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Monitoring Key Reactions in Degradation of Chloroaromatics by In Situ ^{1}H Nuclear Magnetic Resonance: Solution Structures of Metabolites Formed from *cis*-Dienelactone

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A ^{1}H nuclear magnetic resonance (^{1}H NMR) assay was used to study the enzymatic transformation of *cis*-dienelactone, a central intermediate in the degradation of chloroaromatics. It was shown that the product of the *cis*-dienelactone hydrolase reaction is maleylacetate, in which there is no evidence for the formation of 3-hydroxymuconate. Under acidic conditions, the product structure was 4-carboxymethyl-4-hydroxybut-2-en-4-olide. Maleylacetate was transformed by maleylacetate reductase into 3-oxoadipate, a reaction competing with spontaneous decarboxylation into *cis*-acetylacrylate. One-dimensional ^{1}H NMR in ^1H_2O could thus be shown to be an excellent noninvasive tool for monitoring enzyme activities and assessing the solution structure of substrates and products.

A major route for mineralization of chloroaromatic compounds by microorganisms is their transformation into chlorocatechols and their further metabolism by enzymes of the chlorocatechol pathway (24). In this metabolic pathway, chlorocatechols are subject to intradiol cleavage to form the respective chloromuconates, which are converted by chloromuconate cycloisomerases into *cis*- or *trans*-dienelactone. The dienelactones undergo hydrolysis by dienelactone hydrolase. The hydrolysis product formed during the metabolism of 4-chlorocatechol was tentatively identified as "maleylacetate" based on its absorption characteristics (λ_{max} = 243 nm in aqueous alkali) by Evans et al. (11). Similarly, Tiedje et al. (31) postulated maleylacetate and chloromaleylacetate as products formed during the metabolism of 4-chloro- and 3,5-dichlorocatechol, respectively. Those authors noted, however, that UV absorption was essentially quenched upon acidification, a behavior resembling keto-enol tautomerism, raising the question of the actual solution structure of maleylacetate. More recently, Seibert et al. claimed that the enol form (3-hydroxy-2,4-hexadienedioate, 3-hydroxymuconate) is thermodynamically favored under physiological conditions and exhibits an absorption maximum at 243 nm (28). The disappearance of this absorption under acidic conditions was believed to be due to the presence of the keto form, 3-oxo-*cis*-4-hexenedioate (maleylacetate in the strict sense), under these conditions. Despite the authors' assumption that 3-hydroxymuconate was the actual substrate of the purified reductase, the enzyme was termed "maleylacetate" reductase. Later, Prucha et al. (23) showed that the hydrolysis product of 3-methyldienelactone, supposedly 3-methylmaleylacetate or 3-hydroxy-4-methylmuconate, has a cyclic structure under acidic conditions (4-carboxymethylene-4-hydroxy-3-methylbut-2-en-4-olide, 4-hydroxy-3-methylmuconolactone), while no indication of the configuration under physiological conditions was given. Thus, although the structure of the decarboxylation product has been published (27), the actual solution structure of "maleylacetate" remained uncertain, presumably due to its reportedly high instability.

"Maleylacetate" is an intermediate not only in the degradation of chlorocatechols via the chlorocatechol *ortho*-cleavage pathway but also in the degradation of more highly substituted aromatics, such as 2,4,5-trichlorophenoxyacetate (1, 7), 2,4,6-trichlorophenol (16, 20), pentachlorophenol (19, 33), and lindane (18), even though different reaction sequences yielding maleylacetate have been proposed. Moreover, "maleylacetate" is assumed to be an intermediate in the degradation of resorcinolic compounds (5). Thus, "maleylacetate" is a central intermediate in the metabolism of chloroaromatic as well as natural aromatic compounds. Intermediates similar in structure to "maleylacetate" have been found in various other pathways involved in aromatic degradation. 2-Hydroxymuconate (2-hydroxy-2,4-hexadienedioate) was reported to be an intermediate in the degradation of catechols after extradiol cleavage (14, 25). In aqueous solution, this compound is in equilibrium with 2-oxo-4-hexenedioate, a structural isomer of "maleylacetate" (3-oxo-*cis*-4-hexenedioate), and 4-oxalocrotonate tautomerase catalyzes the otherwise slow transformation of 2-oxo-4-hexenedioate to 2-oxo-3-hexenedioate (32). However, the actual solution structures of such intermediates have been investigated in only a few cases. Thus, knowledge of the identity of the substrates for the following reaction and the actual enzyme mechanism involved is limited. Previously, Guthrie (13) showed that acetopyruvate exists in three major forms in aqueous solution and Pokorny et al. (22) proved that the enol form is the actual substrate for hydrolysis by acetopyruvate hydrolase.

