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Detoxification of Protoanemonin by Dienelactone Hydrolase

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Protoanemonin is a toxic metabolite which may be formed during the degradation of some chloroaromatic compounds, such as polychlorinated biphenyls, by natural microbial consortia. We show here that protoanemonin can be transformed by dienelactone hydrolase of *Pseudomonas* sp. strain B13 to *cis*-acetylacrylate. Although similar K_m values were observed for *cis*-dienelactone and protoanemonin, the turnover rate of protoanemonin was only 1% that of *cis*-dienelactone. This indicates that at least this percentage of the enzyme is in the active state, even in the absence of activation. The *trans*-dienelactone hydrolase of *Pseudomonas* sp. strain RW10 did not detectably transform protoanemonin. Obviously, *Pseudomonas* sp. strain B13 possesses at least two mechanisms to avoid protoanemonin toxicity, namely a highly active chloromuconate cycloisomerase, which routes most of the 3-chloro-*cis*,*cis*-muconate to the *cis*-dienelactone, thereby largely preventing protoanemonin formation, and dienelactone hydrolase, which detoxifies any small amount of protoanemonin that might nevertheless be formed.

A major route for mineralization of chloroaromatics is their transformation into chlorocatechols (14) and further metabolism to Krebs cycle intermediates by enzymes of the chlorocatechol pathway (8, 9, 19). In contrast to earlier assumptions that enzymes of this route catalyze reactions analogous to those of the widespread 3-oxoadipate pathway, it has recently been shown that muconate cycloisomerase and chloromuconate cycloisomerase, which act on *cis*,*cis*-muconates formed by intradiol cleavage of catechols, catalyze different reactions with chloromuconates as substrates (Fig. 1). In the case of 2-chloro-*cis*,*cis*-muconate, chloromuconate cycloisomerase catalyzes a dehalogenation to form *trans*-dienelactone (19), whereas muconate cycloisomerase produces a mixture of 2-chloro- and 5-chloromuconolactone (22). In the case of 3-chloro-*cis*,*cis*-muconate, chloromuconate cycloisomerase carries out a dehalogenation reaction to form the *cis*-dienelactone, whereas muconate cycloisomerase simultaneously dehalogenates and decarboxylates to form protoanemonin (3), a toxic metabolite (7, 20).

The formation of protoanemonin has recently been shown to be a major reason for the poor performance of bacterial polychlorinated biphenyl degraders in environmental settings (2). These experiments also indicated that the natural microflora in such settings has the potential to further transform protoanemonin, although the metabolic route involved has not until now been investigated.

Protoanemonin can be regarded as a structural analog of *cis*- and *trans*-dienelactone, which are intermediates in the degradation of chlorocatechols by enzymes of the chlorocatechol pathway. Dienelactones are transformed by dienelactone hydrolase of this pathway into maleylacetate (19). On the basis of crystallographic studies of the dienelactone hydrolase of *Pseudomonas* sp. strain B13 (5, 12), it has been argued that a carboxyl substituent is an essential structural element of the substrate for the fully active enzyme (1).

