Oxidative Degradation of 3,4-Dimethoxybenzyl Alcohol and Its Methyl Ether by the Lignin Peroxidase of *Phanerochaete chrysosporium*

Harald W. H. Schmidt,[‡] Stephan D. Haemmerli,[§] Hans E. Schoemaker,[∥] and Matti S. A. Leisola*[,§]

Givaudan Forschungsgesellschaft AG, 8600 Dübendorf, Switzerland, Department of Biotechnology, Swiss Federal Institute of Technology, ETH-Hönggerberg, 8093 Zürich, Switzerland, and Bio-organic Chemistry Section, DSM Research, P.O. Box 18, 6160 MD Geleen, The Netherlands

Received June 21, 1988; Revised Manuscript Received September 26, 1988

ABSTRACT: The oxidative degradation of 3,4-dimethoxybenzyl alcohol and 3,4-dimethoxybenzyl methyl ether by the lignin peroxidase (ligninase, LiP) of *Phanerochaete chrysosporium* was studied. In addition to previously isolated products of 3,4-dimethoxybenzyl alcohol oxidation (veratraldehyde, two quinones, γ-lactones, and a δ-lactone) three new products—4,5-dimethoxy-3,5-cyclohexadiene-1,2-dione, (*E*)-δ-lactone, and 2,5-dihydroxy-4-methoxybenzaldehyde—were now identified as oxidation products. The relative quantities of the products were determined. Six products formed in the oxidation of 3,4-dimethoxybenzyl methyl ether by LiP in the presence of oxygen, veratraldehyde, veratric acid methyl ester, three quinones, and an aromatic ring cleavage product, 3-(methoxymethyl)-(*Z*,*Z*)-muconic acid dimethyl ester, were identified and their relative quantities determined. Under anaerobic conditions only trace amounts of products other than veratraldehyde were formed. With cerium(IV) ammonium nitrate as the oxidant comparable results were obtained. The influence of pH and of manganese(II) ion on the LiP reaction was also studied. The major oxidation product at pH 3.0 was veratraldehyde and at pH 5.0 veratric acid methyl ester. This is the first time that this kind of compound has been identified in lignin peroxidase catalyzed reactions. Possible mechanisms for the formation of these products which indicate involvement of activated oxygen species are presented, and the results are discussed in relation to lignin degradation by *P. chrysosporium*.

Lignin is a highly irregular, three-dimensional biopolymer composed of oxygenated phenylpropane units interlinked—inter alia—via β-O-4 and α-O-4 aryl ether linkages. It is rather resistant to chemical and microbial attack but is oxidatively degraded by white rot fungi. The best characterized lignin-degrading fungus is the basidiomycete *Phanerochaete chrysosporium*. In 1983 an extracellular lignin peroxidase (ligninase, LiP[1]) was isolated from ligninolytic cultures of this organism (Tien & Kirk, 1983; Glenn et al., 1983). Fifteen isozymic forms of this enzyme have now been isolated (Leisola et al., 1987). While some of these are probably a result of posttranslational modifications evidence has also been given for the presence of LiP gene family (Walther et al., 1988). Lignin peroxidases are glycoproteins having a molecular weight of about 40000 (Tien & Kirk, 1984). All the tested isozymes have a single iron protoporphyrin IX prosthetic group (Renganathan et al., 1985). The heme is in a high-spin ferric state and probably has histidine as an axial ligand as judged from different spectroscopic evidence (Andersson et al., 1985; Kuila et al., 1985). One LiP form has recently been crystallized (Troller et al., 1988), and detailed information on the orientation of the heme and on the three-dimensional structure of this type of peroxidases should soon be available.

Lignin peroxidase has been shown to catalyze a wide variety of reactions important for the degradation of lignin, e.g., C_α–C_β cleavage, cleavage of β-O-4 ether bonds, oxidation of C_α-carbinol to C_α-oxo compounds, hydroxylation of benzylic methylene groups and styrene olefinic bonds, decarboxylation of phenylacetic acid, and aromatic ring opening (Farrell & Kirk, 1987; Palmer et al., 1987). The LiP-catalyzed degradation of veratryl alcohol, a metabolite of *P. chrysosporium* which may be considered to be the most simple model compound of lignin, has been investigated recently by us (Leisola et al., 1985a; Haemmerli et al., 1987). It was shown that under anaerobic conditions veratryl alcohol is oxidized essentially at the C_α-carbinol to give veratric aldehyde, whereas under aerobic conditions an additional oxidative pathway is operating, leading to quinones and aromatic ring cleavage. The ability of LiP to cleave aromatic rings was thus demonstrated for the first time (Leisola et al., 1985a).

Later, aromatic ring cleavage was also reported for β-O-4 lignin substructure models (Umezawa et al., 1986; Umezawa & Higuchi, 1986; Miki et al., 1987), and mechanisms have been proposed for these cleavage reactions based on the products isolated and on experiments made with ^{13}C-labeled substrates and $^{18}O_2$ and $H_2{}^{18}O$ (Palmer et al., 1987; Miki et al., 1987; Umezawa & Higuchi, 1987a,b; Shimada et al., 1987). It has been demonstrated that the *o*-dimethoxybenzene ring in veratryl alcohol and in β-O-4 lignin substructure model compounds is cleaved at the carbon atoms bearing the alkoxy groups, yielding muconic acid derivatives, e.g., diesters (Umezawa & Higuchi, 1987a,b) as primary ring cleavage products and—in the case of veratryl alcohol—lactone esters (Leisola et al., 1985a; Haemmerli et al., 1987; Shimada et al., 1987; Hattori et al., 1988).

3,4-Dimethoxybenzyl methyl ether may be considered to be the most simple model compound for α-O ether lignin substructures. It should not yield lactones as ring cleavage

[‡] Givaudan Forschungsgesellschaft AG.
[§] Swiss Federal Institute of Technology.
[∥] DSM Research.

[1] Abbreviations: LiP, lignin peroxidase; NMR, nuclear magnetic resonance; MS, mass spectrum; GC–MS, gas chromatography–mass spectrometry coupling; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography; mp, melting point; bp, boiling point; DMME, 3,4-dimethoxybenzyl methyl ether; NOE-DIFF, nuclear Overhauser effect, difference spectrum; VA, veratryl alcohol.