To unequivocally identify the structure of the dienelactone hydrolysis product under physiological conditions, we have characterized its structure by in situ ^{1}H nuclear magnetic resonance (^{1}H NMR) analysis, a method recently shown to be a

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powerful tool for investigating the solution structures of such intermediates (2).

Dienelactone hydrolase and maleylacetate reductase in *Ralstonia eutropha* JMP222(pBBR1M-1). In order to characterize the structure of the dienelactone hydrolysis product under physiological conditions, *R. eutropha* JMP222(pBBR1M-1) was grown on 3-chlorobenzoate as recently described (21). *R. eutropha* JMP222 is a derivative of *R. eutropha* JMP134 (9) cured of plasmid pJP4. pJP4 was shown to contain two chlorocatechol gene clusters, namely, module I (8) and module II (17), which both encode a "maleylacetate" reductase (15, 28). Only module I was present in JMP222(pBBR1M-I). Enzyme activities were estimated in cell extracts by methods described previously (21). *R. eutropha* JMP222(pBBR1M-I) expresses high levels of dienelactone hydrolase (1,200 U/g of protein with 80 μM *cis*-dienelactone as the substrate) during growth on 3-chlorobenzoate. In contrast to recent observations (21), we were also able to show the existence of a significant level of maleylacetate reductase activity in cell extracts (up to 700 U/g of protein). We could prove that the respective enzyme was extremely unstable under different conditions; e.g., only a small percentage of activity (usually less than 20%) was recovered when a cell extract was subjected to purification by fast protein liquid chromatography using a MonoQ HR5/5 column with Tris-HCl as the eluent system (data not shown). However, high activities (always higher than 60%) were recovered when phosphate buffer was the eluent. Such a buffer system was necessary for the later ^{1}H NMR analysis to avoid interference of signals originating from any protein fraction added to the in situ transformation NMR assay. A cell extract of strain JMP222 (pBBR1M-I) was therefore prepared in phosphate buffer, and a total of 5 to 8 mg of protein was loaded onto a MonoQ HR5/5 column and eluted with a linear gradient of 0 to 0.5 M NaCl in 50 mM phosphate buffer (pH 7.4) at a flow rate of 0.5 ml/min. Fractions of 0.5 ml were collected and analyzed for dienelactone hydrolase activity as described previously (21). Under these conditions, dienelactone hydrolase eluted at a NaCl concentration of 0.03 mM, whereas the bulk of the maleylacetate reductase activity (approximately 60 to 70% of the applied activity) eluted at a NaCl concentration of 0.35 mM.

The transformation of *cis*-dienelactone (0.08 mM) was analyzed spectrophotometrically as described previously (28) with aliquots of fractions containing dienelactone hydrolase activity, and the formation of a product exhibiting an absorption maximum at 243 nm under physiological conditions was confirmed. This maximum disappeared upon acidification as described previously (28). Thus, the transformation product can be assumed to be identical to that formed by homogeneous maleylacetate reductase (28). Higher concentrations of *cis*-dienelactone (2 mM) were transformed at rates nearly five times those calculated for substrate concentrations of 0.08 mM, as expected for an enzyme with a low affinity (K_m value of approximately 0.14 mM) for *cis*-dienelactone (26). Complete turnover of *cis*-dienelactone was confirmed by high-pressure liquid chromatography analysis using a Shimadzu high-pressure liquid chromatography system equipped with a SC125/Lichrospher 5-μm (Bischoff, Leonberg, Germany) column and an aqueous solvent system (flow rate, 1 ml/min) containing 0.1% (vol/vol) H_3PO_4 (87%), and 20% (vol/vol) methanol.

Solution structure of metabolites formed from *cis*-dienelactone by dienelactone hydrolase as determined by ^{1}H NMR. In order to characterize the substrate structural changes occurring under the various solution conditions described above, the transformation of *cis*-dienelactone was monitored by ^{1}H NMR spectroscopy using an NMR sample containing 30 mM sodium phosphate buffer (pH 7.2), 2 mM *cis*-dienelactone, and 0.14 ml of D_2O in a total volume of 0.7 ml. The one-dimensional ^{1}H NMR spectra were recorded at 300 K on a Bruker Avance DMX 600 NMR spectrometer locked to the deuterium resonance of D_2O in the solution. Spectra were recorded by using the standard Bruker one-dimensional nuclear Overhauser effect spectroscopy suppression sequence with 280 scans, each with a 1.8-s acquisition time and a 1.3-s relaxation delay. The center of the suppressed water signal was used as an internal reference (δ = 4.80 ppm).

