Vol. 118, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 30, 1984 Pages 437-443

FATTY ACYL-COENZYME A OXIDASE ACTIVITY AND H_2O_2 PRODUCTION IN PHANEROCHAETE CHRYSOSPORIUM MYCELIA

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Received November 21, 1983

Mycelia of the lignin-degrading fungus Phanerochaete chrysosporium consume O_2 and produce extracellular H_2O_2 when incubated with fatty acyl-CoA substrates, even in the presence of mitochondrial respiratory chain inhibitors such as antimycin A and cyanide. These results suggest the possibility that peroxisomal fatty acyl-CoA oxidase activity in P. chrysosporium mycelia may be an important metabolic source for the extracellular H_2O_2 believed to be involved in lignin biodegradation.

Accumulating evidence strongly suggests that hydrogen peroxide (H_2O_2) plays a key role in the mechanism of fungal lignin degradation. In Phanerochaete chrysosporium Burds., the most extensively studied lignin-degrading fungus, lignolytic activity is temporally and physiologically correlated with the ability of the organism to produce extracellular H_2O_2 (1-3), and the production of H_2O_2 is enhanced when lignin is present (4). Recently, Tien and Kirk (5) isolated a protein from concentrated P. chrysosporium culture media which cleaves certain linkages in lignin and lignin model compounds, but only in the presence of H_2O_2.

The metabolic source of H_2O_2 excreted by P. chrysosporium mycelia is not known. Lignin degradation apparently requires molecular oxygen and is stimulated by high partial pressures of O_2 in a manner not associated with a stimulation of growth (6). Furthermore, it has been shown that ^{18}O is incorporated into cleavage products of lignin model compounds when the model compounds are incubated with lignolytic P. chrysosporium cultures under $^{18}O_2$ (7). These observations suggest that O_2 is acting as a substrate for the lignin degradative system, possibly for the formation of H_2O_2. One of the more active H_2O_2-producing pathways found in cells is the fatty acyl-CoA oxidizing system of

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peroxisomes and glyoxysomes (8-10). Because fatty acid β-oxidation in these organelles is catalyzed by a flavin-linked enzyme, which reduces O_2 directly to H_2O_2 rather than transferring electrons into the mitochondrial electron transport chain (Fig. 1), peroxisomal acyl-CoA oxidation is insensitive to inhibitors of mitochondrial respiration.

Fatty acids are a major form of energy storage in many fungi (11), and thus could form a convenient substrate reserve for generating H_2O_2 to be used extracellularly in the degradation of lignin. In an effort to identify possible metabolic sources of H_2O_2 in lignolytic organisms, we examined P. chrysosporium for the existence of an acyl-CoA oxidizing system. In this paper we describe antimycin A and cyanide-insensitive, fatty acyl-CoA-dependent H_2O_2 production in P. chrysosporium mycelia.

MATERIALS AND METHODS

Cultures of P. chrysosporium Burds. were grown on limited nitrogen (0.6 mM NH_4NO_3 and 0.6 mM asparagine) following procedures previously described (6,12). The mycelial mat obtained after 10-14 days of growth was broken into smaller pieces with a Waring Blendor and the mycelial pieces were washed according to a procedure described earlier (4). Endogenous substrate pools were depleted by starving the washed mycelial suspension overnight at room temperature. Starved mycelia were harvested by centrifugation (20,000 x g, 5 min). The resulting pellet was gently resuspended in 50 ml of 0.4 M KCl, 20 mM $NaHPO_4$ (pH 6.2). Changes in O_2 concentration of the mycelial preparation were monitored polarographically in a thermostatted (30°C), water-jacketed cell (Gilson Medical Electronics) using a Clark oxygen electrode connected to a strip chart recorder.

