Vol. 102, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 16, 1981 Pages 484-491

INVOLVEMENT OF SINGLET OXYGEN IN THE FUNGAL DEGRADATION OF LIGNIN

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Received July 27,1981

SUMMARY: The possible involvement of singlet oxygen ($^1O_2$) in the degradation of lignin by Phanerochaete chrysosporium was examined. Ligninolytic cultures and photochemically generated $^1O_2$ gave the same oxidation products from the lignin substructure model compound 1,2-bis(3-methoxy-4-alkoxyphenyl)propan-1,3-diol. Fluorescence and near UV absorbance of the specific $^1O_2$ trapping agent anthracene-9,10-bisethanesulfonic acid (AES) disappeared in ligninolytic cultures, indicating that $^1O_2$ was produced. AES strongly inhibited oxidation of ^{14}C-lignin, but not ^{14}C-glucose, to $^{14}CO_2$ in cultures, and also strongly suppressed oxidation of the model compound. These results indicate the $^1O_2$ plays an integral role in lignin biodegradation.

INTRODUCTION

The complex natural aromatic polymer lignin is metabolized efficiently by higher basidiomycetous fungi. Research on this centrally important biodegradative process has accelerated greatly in recent years, and knowledge of its chemistry and physiology has improved considerably. The biochemistry involved, however, has remained largely a mystery (1-7).

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Non-standard abbreviations: AES, anthracene-9,10-bisethanesulfonic acid; $^1O_2$, singlet oxygen.

0006-291X/81/170484-08$01.00/0

Nevertheless, chemical and spectroscopic characterizations of lignin after fungal attack have shown that the polymer-degrading system is largely oxidative (8,9,10), which means that an extracellular oxidizing system is involved (10). The kinds of reactions indicated are those generally catalyzed by oxygenases, including monooxygenases which require NAD(P)H. It is unlikely that fungi would secrete such coenzymes (10). On the basis of the above considerations, it has been hypothesized that the actual polymer-attacking entity might be an active oxygen species such as superoxide anion rather than an enzyme (11). An earlier demonstration of H_2O_2 in cultures of ligninolytic fungi had led to the similar conjecture that H_2O_2 might be involved in lignin degradation (12). No direct evidence in support of these speculations has been presented. The very low specificity of the ligninolytic system, however, increases the attractiveness of the hypothesis that the initial reaction does not occur on an enzyme surface (6).

A consideration of the reactions in the degradation of lignin model compounds by ligninolytic cultures of the wood-decomposing basidiomycete Phanerochaete chrysosporium Burds. (13-18) suggested to us that several might be effected by singlet oxygen. This excited state of the oxygen molecule reacts more rapidly with organic materials than does ground state (triplet) oxygen (19,20), and has been implicated in several biological systems (21).

Consequently, the effects of $^1O_2$ and P. chrysosporium on a lignin model compound were compared, the ability of P. chrysosporium to produce $^1O_2$ was assessed, and the effect of a specific $^1O_2$ scavenger on oxidation of the lignin model compound and of lignin itself by P. chrysosporium were determined.

MATERIALS AND METHODS

Cultures. Cultures (10 ml/125-ml Erlenmeyer) of P. chrysosporium ME 446 (ATCC 35540) were grown at 39° C under O_2 in a nitrogen-limiting, glucose, dilute mineral salts medium (22,23). Cultures 4 days old and older could oxidize ^{14}C-lignin to $^{14}CO_2$ (i.e. they had become ligninolytic (23)), and were used when 6-7 days old.