0006-2960/89/0428-1776$01.50/0

products provided that the benzylic ether linkage is not concomitantly cleaved. Here we present results for the degradation of this compound by LiP. The obtained results confirm the observations made earlier in our study of the degradation of veratryl alcohol (Leisola et al., 1985a; Haemmerli et al., 1987). We further prove for the first time that the actual configuration of the immediate ring cleavage product is *Z,Z*. The results also indicate that more than one mechanism is involved in oxygen incorporation into the degradation products obtained.

Materials and Methods

Enzyme Preparations. Lignin peroxidase was isolated and purified from carbon-limited cultures of *P. chrysosporium* (ATCC 24725) as described previously (Leisola et al., 1985b; Haemmerli et al., 1986). The crude enzyme preparation or the enzyme with p*I* 4.15 was used in the oxidation experiments.

Chemicals. 3,4-Dimethoxybenzyl alcohol (IX, veratryl alcohol), 3,4-dimethoxybenzaldehyde (XI, veratraldehyde), 3,4-dimethoxybenzoic acid (veratric acid), 1,2-dihydroxybenzene, 3-hydroxy-4-methoxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol, 3-hydroxy-4-methoxybenzaldehyde (isovanillin), and cerium(IV) ammonium nitrate were obtained from Fluka (Switzerland). Fremy's salt was from Aldrich (Suchema, Switzerland).

Compounds I, II, V, and VI were isolated and/or prepared as described previously (Leisola et al., 1985a; Haemmerli et al., 1987).

4,5-Dimethoxy-3,5-cyclohexadiene-1,2-dione (III) was prepared from 1,2-dihydroxybenzene by oxidation with PbO_2 in the presence of methanol as described by Wanzlick and Jahnke (1968) to give yellow needles (from methanol), mp 227–230 °C (uncorrected; lit. 225–227 °C): UV (methanol) λ_{max} (log ϵ) 282 (4.089) and 404 (2.761) nm; MS *m/z* (%) 168 (M^+, 1), 153 (2.5), 140 (33), 125 (14), 82 (9), 69 (100), 59 (21), 53 (23); ^1H NMR ($CDCl_3$) δ (ppm) 3.91 (s, 6 H, $-OCH_3$), 5.77 (s, 2 H, ring protons).

(*Z*)-6-Oxo-2*H*-pyran-3(6*H*)-ylideneacetic acid methyl ester (VII) was prepared by oxidation of 4-hydroxy-3-methoxybenzyl alcohol with sodium chlorite as described in the literature (Dence et al., 1962; Ainsworth & Kirby, 1968) to give colorless prisms, mp 87–90 °C (uncorrected, from ether; lit. 96–97 °C): MS *m/z* (%) 168 (M^+, 10), 137 (33), 136 (55), 124 (100), 111 (30), 109 (13), 108 (32), 97 (7), 81 (57), 80 (27), 79 (63), 69 (11), 59 (17), 53 (68), 52 (19), 51 (43), 50 (34); ^1H NMR ($CDCl_3$) δ (ppm) 3.79 (s, 3 H, $-COOCH_3$), 5.02 (d, J = 1.7 Hz, 2 H, $-CH_2O-$), 5.94 [m, 1 H, =CH-CO(OCH_3)], 6.21 (q, J = 10.1 Hz, 1.8 Hz, 1 H, $-OCOCH=$), 8.34 (q, J = 10.1 Hz, 0.8 Hz, 1 H, $=CHC=$).

(*E*)-6-Oxo-2*H*-pyran-3(6*H*)-ylideneacetic acid methyl ester (VIII) was prepared via the corresponding acid from VII as described by Ainsworth and Kirby (1968) to give colorless crystals, mp 147–150 °C (uncorrected, from ether): MS *m/z* (%) 168 (M^+, 10), 137 (20), 136 (100), 124 (13), 111 (12), 109 (9), 108 (37), 81 (22), 80 (39), 79 (22), 59 (16), 58 (21), 55 (10), 53 (31), 52 (17), 51 (42), 50 (12); ^1H NMR ($CDCl_3$) δ (ppm) 3.78 (s, 3 H, $-COOCH_3$), 5.69 (d, J = 2.8 Hz, 2 H, $-CH_2O-$), 5.98 [m, 1 H, $=CHCO(OCH_3)$], 6.19 (d, J = 9.8 Hz, 1 H, $-OCOCH=$), 7.01 (q, J = 9.8 Hz, 0.8 Hz, 1 H, $=CHC=$).

2,5-Dihydroxy-4-methoxybenzaldehyde (X) was prepared from isovanillin as described (Rajagopalan et al., 1949) to give colorless crystals, mp 205–207 °C dec (uncorrected; lit. 209 °C dec); MS *m/z* (%) 168 (M^+, 100), 167 (82), 153 (15), 139 (3), 122 (3), 111 (7), 107 (3), 97 (5), 83 (7), 79 (4), 69 (36), 53 (11); ^1H NMR (acetone-d_6) δ (ppm) 4.010 (s, 3 H, $-OCH_3$), 6.580 and 7.137 (s, 1 H, ArH), 9.837 (s, 1 H, $-CHO$).

3,4-Dimethoxybenzyl methyl ether (XII) was prepared by dissolving 8.4 g (0.05 mol) of veratryl alcohol in 200 mL of absolute methanol containing 0.5 g of *p*-toluenesulfonic acid monohydrate. The solution was kept overnight at room temperature. The solution was filtered via a layer of basic alumina (Woelm, activity grade 1). The colorless liquid obtained after evaporation of the methanol was fractionated in vacuo yielding the 3,4-dimethoxybenzyl methyl ether as a colorless liquid, bp 80–82 °C [(3–4) × 10^{-2} mbar], 7.4 g (81.3% of theory): UV (methanol) λ_{max} (log ϵ) 229 (3.884) and 276 (3.426) nm; MS *m/z* (%) 182 (M^+, 70), 166 (6), 151 (100), 139 (5), 124 (3), 107 (10), 91 (8), 77 (5), 65 (4), 51 (5), 45 (7); ^1H NMR ($CDCl_3$) δ (ppm) 3.361 (s, 3 H, $-CH_2OCH_3$), 3.858 and 3.866 (s, 3 H, $-OCH_3$), 4.382 (s, 2 H, $-CH_2O-$), 6.828 (d, J = 8.1 Hz, 1 H, $-ArC_5H$), 6.857 (q, J = 8.1 Hz, 1.8 Hz, 1 H, $-ArC_6H$), 6.888 (d, J = 1.8 Hz, 1 H, $-ArC_2H$).