Hydrogen peroxide production by the mycelial preparation was monitored as the peroxidation of methanol to form formaldehyde (13). The reaction mixture (0.5 ml, total volume) contained starved mycelial preparation, 0.3 M methanol, 20 μg catalase and various substrates and cofactors as indicated in the text. Reactions were incubated at 37°C for 3 hr and subsequently centrifuged at 6000 x g for 15 min. A 0.45-ml aliquot of the supernatant was removed and acidified with 15 μl 3 M trichloroacetic acid. Occasionally residual cloudiness was observed in the supernatant and was removed by centrifugation at 175,000 x g for 5 min. Formaldehyde formation was determined using the procedure of Nash (14) by adding 0.45 ml of 2 M ammonium acetate, 0.1 M acetic acid, and 0.04 M acetylacetone (pH 6.2) to the supernatant. After 4 hr at room temperature the absorbance of the resulting yellow color was measured at 412 nm with a Bausch and Lomb Spectronic 2000 spectrophotometer.

Protein concentration was determined by the method of Lowry et al. (15), after disrupting the mycelia with a Branson model S125 sonifier. Fatty acyl-coenzyme A's, bovine serum albumin, antimycin A, catalase, NAD, nicotinamide and triton X-100 were obtained from Sigma. All other chemicals used were of reagent grade.

RESULTS

The molecular mechanism of fatty acyl-CoA oxidase is thought to involve the transfer of electrons from fatty acyl-CoA through a prosthetic flavin moiety to O_2, forming enoyl-CoA and H_2O_2 (Fig. 1). This reaction is characterized by O_2 consumption occurring independently of the mitochondrial respiratory chain. When starved P. chrysosporium mycelia were incubated in the presence of KCN (to inhibit mitochondrial cytochrome oxidase activity), O_2 consumption was observed following the addition of stearoyl-CoA to the suspension (Fig. 2). Once the O_2 concentration reached an apparent steady state, the addition of catalase resulted in a brief burst of O_2 evolution. More O_2 was evolved from the mycelial preparation incubated with added stearoyl-CoA (upper trace) than from the mycelial preparation incubated without added stearoyl-CoA (lower trace), indicating that at least some of the O_2 consumed by the mycelia upon addition of stearoyl-CoA had been converted into H_2O_2.

The amount of H_2O_2 produced by starved P. chrysosporium mycelia in the presence of various substrates and inhibitors is shown in Table 1. In these experiments, H_2O_2 production was assayed as catalase-mediated formaldehyde production (see Materials and Methods). To facilitate the entry of acyl-CoA

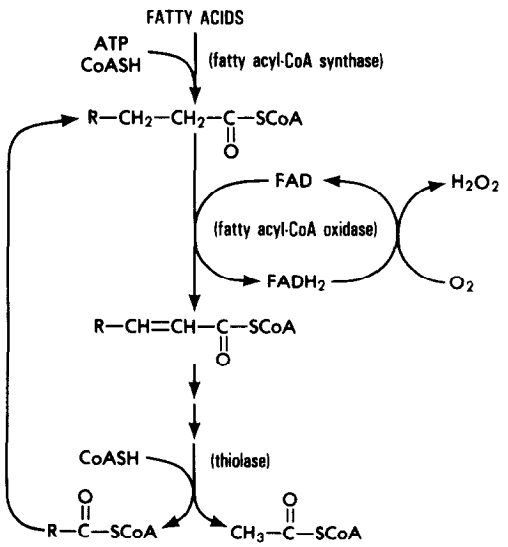

Fig. 1. The role of fatty acyl-CoA oxidase in peroxisomal H_2O_2 production (simplified from Tolbert, Ref. 8).