Chemicals. 1,2-Bis(3-methoxy-4-hydroxyphenyl)-propan-1,3-diol (I') was synthesized by the procedure used for a closely related compound (24). Ethylation of I' with diazoethane in 20% CH_3OH in CH_2Cl_2 yielded I. Similar ethyla-

tion of vanillin and vanillyl alcohol yielded II and III, respectively. Ia was prepared by methylating I' at 0°C with $^{14}CH_3I$/NaH in DMF. Specific activity of Ia was 2.4 x 10^9 dpm/mmole. IV was synthesized as follows: 4-Hydroxy-3-methoxymandelic acid (Aldrich, Milwaukee) was ethylated as above, the product reduced with $NaBH_4$ in CH_3OH, and the resulting phenylglycol oxidized to IV with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone in dioxane. The structure was confirmed by ^1H NMR. Anthracene-9,10-bisethanesulfonic acid (AES) disodium salt was synthesized from 9,10-dibromoanthracene (25); its structure was confirmed by ^1H NMR. Riboflavin was obtained from ICN (Cleveland), rose bengal from Allied Chemicals (New York), xanthine and xanthine oxidase from Sigma (St. Louis), [6-^{14}C]glucose (10 mCi/mmole) from Cal Atomic (Los Angeles), and $^{14}CH_3I$ (30 mCi/mmole) from ICN (Irvine). [Ring-U-^{14}C]lignin (9.2 x 10^5 dpm/mg) had been synthesized previously (23).

Metabolism of I and Ia. Compound I was added to cultures as an aqueous solution (5 mg/culture), and the flasks were flushed with O_2, sealed and incubated at 39° C (17). Metabolites were extracted from whole cultures (17) and separated by thin-layer chromatography (TLC) on silica gel 60 F_{254} aluminum plates (Merck) with 5% CH_3OH in CH_2Cl_2 as solvent. Spots were detected under short-wave ultraviolet (UV) light, by spraying with a solution of 2,4-dinitrophenylhydrazine/HCl, or by heating after dipping the plates in 5% phosphomolybdic acid in ethanol. Structures of the isolated metabolites were confirmed by mass spectrometric comparison with authentic samples. Residual Ia and its metabolites, extracted from cultures, separated by TLC and located (UV), were quantified by scintillation counting after placing cut-up plate pieces directly in a scintillation cocktail (10g 2,5-diphenyloxazole, 100 mg 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene and 100g naphthalene/l dioxane).

Photochemical generation of singlet oxygen. Reactions were performed at 45-50° C in 10 ml of 0.01M poly(acrylic acid) buffer, pH 4.5, in 125-ml Erlenmeyer flasks, with stirring, under an atmosphere of O_2, with 300 µM riboflavin as photosynthesizer(26,27). The flasks were illuminated with two 40-watt incandescent bulbs from a distance of approximately 20 cm. Compounds to be photo-oxidized were added as 0.5 ml of solutions in DMF/H_2O(1/4). Illumination was for 0.5 to 2 hours, and products were extracted and examined as with cultures.

Measurement of lignin degradation. Cultures each received 100 µg of ^{14}C-lignin as an aqueous suspension; they were flushed periodically with O_2, and evolved $^{14}CO_2$ trapped and counted (24).

RESULTS

Degradation of I by P. chrysosporium and by $^1O_2$. Compound I was degraded in ligninolytic cultures to three major products (Figure 1). Traces of other products were observed by TLC and will be reported later. II was reduced to III when added to cultures. The same products were formed from I on incuba-

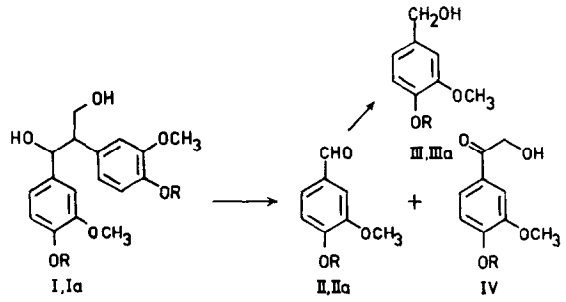

Figure 1.--Products formed from compounds I and Ia. I, II, III, IV:R = CH_2CH_3; Ia, IIa, IIIa:R = $^{14}CH_3$.

tion in the $^1O_2$-generating system, except that the aldehyde II was not reduced to the alcohol III. Most minor products co-chromatographed with those from the cultures. In the absence of light or riboflavin no reaction of I occurred in the system; only a slight reaction (formation of II) occurred in the system under He instead of O_2.