3,4-Dimethoxybenzoic acid methyl ester (XIII) was prepared from veratric acid with methanol/acetyl chloride as described by Freudenberg and Jakob (1941), yielding colorless prisms, mp 61–62 °C (uncorrected, lit. 62 °C): UV (methanol) λ_{max} (log ϵ) 217 (4.384), 258 (4.123), and 288 (3.811) nm; MS *m/z* (%) 196 (M^+, 95), 181 (22), 165 (100), 149 (12), 137 (33), 121 (16), 107 (11), 94 (14), 79 (25), 77 (22), 59 (19), 51 (18); ^1H NMR ($CDCl_3$) δ (ppm) 3.898 (s, 3 H, $-COOCH_3$), 3.936 and 3.940 (s, 3 H, $-OCH_3$), 6.892 (d, J = 8.4 Hz, 1 H, $-ArC_5H$), 7.550 (d, J = 2.0 Hz, 1 H, $-ArC_2H$), 7.685 (9, J = 8.4 Hz, 2.0 Hz, 1 H, $-ArC_6H$).

2-Methoxy-5-(methoxymethyl)-2,5-cyclohexadiene-1,4-dione (XV) was prepared as follows: To a solution of 1.7 g of potassium dihydrogen phosphate in 140 mL of water was added with stirring 2.9 g of Fremy's salt. To the violet solution was added a solution of 0.91 g (5.4 mmol) of 3-hydroxy-4-methoxybenzyl methyl ether in 20 mL of acetone. The solution turned slowly to a dark yellow, and after stirring for about 35 min the quinone crystallized in yellow needles. The solution was extracted three times with methylene chloride. Evaporation of the methylene chloride solution to dryness gave 0.85 g of yellow crystals, mp (after recrystallization from chloroform) 124–125 °C (uncorrected): UV (methanol) λ_{max} (log ϵ) 258 (4.147) and 360 (2.892) nm; MS *m/z* (%) 182 (M^+, 2.5), 181 (7), 167 (38), 153 (100), 139 (15), 125 (8), 111 (28), 95 (11), 83 (8), 69 (44), 53 (15), 45 (22); ^1H NMR ($CDCl_3$) δ (ppm) 3.463 (s, 3 H, $-CH_2OCH_3$), 3.838 (s, 3 H, ring OCH_3), 4.323 (d, J = 2.2 Hz, 2 H, $-CH_2OCH_3$), 5.909 [s, 1 H, $-COCH=C(OCH_3)-$], 6.724 [H, J = 2.2 Hz, 1 H, $-COCH=C(CH_2OCH_3)-$].

2,5-Dimethoxy-2,5-cyclohexadiene-1,4-dione (XVI) was prepared from the 4,5-dimethoxy-3,5-cyclohexadiene-1,2-dione (III) as described by Wanzlick and Jahnke (1968), yielding yellow-orange crystals, mp 298–303 °C dec (uncorrected; lit. 300 °C): MS *m/z* (%) 168 (M^+, 3.5), 153 (11), 139 (25), 125 (12), 111 (10), 95 (19), 69 (100), 59 (21), 53 (39); ^1H NMR ($CDCl_3$) δ (ppm) 3.85 (s, 6 H, $-OCH_3$), 5.88 (s, 2 H, ring protons).

3-Hydroxy-4-methoxybenzyl methyl ether (XVII) was prepared by dissolving 2.0 g (0.013 mol) of 3-hydroxy-4-methoxybenzyl alcohol in 50 mL of absolute methanol containing 0.2 g of *p*-toluenesulfonic acid monohydrate. After standing at room temperature overnight, the solution was filtered via a layer of basic alumina (activity grade 1, Woelm). Evaporation of the filtrate to dryness yielded a slightly yellow liquid which was fractionated in vacuo to give 1.7 g (78.0% of theory) of a colorless liquid, bp 76–77 °C (0.25 mbar): n^{20}_D

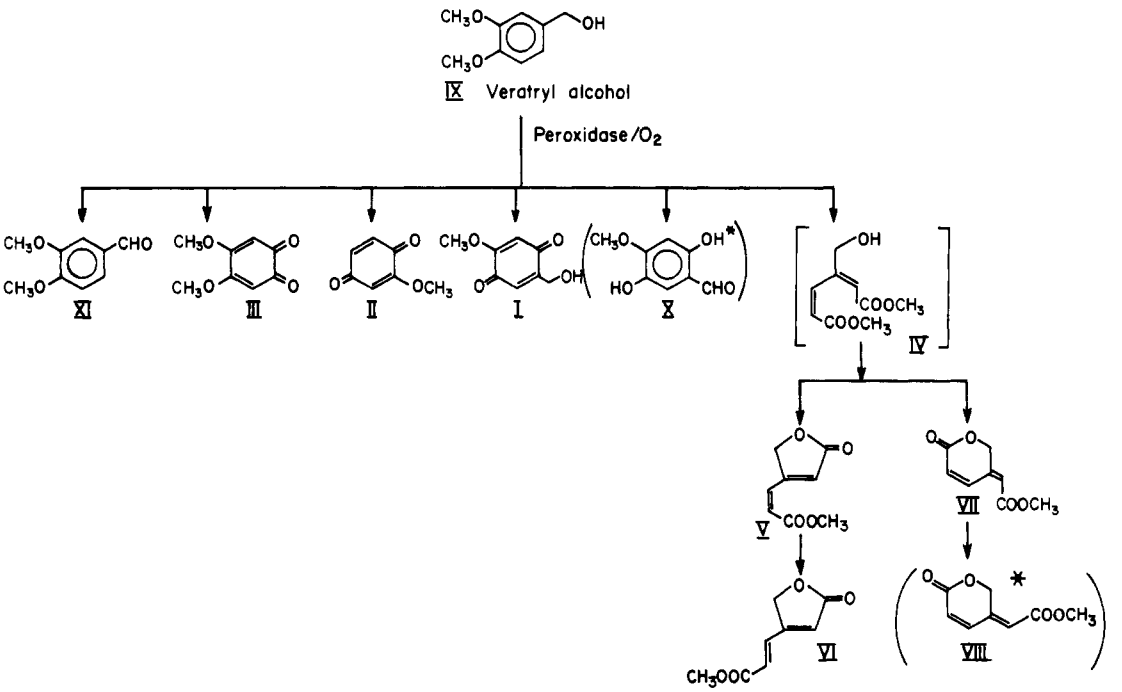

FIGURE 1: Products of veratryl alcohol oxidation by lignin peroxidase. (*) indicates tentatively identified.