It was perhaps unexpected, therefore, that in preliminary experiments we found that cell extracts of 3-chlorobenzoate-grown cells of *Pseudomonas* sp. strain B13 were able to transform protoanemonin with a specific activity of 2 to 4 U/g of protein, which corresponds to about 1% of the activity obtained with *cis*-dienelactone as a substrate. Unless stated otherwise, enzyme activities were assayed spectrophotometrically at 260 nm for protoanemonin as a substrate, with a reaction coefficient of 13.1 mM^{-1} cm^{-1} (calculated from the difference in absorption of protoanemonin with ε_{260} = 15.1 mM^{-1} cm^{-1} and the reaction product *cis*-acetylacrylate with ε_{260} = 2.0 mM^{-1} cm^{-1}), or at 280 nm for *cis*-dienelactone (ε_{260} = 17.0 mM^{-1} cm^{-1}) as a substrate in 10 mM histidine-HCl (pH 6.5) (18) with 50 μM substrate. Protein concentrations were measured by the procedure of Bradford (4). To ascertain whether or not dienelactone hydrolase is responsible for this activity, the enzyme was purified to homogeneity from 3-chlorobenzoate-grown cells by a modification of a previously described procedure (11). Cell extracts were prepared after resuspension in 20 mM ethylenediamine buffer (pH 7.3) containing 1 mM dithiothreitol (DTT). The extract (volume, 5 ml; protein, 146 mg) was initially applied to a Mono Q HR 10/10 column. Proteins were eluted with 10 mM ethylenediamine buffer (pH 7.3)–0.1 mM DTT and a 200-ml 0 to 0.2 M linear gradient of NaCl (flow rate, 2 ml/min; fraction volume, 4 ml). The fractions with the highest activity (12 ml; eluting at ca. 0.1 M NaCl) were pooled and concentrated by ultrafiltration to a final volume of 2.5 ml. Ammonium sulfate was added to 45% saturation. The supernatant was clarified by centrifugation and applied to a Phenyl Superose HR 5/5 column. Proteins were eluted with 50 mM Tris-HCl (50 mM)–2 mM $MnSO_4$–0.1 mM DTT and a 12-ml linear gradient from 2 to 0.8 M $(NH_4)_2SO_4$ followed by a 30-ml linear gradient of 0.8 to 0 M $(NH_4)_2SO_4$ (flow rate, 0.5 ml/min; fraction volume, 0.5 ml). Fractions with the highest activity (eluting at ca. 0.6 M) were pooled, concentrated by ultrafiltration to a final volume of 0.27 ml, and applied to a Superose 6 HR 10/30 column. Elution of proteins

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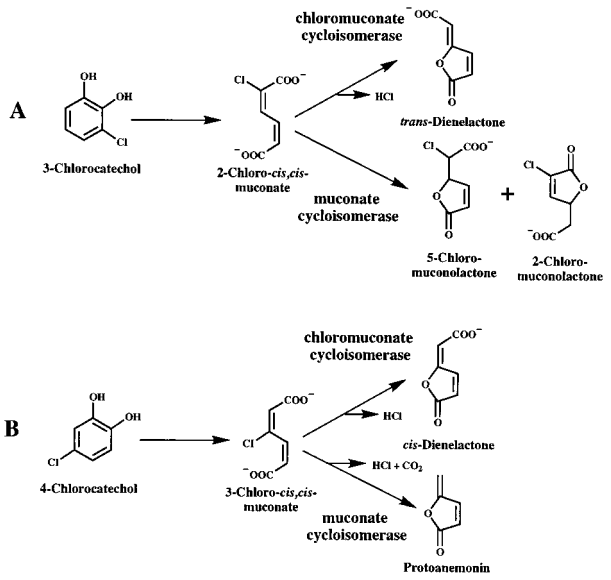

FIG. 1. Bacterial metabolism of 2-chloro-*cis*,*cis*-muconate (A) and 3-chloro-*cis*,*cis*-muconate (B) according to Blasco et al. (3) and Vollmer et al. (22).

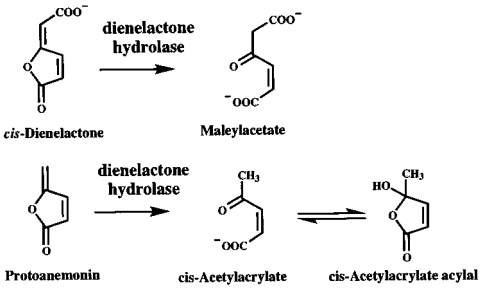

FIG. 2. *cis*-Dienelactone and protoanemonin conversion by the dienelactone hydrolase of *Pseudomonas* sp. strain B13. *cis*-Acetylacrylate under acidic conditions is present in a cyclic form (21).

was performed with 50 ml of Tris-HCl (50 mM [pH 7.5]) containing 100 mM NaCl at a flow rate of 0.5 ml/min. The purification yielded a product which showed a single band at 28 to 29 kDa on sodium dodecyl sulfate gels. There were no impurities observed on sodium dodecyl sulfate gels, which indicates that the preparation was highly homogeneous (>95%).