Production of $^1O_2$ by P. chrysosporium. When added to ligninolytic cultures, the $^1O_2$ scavenger AES disappeared. This was easily followed in the intact cultures by the disappearance of fluorescence under long-wave UV light, and was verified by the disappearance of the characteristic absorbance peaks at 360, 378, and 400 nm in the culture fluid. Heat-killed cultures did not affect AES. AES disappeared rapidly in the photochemical system.

Inhibition of oxidations by AES. Addition of AES to the $^1O_2$-generating systems markedly inhibited degradation of Ia (Table 1).

AES had no effect on the oxidation of ^{14}C-glucose to $^{14}CO_2$, but strongly inhibited the oxidation of ^{14}C-lignin to $^{14}CO_2$; after the AES had been depleted, the rate of lignin oxidation increased (Figure 2).

DISCUSSION

The facts that a) the $^1O_2$-generating system and ligninolytic cultures of P. chrysosporium gave the same products from compound I, and b) the $^1O_2$ scavenger AES disappeared in the ligninolytic cultures (as well as in the riboflavin/light/O_2 system) provide evidence that P. chrysosporium produces

Table 1.--Effect of the $^1O_2$ scavenger AES on oxidation of Ia

Oxidizing system	% of Ia converted to IIa + IIIa[1/]	
	-AES	+AES[2/]
Riboflavin/light[3/]	65.2	1.2
Ligninolytic cultures[4/]	24.3	5.7

1/ One mg (7×10^6 dpm) of Ia was added to the 10-ml oxidizing systems. After incubation and extraction, residual Ia and products were separated by TLC and quantified (Materials and Methods). Principal products were IIa (riboflavin system) and IIIa (cultures).

2/ 2.2 μmoles of the sodium salt of AES was added in 0.5 ml of water.

3/ Illuminated for 0.5 hours.

4/ Seven-day old cultures, incubated at 39°C for 4 hours under an atmosphere of O_2.

$^1O_2$. AES was first synthesized by Botsivali and Evans (25) as a specific, water-soluble scavenger of $^1O_2$, with which it reacts to form the 9,10-endoperoxide (25). Disappearance of fluorescence and of the absorbance peaks at 360, 378, and 400 nm was consistent with formation of the endoperoxide. Work is underway to isolate and characterize this expected product.

The possibility was considered that the degradation of I in the photochemical system was not due to $^1O_2$. The riboflavin/light system reportedly generates not only $^1O_2$ when O_2 is present, but also superoxide, $O_2^{\cdot-}$ (26). In addition, the system exhibits hydrogen atom-abstracting activity (27). Substitution of rose bengal for riboflavin reportedly provides a cleaner supply of $^1O_2$ (27), and was found to give the same products from I, albeit at a lower rate. A xanthine/xanthine oxidase system, which produces $O_2^{\cdot-}$ (26), did not affect I. These results with $O_2^{\cdot-}$ are not suprising; it is a very weak oxidant (28). The absence of a significant reaction in the helium system ruled out a direct reaction of I with riboflavin in the presence of light. Finally, the $^1O_2$ scavenger AES suppressed degradation of I in the photochemical system.