After the in situ ^{1}H spectrum of *cis*-dienelactone had been recorded (Fig. 1A), 10 μl of dienelactone hydrolase (corresponding to an activity of 30 mU) was added. Figure 1B shows the spectrum after complete transformation of *cis*-dienelactone. The sample was then adjusted to a pH of 2.5 by the addition of HCl, and a spectrum was recorded under acidic conditions (Fig. 1C). The spectrum of the sample was then recorded at appropriate time intervals to monitor the expected decarboxylation reaction, which should yield *cis*-acetylacrylate acylal (Fig. 1D), the ^{1}H NMR spectrum of which has already been reported (30). After complete decarboxylation, the sample was neutralized by addition of NaOH to give a final pH of 8, and the structure of the product was analyzed under neutral conditions (Fig. 1E). In all cases, the structures of the various products were evident from the intensity of the ^{1}H signals, their characteristic chemical shifts, and the magnitude of the coupling constants (Fig. 1).

Only two olefinic protons (δ = 6.32 and 6.47 ppm) were present in the product formed from *cis*-dienelactone by dienelactone hydrolase. Furthermore, the presence of a methylene function in the product (δ = 3.52 ppm) excluded its identity with 3-hydroxymuconate (Fig. 1B). The vicinal coupling of 12 Hz observed for the olefinic systems is characteristic of their *cis* configuration in an open-chain system and thus indicates the identity of the product with maleylacetate in the strict sense. Acidification resulted in a downfield shift of the resonance line of the methylene function (δ = 3.15 ppm) and a high-field shift of the resonance lines of one olefinic proton (two olefinic protons resonate at 6.33 and 7.65 ppm). The small coupling constant of 5.8 Hz indicates the presence of the olefinic system in a closed five-membered ring system (4). These data (Fig. 1C) indicate that maleylacetate, under acidic conditions, is 4-carboxymethyl-4-hydroxybut-2-en-4-olide (4-hydroxymuconolactone). There were no indications of the presence of 3-hydroxymuconate under either physiological or acidic conditions. However, a mechanism of dienelactone hydrolase catalysis involving a nucleophilic attack by a cysteine residue has been proposed (6). The enolate anion generated after collapse of the first tetrahedral intermediate is supposed to abstract a proton from a nearby water molecule, leaving a hydroxyl which is appropriately placed for attack of the acyl carbon in the subsequent deacylation step, which should generate 3-hydroxymuconate. Obviously, in contrast to 2-hydroxymuconate (32), 3-hydroxymuconate belongs to the class of fast-reacting enols (10), giving rise in milliseconds to the corresponding

