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FURTHER STUDY DISCOUNTS ROLE FOR SINGLET OXYGEN IN FUNGAL DEGRADATION OF LIGNIN MODEL COMPOUNDS

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SUMMARY: This study reexamined our contention that singlet oxygen ($^1O_2$) plays a role in the fungal degradation of lignin (BBRC 102(1981)484). Cultures of Phanerochaete chrysosporium and a photochemical $^1O_2$-generating system (riboflavin/light/O_2) cleaved a lignin substructure model compound, 1,2-bis(4-methoxyphenyl)propane-1,3-diol (I), by indistinguishable mechanisms. However, the rate of cleavage of I in D_2O was the same as in H_2O in the photochemical $^1O_2$-generating system, indicating that $^1O_2$ was not involved. Furthermore, products formed from I in a chemical system for generating $^1O_2$ (H_2O_2 + NaOCl) differed from those produced by cultures or the photochemical system. It is concluded that $^1O_2$ is not responsible for cleavage of I or related compounds in the fungal cultures or in the photochemical system.

Although lignin is degraded efficiently by basidiomycetous fungi, the biochemical mechanism is largely unknown (1-4). Past research has provided strong evidence that lignin is attacked by extracellular non-specific oxidizing agents (4,5), which has led to the hypothesis that active oxygen species rather than specific enzymes are involved (6).

We recently presented evidence indicating that singlet oxygen, $^1O_2$, is involved in the fungal metabolism of lignin (7), as it has been proposed to be in several other biological processes (8). Our evidence was as follows: a) photochemical $^1O_2$-generating system and ligninolytic cultures of

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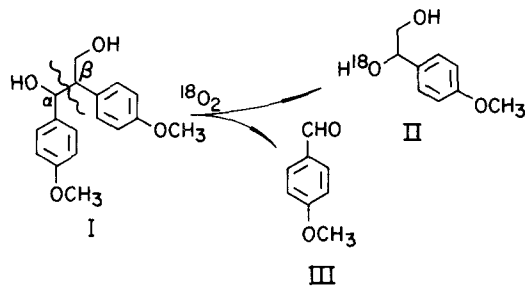

Figure 1.--Oxygenative cleavage between C_α and C_β initiates degradation of β-1 model compounds by Phanerochaete chrysosporium. $^{18}O_2$ is incorporated into the phenylglycol product as shown (10,11). (M153017)

Phanerochaete chrysosporium both cleaved a lignin substructure model compound of the nonphenolic β-1 (1,2-diarylpropane-1,3-diol) type, yielding the same products; b) a $^1O_2$ scavenger, anthracene-9,10-bisethanesulfonate (9), inhibited degradation of the model by cultures and by the photochemical system; c) the scavenger inhibited lignin oxidation but not glucose oxidation by the cultures; and d) UV fluorescence and long-wave UV absorbance of the scavenger were reduced in cultures, in accord with reaction with $^1O_2$.

Investigations (10,11) of the fungal degradation of lignin substructure model compounds of the nonphenolic β-1 type have shown initial cleavage between C_α and C_β. Under $^{18}O_2$, the cultures cleave the compounds with incorporation of ^{18}O into the diol product (Figure 1). Deuterium atoms at C_α and C_β are retained in the cleavage products (11).

The purpose of the present study was to investigate further the possible involvement of $^1O_2$ in this cleavage by examining a) $^{18}O_2$-incorporation and deuterium retention patterns during cleavage of I in the photochemical system riboflavin/light/O_2 (R/hν/O_2), b) the effect of D_2O on the rate of cleavage of I in the photochemical system, and c) the fate of β-1 model compound I in the presence of $^1O_2$ generated chemically.

MATERIALS AND METHODS

Experiments with ligninolytic cultures of Phanerochaete chrysosporium. Procedures are described elsewhere (11).

Chemicals. 1,2-Bis(4-methoxyphenyl)propane-1,3-diol (I) and the corresponding C_α-deuterated and C_β-deuterated compounds were prepared from desoxyanisoin (11).

Diol II was prepared from 4'-hydroxyacetophenone (11). Anisaldehyde (III), vanillin and methoxyhydroquinone were purchased from Aldrich Chemical Co., Milwaukee, Wis. Anthracene-9,10-bis-ethanesulfonic acid (AES) was prepared earlier (7).

Photochemical reactions. These were conducted at 45-50° C in 10 ml of poly(acrylic acid) buffer, 0.01 M in carboxyl, pH 4.5, in 125-ml Erlenmeyer flasks under an atmosphere of O_2, with 300 μM riboflavin (Sigma) (Chemical Co., St. Louis, Mo)., as photosensitizer. Compound I, 10% in N,N-dimethylformamide (DMF), was added to H_2O to give a solution for addition to the flasks (0.5 ml, 11 μmol/flask). Flasks were illuminated with two 15-watt fluorescent bulbs (Westinghouse "Cool White," 45 cm long) at approximately 20 cm. Comparison of H_2O and D_2O as solvent was in 0.01 M acetate buffer, pH 4.5, and with 22 μmol compound I. Reaction under $^{18}O_2$ was in the polymeric buffer in a 50-ml Warburg flask; compound I (11 μmol) was added from a sidearm after the flask and contents had been evacuated and filled (1 atm.) with $^{18}O_2$ (97%, KOR Inc., Cambridge, Mass.).