1.5418; MS *m/z* (%) 168 (M^+, 54), 151 (7), 137 (100), 122 (12), 107 (5), 94 (8), 81 (4), 65 (9), 53 (6).

Oxidations. All the oxidations were carried out at room temperature. In aerobic oxidations the reaction mixture and all solutions were flushed with oxygen 15 min prior to and during reactions. The anaerobic reaction mixtures were flushed with nitrogen, respectively. Hydrogen peroxide was added in small portions. The progress of the reaction was monitored by HPLC analysis (direct injection of samples of the reaction media). After the reaction was completed (usually after 30 min), the products were immediately extracted with methylene chloride at pH 2. The extracts were evaporated to dryness under nitrogen and analyzed.

(A) Oxidation by Lignin Peroxidase. The reaction mixtures contained 0.4 mM (veratryl alcohol) or 7 mM (3,4-dimethoxybenzyl methyl ether) substrate, 10 mM sodium succinate (pH 3–5), 0.4 or 7 mM H_2O_2 (final concentration), and 30–50 units/L lignin peroxidase. In the experiments to determine the influence of pH, O_2, and Mn(II), the substrate and hydrogen peroxide concentrations were 0.2 mM each.

(B) Oxidation by Ce(IV). Under a steady flow of oxygen 5.0 mM cerium(IV) ammonium nitrate in 1.5 mL of water was slowly added dropwise to a mixture containing 1.0 mM 3,4-dimethoxybenzyl methyl ether in 1.5 mL of water. The reaction was analyzed by HPLC (direct injection of samples of the reaction mixture).

Analytical Methods. Lignin peroxidase activity was measured according to Tien and Kirk (1984). One unit of enzyme produced 1 μmol of veratraldehyde from veratryl alcohol in a minute at room temperature.

Thin-layer chromatography was carried out by using silica gel 60 F_{254} TLC plates (Merck) which were developed in hexane/ethyl acetate, 3:1. R_f values for the compounds II, III, and XI–XV (see Figures 1 and 5) were 0.45, 0.08, 0.52, 0.31, 0.67, 0.67, and 0.45, respectively. For preparative separations the plates were developed once, dried in air, and developed a second time in the same solvent system. The bands were then scratched off and the products eluted with ethyl acetate. To achieve further purity, the products were rechromatographed either in hexane/ethyl acetate as above, in methylene chloride (R_f values: XII, 0.22; II, 0.14; XV, 0.31), or in benzene (R_f values: XII, 0.13; XIII, 0.31; XIV, 0.16).

Gas chromatograms were run on a Carlo Erba gas chromatograph type Fractovap GI on a 2 m × 3.0 mm glass column filled with 3% OV-17 on Gaschrom Q, 100–120 mesh; N_2 flow, 55 mL/min; oven temperature, isothermal 160 °C; injector temperature, 210 °C; FID. The following retention times were recorded (min): II, 1.05–1.09; III, 15.09–16.05; VII, 2.90–2.98; VIII, 3.09–3.18; IX, 2.67–2.74; XI, 2.48–2.52; XII, 1.79–1.85; XIII, 3.78–3.87; XIV, 1.92–1.99; XV, 3.40–3.49; XVI, 6.03–6.09.

GC–MS couplings were run on a Carlo Erba gas chromatograph type HRGC 5160 combined with a Varian MAT CH5 mass spectrometer.

Reversed-phase HPLC was performed on a Merck-Hitachi 655A-12 system. A 0.46 × 12.5 cm Hyperchrome column filled with Shandon ODS Hypersil (5 μM, RP 18) was used. Samples were injected in methanol, and the system was run at a flow rate of 1.0 mL/min with a methanol/water gradient (20% methanol for 8 min, 20%–50% methanol in 2 min, 50% methanol for 10 min, 50%–100% methanol in 5 min, 100% methanol for 10 min). The absorbance detector was operated at 254 nm and/or 275 nm and/or 282 nm, respectively.

UV spectra were taken in methanol on a Perkin-Elmer 557 spectrometer. ^1H NMR spectra were obtained on a 400-MHz nuclear magnetic resonance spectrometer (Bruker WH 400). MS spectra were recorded on a Varian MAT CH5B spectrometer.

RESULTS

Veratryl Alcohol Oxidation. When veratryl alcohol (IX) was oxidized by LiP in the presence of oxygen, a series of products has been observed (Figure 1). Some of them have been isolated and identified (Leisola et al., 1985a; Haemmerli et al., 1987), e.g., veratraldehyde (XI), the two quinones I and II, and the γ-lactones V and VI. Shimada et al. (1987) also detected the (*Z*)-δ-lactone VII among the ring cleavage products. By GC–MS of the isolates we could also detect the lactone VII, and in addition we could tentatively identify the (*E*)-δ-lactone VIII and the 2,5-dihydroxy-4-methoxybenz-

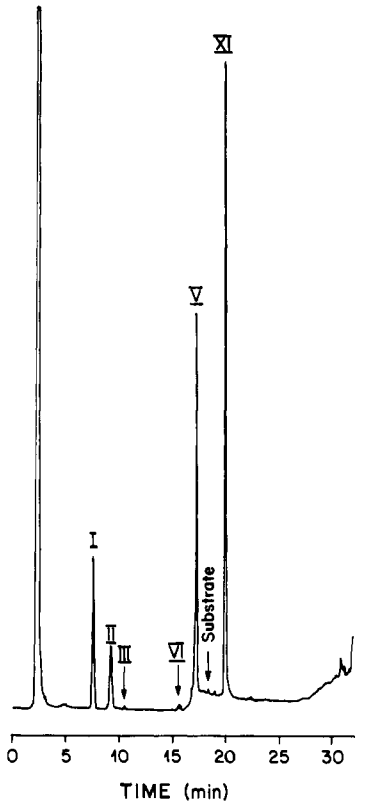

FIGURE 2: HPLC chromatogram of the reaction mixture of the LiP oxidation of veratryl alcohol.