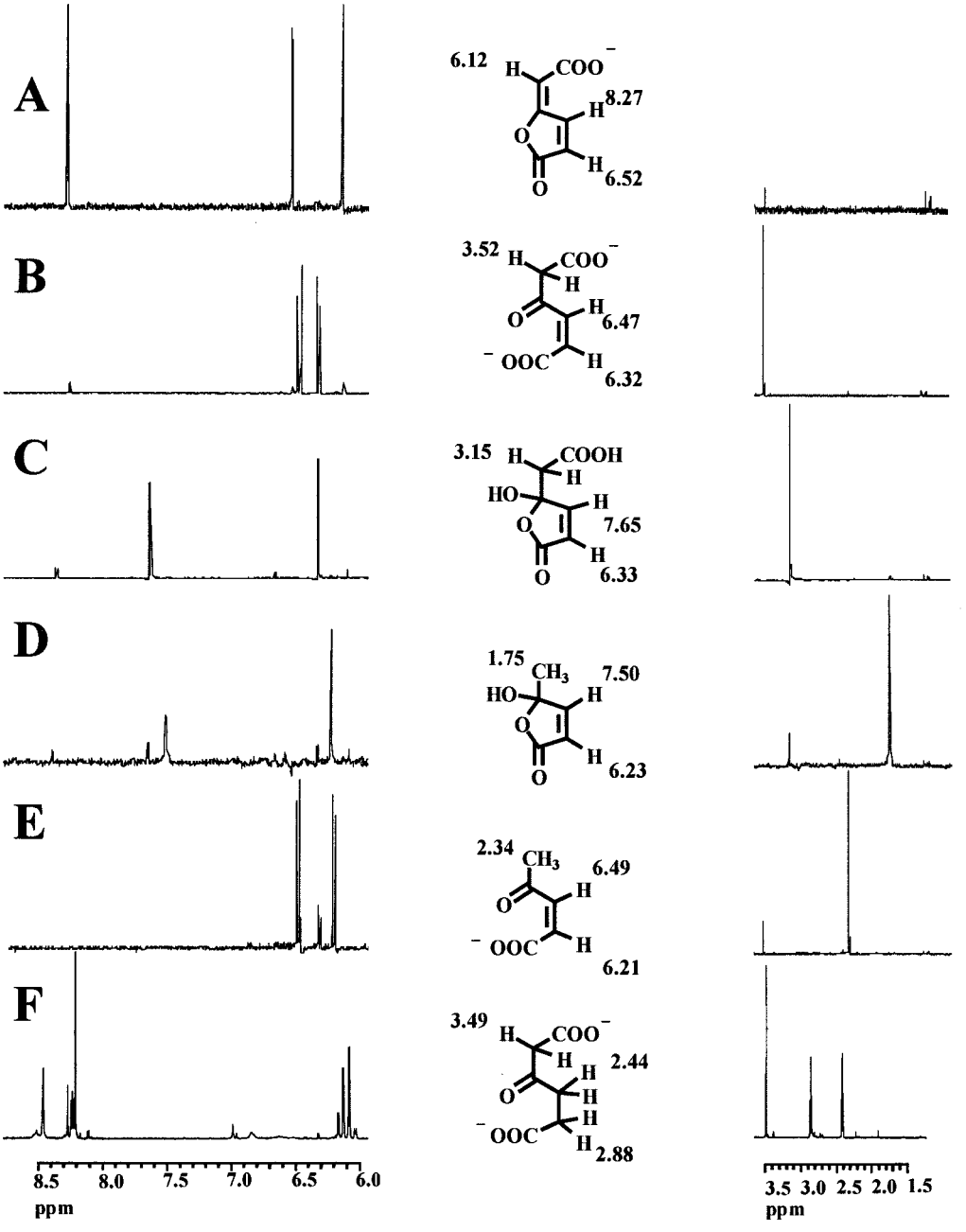

FIG. 1. ^{1}H NMR spectra of *cis*-dienelactone and transformation products. *cis*-Dienelactone (2 mM) in 50 mM phosphate buffer (pH 7.4) (A) was incubated for 1 h with partially purified dienelactone hydrolase (43 mU/ml) (B), acidified to pH 2.5 (C), and kept under acidic conditions for 48 h (D), and the resulting solution was neutralized (E). (F) ^{1}H NMR spectrum of *cis*-dienelactone after 1 h of incubation with partially purified dienelactone hydrolase (29 mU/ml) plus partially purified maleylacetate reductase (71 mU/ml). The proposed solution structures *cis*-dienelactone (A), 3-oxo-*cis*-4-hexenedioate (maleylacetate) (B), 4-carboxymethyl-4-hydroxybut-2-en-4-olide (4-hydroxymuconolactone) (C), *cis*-acetylacrylate acylal (D), *cis*-acetylacrylate (E), and 3-oxoadipate (F) are indicated.

keto-derivative, maleylacetate, and thus, the initial dienelactone hydrolysis product could not be observed. Even when *cis*-dienelactone was transformed in the NMR tube with 200 mU of enzyme, generating a 0.3 mM concentration of product/min, no signals corresponding to the formation of 3-hydroxymuconate were visible. However, the presence of such a fast equilibrium step and the production of 3-hydroxymuconate as the initial hydrolysis product remain to be proven.

Under acidic conditions, a slow decarboxylation was observed with *cis*-acetylacrylate acylal as the product (Fig. 1D), as reported by Schlömann (27). The small coupling constant of 5.8 Hz between the two olefinic protons resonating at 6.23 and 7.50 ppm indicates their presence in a closed-ring system. Three protons of a methyl group resonate at 1.75 ppm. These values are in accordance with those previously reported for *cis*-acetylacrylate acylal (30).

At neutral pH (Fig. 1E), the compound is present in the open-ring form, as evidenced by the high coupling constant of 12.3 Hz between the two olefinic protons (6.21 and 6.49 Hz), the downfield shift of the proton neighboring the oxygen-substituted carbon atom compared to the closed form, and the respective upfield shift of the signal of the methyl group (2.34 ppm). These values are again very similar to those reported previously for *cis*-acetylacrylate (30).

