K165A. In fact, these observations provide experimental evidence for the inference from comparisons with mandelate racemase (15) and modeling studies on CatB (32) that Lys169 should be the protonating amino acid.

The most direct evidence for the validity of our basic hypothesis that protoanemonin formation from 3-chloro-cis,cis-muconate should require a protonation step, whereas cis-dienelactone formation should not, came from the observed shifts of product formation: while wild-type CatB formed protoanemonin from 3-chloro-cis,cis-muconate and 2-chloroprotoanemonin from 2,4-dichloro-cis,cis-muconate, the K169A variant formed cis-dienelactone and 2-chloro-cis-dienelactone, respectively (Fig. 7). Thus, a protonation reaction is definitely necessary for (chloro-)protoanemonin formation but not for (chloro-)cis-dienelactone formation.

The findings just discussed cast a new light on the catalytic differences between muconate and chloromuconate cycloisomerases (Fig. 7). One might speculate that after the protonation of the (di-)chlorinated enol/enolate intermediate to 4-chloromuconolactone or 2,4-dichloromuconolactone, as catalyzed by muconate cycloisomerases, the decarboxylation and chloride elimination to (chloro-)protoanemonin occur as spontaneous, nonenzymatic reactions. Then the difference between muconate and chloromuconate cycloisomerases, with respect to product formation from 3-chloro- and 2,4-dichloro-cis,cis-muconate, would be due to the fact that of the competing possible reactions of the (di-)chlorinated enol/enolate, different ones are favored: protonation by Lys169 in case of the muconate cycloisomerases, and chloride elimination in case of the chloromuconate cycloisomerases.

It is not clear how this shift in favored reactions was accomplished during the divergence of the chloromuconate cycloisomerases from the muconate cycloisomerases. Obviously the evolutionary solution was different from our experimental one: the Lys169 of \( \textit{P. putida} \) muconate cycloisomerase is conserved as Lys165 in the chloromuconate cycloisomerases of \( \textit{pJP4}, \textit{pAC27}, \textit{and pP51} \) (14, 28, 39) and as Lys168 in the chloromuconate cycloisomerase of \( \textit{Rhodococcus opacus} \) 1CP (10). In principle, two possibilities exist: in chloromuconate cycloisomerases, (i) protonation of the (di-)chlorinated enol/enolate intermediate could be slowed down or (ii) chloride elimination could be accelerated. The former possibility appears to be less likely because the chloromuconate cycloisomerase TfdD converts 2- and 3-methyl-cis,cis-muconate to 4- and 3-methylmuconolactone almost as fast as the muconate cycloisomerase of \( \textit{P. putida} \) converts cis,cis-muconate to muconolactone (43, 44). Thus, TfdD is fully capable of catalyzing a fast protonation. An enhanced rate of chloride elimination from the enol/enolate intermediate is therefore more probable.

In recent modeling studies with TfdD, a residue which could accelerate chloride elimination and which is in the correct position and geometry to the chlorine substituent has not yet been identified. At first sight, an active-site tryptophan appears to be a likely candidate (U. Schell, H.-J. Hecht, and M. Schlo-
mann, unpublished data), because a chloride binding site comprising two tryptophan residues has also been found in haloalkane dehalogenase of *Xanthobacter autotrophicus* GJ10 (40). However, a replacement of Tyr59 in *P. putida* muconate cycloisomerase by tryptophan, the corresponding amino acid of chloromuconate cycloisomerase, did not avoid protoamino-nin formation (43), nor did a reciprocal replacement of Trp55 in pJP4-encoded chloromuconate cycloisomerase by tyrosine abolish productive dehalogenation to dienelactones (Schell et al., unpublished). Thus, it remains to be elucidated which other amino acid residues are, in fact, responsible for (chloro-)cis-dienelactone formation.

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