Table I: Relative Distribution[a] of Major Products Formed by LiP Oxidation from Veratryl Alcohol (VA) and 3,4-Dimethoxybenzyl Methyl Ether (DMME) under Aerobic Conditions at pH 3.0

	major oxidation products (%)							
substrate	XI[b]	II	III	I	XV	V	XIV	XIII
VA (IX)	70.0	3.5	0.5	7.0		19.0		
DMME (XII)	53.5	3.3	2.5		9.2		30.3	1.2

[a] Calculated from HPLC data taking into account the individual UV absorptions at 254, 275, and 282 nm. [b] For structures, see Figures 1 and 5.

aldehyde (X) among the minor oxidation products. A minor polar yellow compound isolated could now be identified as 4,5-dimethoxy-3,5-cyclohexadiene-1,2-dione (III).

From HPLC data obtained (by direct injection) from samples of the reaction mixtures after consumption of the starting material (Figure 2) the relative distribution of the identified degradation products formed was calculated (Table I). Monitoring of the reaction by HPLC at different pH (3.0–5.0) did not give any indication of another product attributable to an intermediate dimethyl (hydroxymethyl)muconate (IV) and confirmed that the (E)-γ-lactone VI is a secondary product which is slowly formed from the *Z* isomer V in the reaction medium.

3,4-Dimethoxybenzyl Methyl Ether Oxidation. When DMME (XII) was subjected to the LiP oxidation under aerobic conditions at pH 3.0, HPLC and TLC analysis revealed the formation of at least six products (Figure 3). Under anaerobic conditions, however, only trace amounts of products other than veratraldehyde (XI) could be detected. Oxidation of DMME with the one-electron oxidant cerium(IV) ammonium nitrate gave comparable results. In the presence of oxygen the same series of oxidation products were formed, however, in a somewhat different proportion. In a nitrogen atmosphere, veratraldehyde was again practically the only product formed.

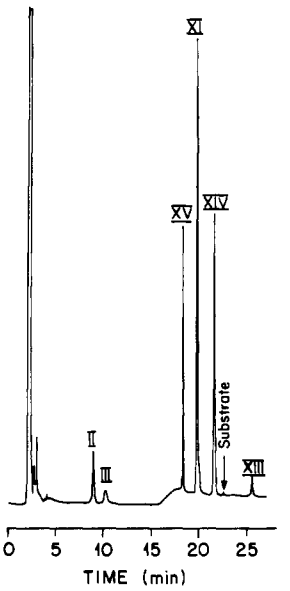

FIGURE 3: HPLC chromatogram of the reaction mixture of the LiP oxidation of 3,4-dimethoxybenzyl methyl ether.

The products of DMME oxidation were isolated from the reaction mixture by exhaustive extraction with methylene chloride. Individual compounds and/or compound mixtures were isolated from these extracts by preparative and analytical TLC. The isolates thus obtained were subsequently analyzed by ^{1}H NMR, MS, and eventually GC–MS. With the exception of the diester XIV, the identity of the products was finally confirmed by comparison of their spectra and their retention times in HPLC and GC analysis and their R_f values in TLC with authentic samples prepared by independent synthetic routes.

Veratraldehyde (XI) was the most prominent product in DMME oxidation. Two yellow compounds showing up in the polar region of HPLC chromatograms had retention times comparable to compounds also detected among the oxidation products of veratryl alcohol. The first was identified as the 2-methoxy *p*-quinone II. The second was a dimethoxy quinone for which the *o*-(III) or the *p*-quinone (XVI) structure was, in principle, possible. However, by comparison with the analytical data collected for the two reference materials the second quinone was identified as the 4,5-dimethoxy-3,5-cyclohexadiene-1,2-dione (III). A third rather prominent quinone was identified as the 2-methoxy-5-(methoxymethyl)-2,5-cyclohexadiene-1,4-dione (XV), a compound analogous to the quinone I found among the oxidation products of veratryl alcohol. Another rather prominent degradation product was identified as dimethyl 3-(methoxymethyl)-(*Z*,*Z*)-muconate (XIV) (Figure 4). The *Z* configuration of the C_4–C_5 double bond of this compound, a colorless liquid, was derived from its NMR spectrum. The coupling constant measured for the H_4–H_5 coupling was 12.3 Hz which is in agreement with coupling constants reported for muconic acid derivatives with comparable *Z* configurations (Ainsworth & Kirby, 1968). The *Z* configuration of the C_2–C_3 double bond was derived from NOE-DIFF experiments. On irradiation at the site of the methylene protons of the methoxymethyl group at 4.16 ppm small effects were observed on the methyl protons of this group at 3.40 ppm as well as on the H_4 at 7.05 ppm, and a large effect was observed on H_2 at 6.05 ppm. These observations are in agreement with a *Z* configuration of the C_2–C_3 double bond. A colorless crystalline compound showing up in the apolar region of HPLC chromatograms was identified as the methyl ester (XIII) of veratric acid.