Transformation of maleylacetate by maleylacetate reductase as determined by ^{1}H NMR. Maleylacetate prepared in situ from *cis*-dienelactone by partially purified dienelactone hydrolase is further transformed by maleylacetate reductase, supposedly yielding 3-oxoadipate by simultaneous oxidation of NADH. This reaction was monitored by NMR analysis as follows. In a first reaction, *cis*-dienelactone (2 mM) was transformed by partially purified dienelactone hydrolase (corresponding to 20 mU) as described above, and after complete transformation to maleylacetate, the reaction mixture was supplemented with NADH (3 mM) and partially purified maleylacetate reductase (corresponding to 50 mU). In addition to the reaction product, the instability of maleylacetate under neutral conditions (27) led to *cis*-acetylacrylate through spontaneous decarboxylation of maleylacetate. In a second experiment, *cis*-dienelactone (2 mM) was incubated with dienelactone hydrolase (corresponding to 20 mU) and an excess of maleylacetate reductase (corresponding to 50 mU) in the presence of NADH (3 mM), thus avoiding the intermediate accumulation of maleylacetate. Besides the appearance of signals from the oxidation of NADH to NAD, the formation of a single reaction product which was identical to the one formed during successive transformation of *cis*-dienelactone via maleylacetate was clearly visible. Its identity with 3-oxoadipate (Fig. 1F) is evident from the presence of three methylene functions (2.44 and 2.88 ppm [vicinal coupling constant = 7.0 Hz] and 3.49 ppm). These signals were clearly separated from those originating from NAD and residual NADH.

3-Oxo-*cis*-4-hexenedioate as a stable intermediate in the degradation of chloroaromatics. Monitoring biocatalyzed reactions and metabolic pathways using NMR spectroscopy is of growing interest (2). The method is noninvasive, and analyses can be performed in ^1H_2O or appropriate buffer systems, which allows one to monitor transformations under physiological conditions. It allows characterization of substrates and products without any manipulation, thus avoiding structural changes during extraction or derivatization procedures. The ^{1}H NMR analysis also allows the identification of solution structures of substrates and intermediates, as shown by Pokorny et al. (22). In the present study, we showed that maleylacetate exists in two stable solution structures, maleylacetate in the strict sense under physiological conditions and its cyclic lactone structure, 4-hydroxymuconolactone, under acidic conditions. The enol form, 3-hydroxymuconate, was not observed in the present study. This is in contrast to previous assumptions (28) of an equilibrium between the enol form (supposed to be the major solution structure under physiological conditions) and maleylacetate (supposed to be the major solution structure under acidic conditions).

The observed absorption maximum at 243 nm is evidently due to maleylacetate. All related keto acids, the solution structures of which have been elucidated, show similar absorption maxima, e.g., 2-oxo-3-hexendioate (λ_{max} = 236 nm [32]), 4,6-dioxo-2-heptenoate (maleylacetone, λ_{max} = 243 nm [29]), and *cis*-acetylacrylate (λ_{max} = 240 nm [30]), due to their conjugated double-bond system, whereas enol tautomers, such as 2-hydroxymuconate (λ_{max} = 295 nm [32]), 4-hydroxy-6-oxo-2,4-heptadieneoate (λ_{max} = 312 nm [29]), and 4-hydroxy-6-oxo-2-methyl-2-4-heptadienoate (λ_{max} = 325 nm [3]), absorb at significantly higher wavelengths due to extended conjugation.

In contrast to maleylacetate which, depending on the pH, exists as the keto and the lactol forms, different solution structures have been reported for maleylacetone, methylmaleylacetone, and acetopyruvate, including an enol tautomer, which under physiological conditions accounts for approximately 50% of the solution structure mixture (3, 12, 22, 29). The only apparent difference between maleylacetate and (methyl) maleylacetone is the higher acidity of the methylene protons in the latter case, favoring formation of the enol tautomer.

Beside maleylacetate, 2-chloromaleylacetate has been proposed as an intermediate in various chloroaromatic-degradative pathways. However, the identification of 2-chloromaleylacetate as an intermediate in the degradation of chloroaromatic compounds via hydroquinones as intermediates is as yet only tentative and based on mass spectrometric data of presumably 2-chloromaleylacetate or further reaction products (18, 19, 33). Moreover, new unidentified reaction products were recently observed during the enzyme-catalyzed transformation of 3,5-dichlorocatechol via 2-chloromaleylacetate. At least some of these products were assumed to be formed by spontaneous reactions of 2-chloromaleylacetate. We propose that in situ NMR is an appropriate tool for monitoring such reactions and for characterizing products formed by enzyme-catalyzed and spontaneous reactions. Experiments to monitor the metabolic fate of 2-chloromaleylacetate are being performed.

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