As shown in Table 1, activities for *cis*-dienelactone and protoanemonin copurified, indicating that dienelactone hydrolase is responsible for protoanemonin transformation in B13. By analogy with the hydrolysis of dienelactone, *cis*-acetylacrylate could be expected as the reaction product (Fig. 2). This compound was synthesized as a standard from *trans*-acetylacrylate according to the method of Schlömann et al. (16). The transformation of protoanemonin by purified dienelactone hydrolase was followed by high-performance liquid chromatography (HPLC) by a previously described procedure (13). The reaction mixture contained (in a total volume of 300 μl) Bis-Tris (10 mM [pH 6.5]), 220 μM protoanemonin, and 20 μl of purified dienelactone hydrolase (corresponding to 0.077 U, when the activity was measured with *cis*-dienelactone as a substrate). With an aqueous solvent system of 4.5% methanol and 0.1% H_3PO_4, the retention volume of protoanemonin was 10.6 ml, that of *cis*-acetylacrylate was 3.4 ml, and that of *trans*-acetylacrylate was 7.0 ml. Formation of a single product which coeluted with and showed an absorption spectrum (λ_{max} = 205 nm) identical to that of authentic *cis*-acetylacrylate was observed. Assuming the identity of the reaction product with *cis*-acetylacrylate, transformation of protoanemonin to this product by dienelactone hydrolase was essentially quantitative (88% ± 5%).

TABLE 1. Purification of dienelactone hydrolase of *Pseudomonas* sp. strain B13

Purification step	Vol (ml)	Amt of protein (mg)	Total activity (U)[a]	Sp. act. (U/g)[a]	Recovery (%)	Purification (fold)
Cell extract	5	146	37.9 (0.35)	260 (2.4)	100	1
Mono Q eluate	2.5	1.25	25.0 (0.25)	20,000 (200)	66	77
Phenyl-Sepharose eluate	0.27	0.28	7.3 (0.067)	26,500 (240)	19	102
Superose 6 eluate	1.5	0.10	5.8 (0.058)	58,800 (580)	15	226

[a] Values are given for *cis*-dienelactone as the substrate. Values in parentheses are the corresponding values for protoanemonin as the substrate.

Because the same product was formed by cell extracts of 3-chlorobenzoate-grown cells and not further metabolized, a preparative transformation was performed. Freshly prepared protoanemonin (80 μmol in 500 ml of Bis-Tris [pH 6.5]; 30 mM) (3) was incubated with a cell extract corresponding to 160 mg of protein, and transformation was monitored by HPLC. After complete conversion of the substrate, the reaction mixture was acidified to pH 2 and extracted with ethyl acetate (three times at 200 ml each). The dried residue was redissolved in 2 ml of 5% methanol (in H_2O) and in portions of 200 μl purified by preparative HPLC with an aqueous solvent system containing 5% methanol and 0.1% H_3PO_4 at a flow rate of 6 ml/min on a GG350 column (16 by 250 mm) filled with Lichrosorb RP8 (10 μm). Fractions containing the reaction product were extracted with ethyl acetate as described above. The dried product was further analyzed by ^{1}H-nuclear magnetic resonance. The recorded spectrum (in d_6-acetone) was identical to that reported for *cis*-acetylacrylate acylal, the tautomeric form of *cis*-acetylacrylate (21). Besides protons of a methyl substituent with a chemical shift of δ = 1.63 ppm, two single protons (6.07 and 7.44 ppm) showing a vicinal coupling of 5.6 Hz were identified.

Typical Michaelis-Menten kinetics were observed with both *cis*-dienelactone and protoanemonin. Transformation was recorded at 285 nm for protoanemonin as the substrate (ε_{285} = 3.8 mM^{-1} cm^{-1}) or at 310 nm for *cis*-dienelactone as substrate (ε_{310} = 4.3 mM^{-1} cm^{-1}). Whereas the K_m values for protoanemonin (415 ± 46 μM) and *cis*-dienelactone (381 ± 28 μM) were similar, the V_{max} for protoanemonin transformation was only 0.8% of that for *cis*-dienelactone. The k_{cat} values for protoanemonin and *cis*-dienelactone were calculated to be 125 ± 8 min^{-1} and 15,600 ± 660 min^{-1}, respectively (assuming a molecular weight of 25,489 as calculated from the nucleotide sequence [10]). Inhibition experiments involving the addition of up to 300 μM protoanemonin to a reaction mixture containing 25 to 200 μM *cis*-dienelactone demonstrated that protoanemonin acts as a competitive inhibitor of the transformation of *cis*-dienelactone. K_i was calculated to be 430 μM.