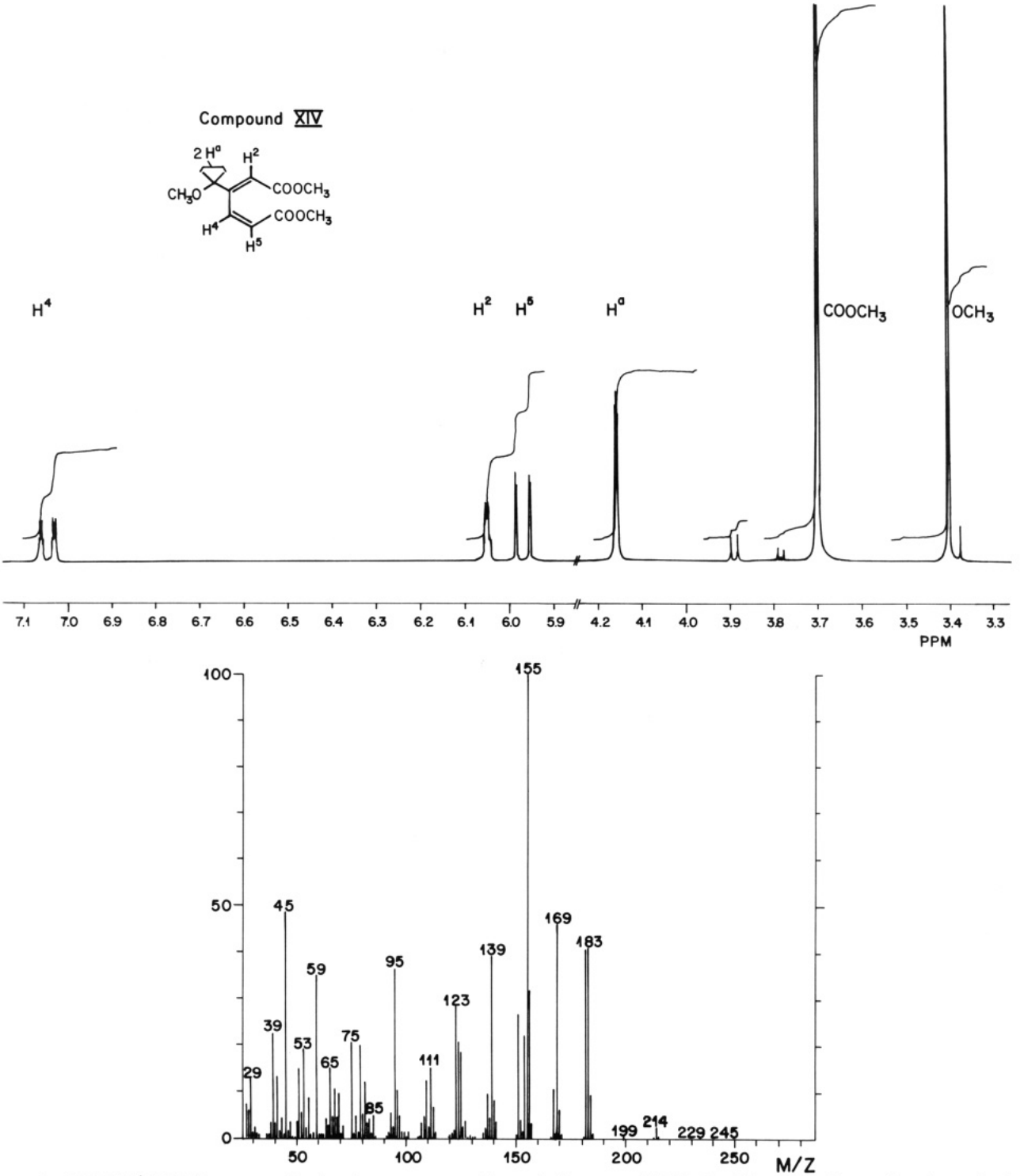

FIGURE 4: 400 MHz ^{1}H NMR spectrum (top) and mass spectrum (bottom) of compound XII isolated from the mixture of products obtained by LiP oxidation of 3,4-dimethoxybenzyl methyl ether.

The relative amounts of these degradation products formed were calculated from HPLC data obtained from samples of the reaction mixture taken after practically all starting material had been consumed. The data are presented in Table I.

Effect of pH and Mn(II) on DMME Oxidation. The effect of pH between 3.0 and 5.0 on the formation of the oxidation products was investigated. With increasing pH there was a slight decrease in the formation of the quinones II, III, and XV. The decrease in the formation of the ring cleavage compound XIV was more pronounced whereas the relative production of veratraldehyde XI seemed to be rather independent on the reaction pH. There was, however, a strong pH influence on the formation of the ester XIII, which increased with increasing pH and became the most prominent product at pH 5.0 (e.g., aldehyde/ester ratio: pH 3.0 = 1:0.37; pH 4.0 = 1:0.80; pH 5.0 = 1:1.18).

When the reactions were run in the presence of 0.2 and 1.0 mM manganese(II) sulfate, respectively, a pronounced decrease in the formation of the ring cleavage compound XIV could be observed with increasing Mn(II) content. Also a decrease in the formation of the quinones II and III was evident. These effects were more pronounced at higher pH values. The formation of the ester XIII was only slightly hampered by the presence of Mn(II) ions. At pH 3.0 the

presence of Mn(II) ions seemed to promote the formation of the quinone XV which became the most prominent product with increasing Mn(II) content. However, at pH 4.0–5.0 no such effect could be observed.

Discussion

In previous papers we have shown that veratryl alcohol is oxidized by the lignin peroxidase of *P. chrysosporium* (Leisola et al., 1985a; Haemmerli et al., 1987). In the presence of oxygen a series of degradation and oxidation products have been isolated and identified, e.g., γ-lactone esters V and VI as products of a ring cleavage reaction and quinones I and II as products of oxygenation/hydroxylation of the aromatic ring. Quinone II may only be formed from veratryl alcohol by loss of the benzylic side chain.

Here, we show that a further product of this type—the dimethoxy *o*-quinone III—is also among the primary degradation products. In addition, we could tentatively identify the δ-lactones VII and VIII and 2,5-dihydroxy-4-methoxybenzaldehyde X in the isolates obtained by preparative TLC separation. All these are minor degradation products, and up to now we cannot decide whether these compounds are primary or secondary products. The aldehyde X is the tautomeric form of quinone I and is formed from it as has been demonstrated by analyzing solutions (e.g., in methylene chloride) of compound I that have been left at room temperature under a nitrogen atmosphere for a longer period of time. Analogously, quinone I was shown to be formed from synthetically prepared aldehyde X.

Shimada et al. (1987) have shown that the (*Z*)-δ-lactone VII is also a ring cleavage product obtained by oxidation of veratryl alcohol with a crude LiP preparation from *P. chrysosporium*. They could demonstrate by ^{18}O incorporation studies that the ring cleavage includes regiospecific attack of water at the C_3 position and of dioxygen (or its active form) at the C_4 position of the 3,4-dimethoxybenzyl alcohol substrate. The lactones are then believed to be formed from a hypothetical primary ring cleavage compound, e.g., dimethyl (*Z*,*Z*)-3-(hydroxymethyl)muconate (IV).

So far, we have not been able to detect this diester in the reaction mixture. However, using diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)propane as a β-O-4 lignin substructure model, Umezawa and Higuchi (1987) were able to isolate the methyl muconate of 1,3-diethoxy-1-(4-ethoxy-3-methoxyphenyl)-2-propanol as a primary ring cleavage product of the oxidation of this model compound by the LiP. They could not, however, prove the *Z*,*Z* configuration that would be expected from the suggested mechanism of its formation (Umezawa & Higuchi, 1986, 1987a,b). The isolation of this methyl muconate indicated that the ring cleavage is not preceded by a demethylation or demethoxylation step.

If the diester IV is in fact the primary ring cleavage product from veratryl alcohol, then an isomerization around the C_2–C_3 or the C_4–C_5 double bond must precede the formation of lactone rings. We are presently studying this problem in detail. We could show that under the reaction conditions used the (*Z*)-γ-lactone V slowly isomerizes to the (*E*)-γ-lactone VI and in a similar way the (*E*)-δ-lactone VIII seems to be formed from its *Z* isomer VII. So far we have no indication that the δ-lactones might be formed from the γ-lactones under the oxidation conditions used.