After reaction times as noted, the solutions were extracted with ethyl acetate, dried over Na_2SO_4, and solvents evaporated off under vacuum. The extracts were examined by thin layer chromatography (tlc; 10,11) or dissolved in 50 μl DMF to which 50 μl of bistrimethylsilyltrifluoroacetamide containing 1% trimethyl chlorosilane (Regis Chemical Co., Morton Grove, Ill.) was added. The latter solutions were heated (~50° C for ~1 min), and the resulting trimethylsilyl derivatives examined by gas chromatography (GC) or GC/mass spectrometry (GC/MS). In some experiments tetracosane was added as internal standard for quantification. All identifications were based on GC/MS.

Chemical generation of singlet oxygen (12). Compound I (50 μmol in DMF/H_2O as above) was added to 25 ml of 100 mM phosphate buffer, pH 5.0,and 17 ml of 3% H_2O_2 added. Sodium hypochlorite (925 mg; 12.4 mmol) in 20 ml H_2O was added dropwise over 40-60 min to the stirred solution. The mixture was extracted and examined as above. The same procedure was used with vanillin as substrate (cf. 12).

GC and GC/MS. GC was with a 2-m column packed with 3% OV 101 on Ultrabond 20 M, 100/200 mesh, and a Hewlett-Packard 5750 instrument (11). GC/MS employed a Finnigan 4500 instrument operated at 70 eV and equipped with a 180-cm x 2-mm i.d. glass column packed with OV-1 on Chromosorb W, 60-80 mesh. Operating parameters: He, 20 ml/min; column T = 165° C (compound III) or 180° C (compound II as TMS derivative); injection T = 225° C; transfer line T = 235° C.

RESULTS AND DISCUSSION

Both the riboflavin/light/O_2 (R/hν/O_2) system and the fungal cultures cleaved compound I with formation of 1-(4-methoxyphenyl)-ethane-1,2-diol (II) and anisaldehyde III. The diol was further oxidized in both systems to form 2-hydroxy-1-(4'-methoxyphenyl)-1-keto-ethane (benzyl alcohol oxidation) and anisaldehyde (intradiol cleavage) (see 10). In the cultures, anisaldehyde was

Table 1.--$^{18}O_2$-incorporation and deuterium retention in products formed on cleavage of compound I in fungal cultures and in a photochemical system

Oxidizing system	$^{18}O_2$ incorporation in diol II (% of diol)	Deuterium in diol II (C_β-D retention) (% of II)	Deuterium in anisaldehyde III[1/] (C_α-D retention) (% of III)
Ligninolytic cultures	89	100	[2/]46
Riboflavin/light/O_2	82	96	58

1/ In both systems, anisaldehyde (III) is formed from both aromatic moieties of compound I, directly from the C_α-linked nucleus, and indirectly by further degradation of diol II from the C_β-linked nucleus. Consequently, complete retention of C_α-D is reflected in a 50% or greater yield deuterated anisaldehyde.

2/ Aldehyde III is rapidly reduced in cultures to anisyl alcohol. Deuterium retention was evaluated using the alcohol (11).

reduced to anisyl alcohol. Other products were not apparent in either system (tlc, GC). The R/hν/O_2 and fungal systems gave very similar $^{18}O_2$-incorporation and deuterium-retention patterns in the degradation of compound I (Table 1).

When the R/hν/O_2 reaction was done in D_2O there was no rate enhancement over that in H_2O (Figure 2). Enhancement of the rates of $^1O_2$ reactions (often tenfold) is considered to be diagnostic for $^1O_2$ involvement (13). Thus,

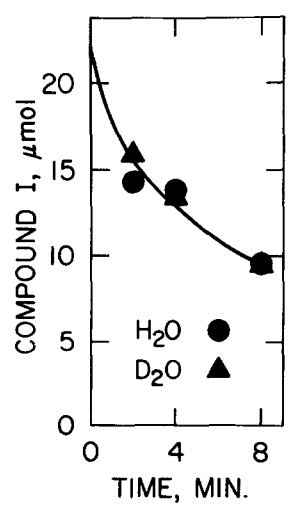

Figure 2.--Oxidation of compound I by the riboflavin/light/oxygen system in D_2O and in H_2O. (M153016)

although the R/hν/O_2 system mimics the fungal system, $^1O_2$ is not involved in the former, as we had earlier concluded (7).

The $^1O_2$-generating system H_2O_2/sodium hypochlorite (12,14) degraded compound I (26%), but the diol and aldehyde products observed in cultures (Fig. 1) and in the R/hν/O_2 system were not detected (tlc, GC). Products were not pursued. Using identical conditions, vanillin was oxidized to methoxyhydroquinone, a reaction attributed to $^1O_2$ (12). We conclude that $^1O_2$ is not responsible for cleavage of β-1 model compounds.

In light of these findings, our earlier results with the $^1O_2$ scavenger AES must be reinterpreted. Obviously AES can interfere with the true oxidative cleavage agent(s) in both the fungal cultures and the R/hν/O_2 system. Whether the biological and photochemical systems employ the same or similar oxidizing agent is not clear. In this regard, we have noted that lignin is rapidly depolymerized in the fungal cultures (15) but not in the R/hν/O_2 system.

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