Dienelactone hydrolases have been classified into three distinct groups based on their substrate specificity (15, 18). The dienelactone hydrolase of B13 and the pJP4-encoded enzyme for chlorocatechol metabolism showed turnover of both *cis*- and *trans*-dienelactone (18). The dienelactone hydrolase of *Burkholderia cepacia*, however, hydrolyzes only the *cis* isomer with significant activity and differs in basic properties from the

enzymes described above (17). A third class of enzymes convert *trans*-dienelactone, but not the *cis* isomer (15). Both *cis*-dienelactone hydrolase from *B. cepacia* and *trans*-dienelactone hydrolase from *Pseudomonas putida* RW10 (3) were partially purified by anion-exchange chromatography and analyzed for their activity on protoanemonin. Cell extracts (1 ml, ca. 20 mg each) were applied to a Mono Q HR 5/5 column, and proteins were eluted with a 20-ml linear gradient from 0 to 0.4 M NaCl in Tris-HCl (pH 7.5) plus 2 mM $MnCl_2$ (flow rate, 1 ml/min; fraction volume, 0.5 ml). Fractions exhibiting the highest activities were analyzed. The activity of *cis*-dienelactone hydrolase from *B. cepacia* with 50 μM protoanemonin was 0.08% of that observed with 50 μM *cis*-dienelactone. No activity of *trans*-dienelactone hydrolase with protoanemonin was detected (detection limit about 0.1% of the activity observed with 50 μM *trans*-dienelactone). The K_m value of this enzyme for *trans*-dienelactone was calculated to be 1.8 mM.

The dienelactone hydrolase of B13 has been intensively studied. The hydrolysis of dienelactones involves a catalytic triad comprised of Cys123, His202, and Asp171. The crystal structure suggests that native enzyme exists predominantly in a catalytically inert configuration in which the cysteine is neutral and points away from the active site binding cleft (1, 6). It has been suggested that substrate binding induces two conformational changes at the active site. On one hand, the anionic side chain interacts with Arg206, which leads to a conformational shift in Glu36. On the other hand, the carbonyl oxygen forms a hydrogen bond with Leu124, thus allowing the thiol group of Cys123 to rotate. Whereas protoanemonin can be assumed to form the necessary hydrogen bond with Leu124, it should not induce the necessary conformational changes allowing the Glu36 to abstract the thiol proton. Comparison of the K_m and V_{max} values seems to confirm this. *trans*-Dienelactone is bound most efficiently (K_m = 15 μM [11, 19]), while *cis*-dienelactone and protoanemonin bind less efficiently, most probably due to lack of or inefficient interaction with Arg206 and Arg81. *cis*-Dienelactone, however, still seems to trigger the activation mechanism, as evidenced by a V_{max} similar to that of *trans*-dienelactone (11, 19), whereas protoanemonin is converted by the naturally existing population of active state enzyme at a much slower rate.

It has been reported that both the 3-oxoadipate pathway and the chlorocatechol pathway are induced in B13 cells growing on 3-chlorobenzoate (3). Consequently, the muconate and chloromuconate cycloisomerases compete for the intermediate 3-chloro-*cis*,*cis*-muconate under these conditions. Blasco et al. (3) reported the accumulation of only *cis*-dienelactone, when a mixture of muconate and chloromuconate cycloisomerases was used for transformation of 3-chloro-*cis*,*cis*-muconate, presumably due to the high activity of chloromuconate cycloisomerase for this substrate. However, it is likely that some protoanemonin can be formed from 3-chloro-*cis*,*cis*-muconate under certain inducing conditions and by other microorganisms according to the relative affinities of the isoenzymes for the substrate. It seems likely, therefore, that dienelactone hydrolase has the additional function of detoxification of minor amounts of protoanemonin that may be formed during chloroaromatic degradation.

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