The observations made when 3,4-dimethoxybenzyl methyl ether was used as a substrate in the oxidative degradation experiments with LiP essentially parallel those made already in the analogous study using veratryl alcohol as a substrate. Again, oxidation at the benzylic position is the most prominent reaction, leading to veratraldehyde as the major reaction product. However, in the presence of oxygen additional reaction pathways are operating, leading to other degradation products, e.g., quinones, methyl veratrate, and a ring cleavage product. These reactions seem to be even more preferred at pH 3.0 with the methyl ether than with the alcohol since about 45% of the degradation products are different from veratraldehyde in the case of the methyl ether compared to about 30% with the alcohol.

The formation of benzaldehydes from benzyl methyl ether lignin model compounds in LiP-catalyzed degradation experiments (Kirk et al., 1986) and of benzyl alcohols (as reduction products from the corresponding aldehydes) in degradation studies using cultures of *P. chrysosporium* (Kirk & Nakatsubo, 1983) has already been reported earlier. It has been pointed out that the aldehyde formation must involve a benzyl ether cleavage, and our results confirm the view (Kirk et al., 1986) that LiP may also cleave C_α–O linkages in lignin in this way (Figure 5). The extent of this cleavage is obviously pH dependent since oxidation at the benzylic methylene group yielding the methyl ester of veratric acid seems to be an important reaction pathway especially at higher pH values. It is known that LiP can hydroxylate the benzylic position and that it will oxidize benzylic alcohols to carbonyl compounds [compare Kirk and Shimada (1985)]. Aromatic carboxylic acids have been shown to be products of the degradation of lignin [compare Chen and Chang (1985)] and lignin model compounds by ligninolytic cultures of *P. chrysosporium* [compare Higuchi (1986)]. However, aromatic carboxylic acid formation has not been observed to occur in LiP-catalyzed reactions so far. In the present study we were able to demonstrate, for the first time, that LiP will oxidize benzylic ethers to carboxylic esters.

Furthermore, the degradation of lignin may also proceed to a considerable extent via cleavage of the benzylic side chain bond under aerobic conditions as can be concluded from the isolation of the quinones II and III.

The isolation of the (*Z*,*Z*)-3-(methoxymethyl)muconate XIV as one of the most prominent degradation products from 3,4-dimethoxybenzyl methyl ether supports the view that the cleavage of aromatic rings by LiP is an important reaction in the degradation of lignin by *P. chrysosporium* under aerobic conditions. It also confirms the results of Umezawa and Higuchi (1986, 1987a,b) that diesters of muconic acid are the primary products of the cleavage of the aromatic rings and that the ring cleavage reaction proceeds without a prior demethylation or demethoxylation step. Our results also for the first time give evidence for the *Z*,*Z* configuration of the primary ring cleavage product.

It is now generally accepted that the initial step in the LiP oxidation involves an one-electron transfer from an aromatic ring of lignin or a model compound to form an intermediate aryl cation radical (Kersten et al., 1985; Habe et al., 1985; Schoemaker et al., 1985; Renganathan et al., 1986; Palmer et al., 1987; Haemmerli et al., 1987). Our observations that the reaction of 3,4-dimethoxybenzyl methyl ether with the one-electron oxidant cerium(IV) ammonium nitrate strongly parallels the oxidation pattern by LiP supports the view also that the LiP-catalyzed reaction proceeds via one-electron oxidation steps.

The intermediate cation radical XVIII (Figure 5) may then react in different ways. It may react with H_2O to incorporate a hydroxyl group, or it may lose a proton to yield in both cases carbon-centered radicals (e.g., XIX–XXII). These radicals may then split off methanol, they may react with dioxygen

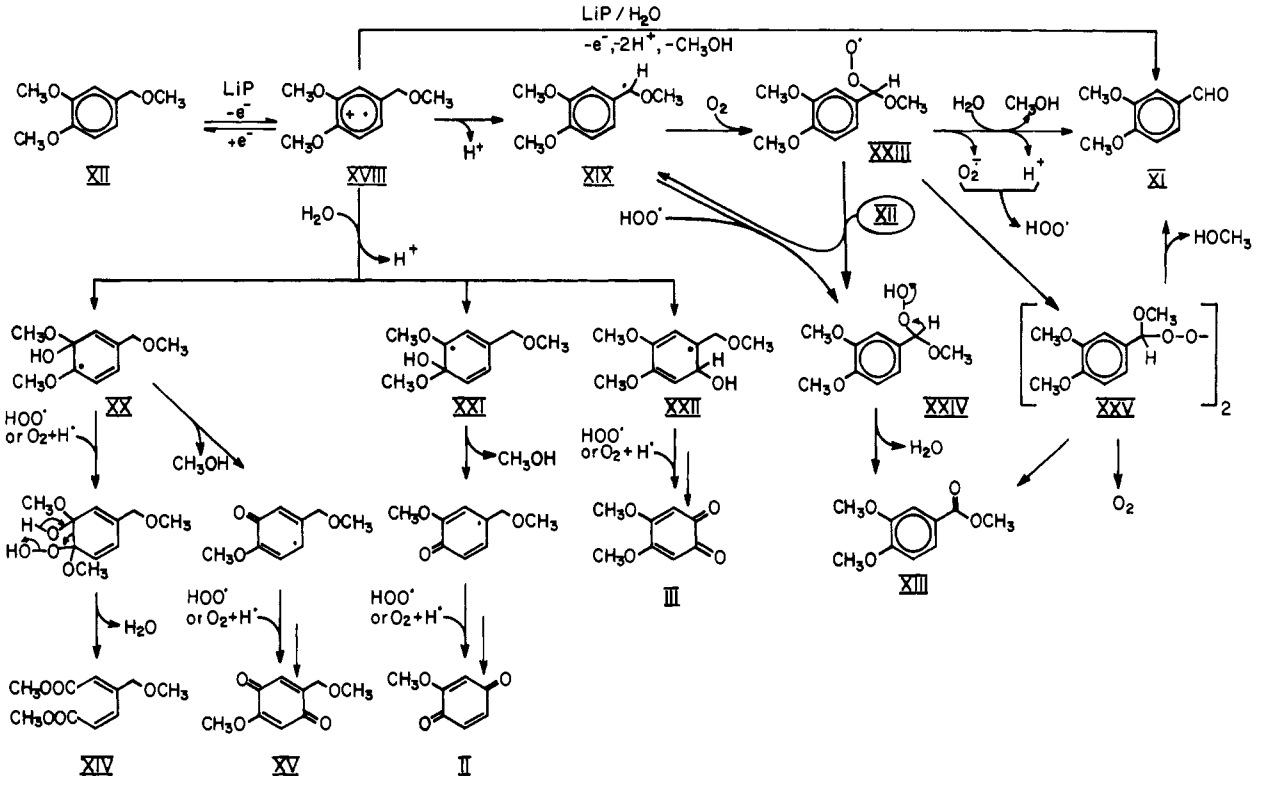

FIGURE 5: Hypothetical mechanisms for the formation of the degradation products of 3,4-dimethylbenzyl methyl ether.