Our results provide two independent indications that $^1O_2$ is involved in lignin degradation by P. chrysosporium. First, the products formed from the

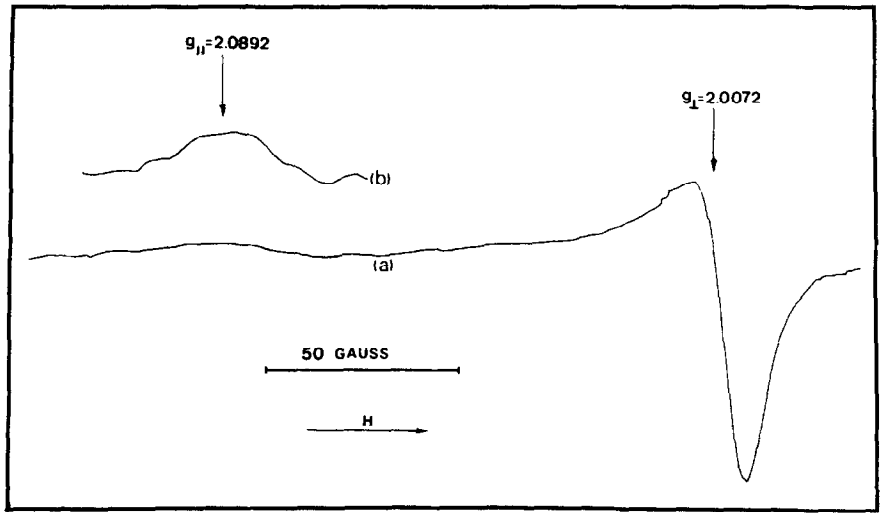

Fig. 4 EPR. spectra of $O_2^{\cdot -}$ generated on the addition of 10 µl of 0.5M NaOH/ml of DMSO. Spectra were performed at 130.K with a microwave frequency of 9.199 GHz, 1.6 Gauss modulation and 2.5mW microwave power. (a) gain = 5×10^4 ; (b) gain = 2.5×10^5.

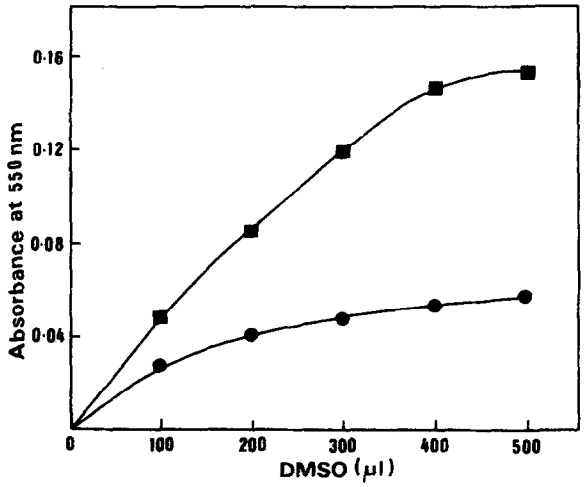

Fig. 5 SOD inhibitable reduction of cytochrome c by $O_2^{\cdot -}$ generated by OH^- in DMSO. $O_2^{\cdot -}$ was prepared by addition of 10 µl of 0.5M NaOH/ml of DMSO. These solutions were stored in tightly stoppered bottles for thirty minutes before use (to prevent H_2O absorbtion). Various volumes were added to buffer containing cytochrome c, (see methods). ●——● + SOD ; ■——■ − SOD.

that many man-made and other compounds considered to be recalcitrant might actually be biodegradable. The question of how $^1O_2$ is produced in biological systems has been discussed by Krinsky (21,32). Studies are in progress to determine its origin in P. chrysosporium, and the effects of $^1O_2$ on other substructure models. Results already have shown that 1-(3-methoxy-4-ethoxyphenyl)-2-(4-formyl-2-methoxyphenoxy)propan-1,3-diol is oxidized in the photochemical system with formation of II. Also being pursued is the relationship of the present results to the powerful, dual role that O_2 plays in lignin degradation by P. chrysosporium (22), and to the unusual physiological features of ligninolytic activity (4,6) in this organism.

ACKNOWLEDGEMENTS

The authors thank Mr. Michael Mozuch for expert technical assistance, and Mr. John Ralph for bringing AES to their attention. F. N. was supported by a U.S.-Japan Cooperative Research grant from the Japan Society for the Promotion of Science and the U.S. National Science Foundation. I. R. was on Professional Development Leave from the National Research Council of Canada.

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