or with hydroperoxy radicals to form peroxy radicals and/or hydroperoxides, or they may be oxidized by a second one-electron transfer to give the products observed (Kersten et al., 1985; Habe et al., 1985; Schoemaker et al., 1985; Renganathan et al., 1986; Palmer et al., 1987). Hydroperoxides may also be formed from peroxy radicals by hydrogen abstraction from, e.g., the benzylic position of 3,4-dimethoxybenzyl methyl ether (XII), thus regenerating a benzylic radical XIX (Palmer et al., 1987) as outlined in the formation of the hydroperoxide XXIV from the peroxy radical XXIII (Figure 5). Under the acidic conditions used, the hydroperoxide XXIV will decompose to yield the ester XIII.

Starting from the peroxy radical XXIII, a third pathway to the ester XIII may be envisaged. Coupling of two peroxy radicals will give a tetroxide XXV which will undergo fragmentation to give dioxygen, the ester XIII, and a hemiacetal which will yield the aldehyde XI after loss of methanol. A mechanism of this type has been proposed (Renganathan et al., 1986) for the LiP degradation of a diarylpropanediol model compound. The fact that the aldehyde XI/ester XIII ratio observed is close to—but different from—1:1 (e.g., 1:0.80 and 1:1.18) at pH 4–5 may be in support of such a mechanism but also shows that additional reaction pathways must be operating. The results that we obtained in the analogous study of the aerobic degradation of veratryl alcohol are, however, not in favor of such a mechanism since no formation of veratric acid—expected in the fragmentation of the respective tetroxide (Russell, 1957)—could be observed. Our findings rather suggest that an oxygen activation mechanism is operating (Haemmerli et al., 1987). The observations made in the present study using the corresponding benzyl methyl ether as a substrate—especially in the formation of the ring cleavage product XIV—may also be interpreted by similar mechanisms involving hydroperoxy radicals generated in the formation of veratraldehyde XI (see Figure 5). However, several oxidative pathways seem to be involved in the formation of the observed products, and additional studies will be necessary to substantiate the proposed mechanisms. Further work in this direction is in progress.

Registry No. I, 50827-56-0; II, 2880-58-2; III, 21086-65-7; IV, 19804-73-0; V, 111093-43-7; VI, 111093-42-6; VII, 115044-07-0; VIII, 118297-95-3; IX, 93-03-8; X, 29865-97-2; XI, 120-14-9; XII, 3840-28-6; XIII, 2150-38-1; XIV, 118297-96-4; XV, 118297-97-5; XVII, 118297-98-6; LiP, 42613-30-9; Mn, 7439-96-5; cerium(IV) ammonium nitrate, 16593-75-2; lignin, 9005-53-2.

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Mechanism of GTP Hydrolysis in Tubulin Polymerization: Characterization of the Kinetic Intermediate Microtubule–GDP–P_i Using Phosphate Analogues

Marie-France Carlier,* Dominique Didry, Colette Simon, and Dominique Pantaloni

Laboratoire d'Enzymologie du CNRS, 91198 Gif-sur-Yvette, France

Received July 14, 1988; Revised Manuscript Received October 5, 1988

ABSTRACT: Beryllium fluoride (BeF_3^-) has previously been shown to bind tightly to microtubules as a structural analogue of P_i and to mimic the GDP–P_i transient state in tubulin polymerization [Carlier, M.-F., Didry, D., Melki, R., Chabre, M., & Pantaloni, D. (1988) *Biochemistry 27*, 3555–3559]. The interaction of BeF_3^- with tubulin is analyzed here in greater detail. BeF_3^- binds to and dissociates from microtubule GDP subunits at very slow rates ($k_+ \simeq 100\ M^{-1}\ s^{-1}$; $k_- \simeq 6 \times 10^{-4}\ s^{-1}$), suggesting that a slow conformation change of tubulin, linked to the stabilization of the microtubule structure, follows BeF_3^- binding. The possibility is evoked that BeF_3^- acts as a transition-state analogue in the GTPase reaction of tubulin. BeF_3^- does not bind to dimeric nor to oligomeric GDP–tubulin with high affinity. Substoichiometric binding of BeF_3^- to microtubules provides extensive stabilization of the structure. An original mechanistic model that accounts for the data is proposed. The kinetic parameters for microtubule elongation in the presence of GTP– and GDP–tubulin with and without BeF_3^- have been determined. Data support the following views: (i) Microtubules at steady state and in a regime of slow growth in the presence of GTP are stabilized by a cap of GDP–P_i subunits functionally similar to GDP–BeF_3 subunits. (ii) In the presence of BeF_3^-, microtubules elongate from GDP–tubulin within the following sequence of reactions: initial nonproductive binding of GDP–tubulin to microtubule ends is followed by the binding of BeF_3^- and the associated conformation change allowing sustained elongation.

The hydrolysis of tightly bound GTP that accompanies tubulin polymerization occurs on microtubules, following the incorporation of GTP subunits in the polymer (Weisenberg et al., 1976; Carlier & Pantaloni, 1981). GTP hydrolysis follows the elongation process closely when the rate of growth is slow, at low tubulin concentration and close to steady state; stretches of terminal GTP subunits develop at high tubulin concentration when the microtubule grows at a rate faster than the intrinsic rate of GTP hydrolysis (40 s^{-1}; Carlier et al., 1987a). In a regime of slow growth, terminal subunits dissociate from microtubules at a rate of 2 orders of magnitude slower than internal GDP subunits, therefore forming a stabilizing "cap" (Carlier, 1982; Hill & Carlier, 1983; Carlier et al., 1984a), initially thought to be a GTP cap. The pos-

0006-2960/89/0428-1783$01.50/0