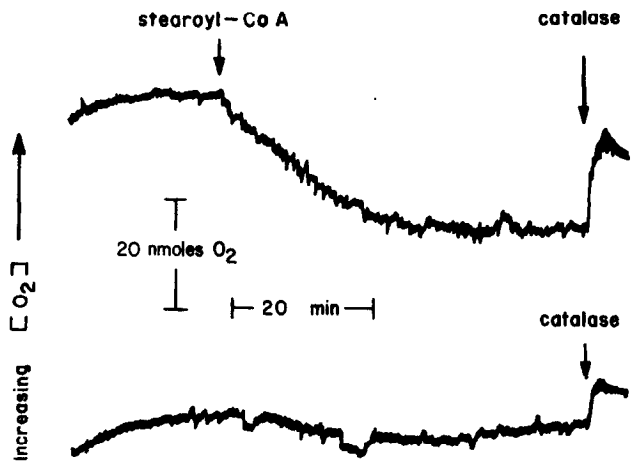

Fig. 2. Stearoyl-CoA-mediated oxygen consumption and H_2O_2 production in P. chrysosporium mycelia. Oxygen concentration of mycelial preparations, containing 0.025% Triton X-100 and 2.5 mM KCN, was monitored with a Clark electrode. Additions designated by the arrows were 300 µg stearoyl-CoA and 300 µg catalase. Final protein concentration and vessel volume were 1.7 mg·ml^{-1} and 1.3 ml, respectively.

molecules into the mycelia, a small amount of detergent (0.02% triton X-100) was added to the reaction mixture. Triton caused a slight stimulation in the basal level of formaldehyde production, which, in this experiment, was reduced to 2.13 nmoles formaldehyde/mg protein by addition of the respiratory chain inhibitor antimycin A. Addition of stearoyl-CoA to antimycin A-poisoned mycelia resulted in approximately a 2.4-fold stimulation of formaldehyde production. In other experiments, using extensively starved mycelia, we have observed stimulations in activity as large as seven-fold resulting from fatty acyl-CoA addition (not shown). These larger stimulations, however, appear to represent a more efficient lowering of endogenous H_2O_2 production rather than an actual increase in the maximum amount of H_2O_2 produced in response to added fatty acyl-CoA. Bovine serum albumin (BSA) enhanced formaldehyde production in the presence of stearoyl-CoA (Table 1), possibly by increasing the solubilization of the fatty acyl-CoA (16). NAD, which is a cofactor for β-hydroxyacyl-CoA dehydrogenase in the peroxisomal β-oxidation pathway (8), also stimulated formaldehyde production in the presence of stearoyl-CoA, as did nicotinamide.

The length of the acyl chain of the fatty acyl-CoA also affected the amount of H_2O_2 produced by P. chrysosporium mycelia (Fig. 3). Lauroyl-CoA

Table 1. Effect of Substrate and Cofactors on Mycelial H_2O_2 Production.

Addition[a]	H_2O_2 Generation[b] (nmoles CH_2O·mg protein^{-1})
None (basal)	2.42 ± 0.43
Triton	2.93 ± 0.30
Triton, antimycin A	2.13 ± 0.10
Triton, antimycin A, stearoyl-CoA	5.13 ± 0.67
Triton, antimycin A, stearoyl-CoA, BSA	5.78 ± 0.68
Triton, antimycin A, stearoyl-CoA, BSA, NAD	6.48 ± 0.54
Triton, antimycin A, stearoyl-CoA, BSA, NAD, nicotinamide	6.70 ± 0.83

[a]When added, the final concentrations were: triton X-100, 0.02%; antimycin A, 20 μm; stearoyl-CoA, 0.3 mM; BSA, 200 μg/ml; NAD 0.2 mM; nicotinamide, 40 mM.

[b]Relative H_2O_2 generation was measured after 3 hr by formaldehyde production as described in Materials and Methods. Protein concentration of the mycelial preparation was 1.2 mg·ml^{-1}. Standard deviations were calculated from 4 determinations.

(C_{12}) appeared to be the best substrate, although significant activity was also observed for C_8-C_{18} substrates. Almost no activity was observed when butyryl-CoA (C_4) was added as substrate. Other acyl-CoA oxidizing systems and acyl-CoA oxidases have been reported to exhibit similar dependences upon acyl chain length (16-18).

DISCUSSION

The occurrence of fatty acyl-CoA-dependent oxygen consumption (Fig. 2) and H_2O_2 production (Fig. 2 and Table 1) in the presence of respiratory chain inhibitors such as antimycin A and cyanide strongly suggests the existence of fatty acyl-CoA oxidase activity in P. chrysosporium mycelia. This conclusion is further supported by the fact that acyl-CoA dependent H_2O_2 production is contingent upon acyl chain length (Fig. 3) in a fashion similar to that already observed for fatty acyl-CoA oxidase activity in other organisms (16-18).

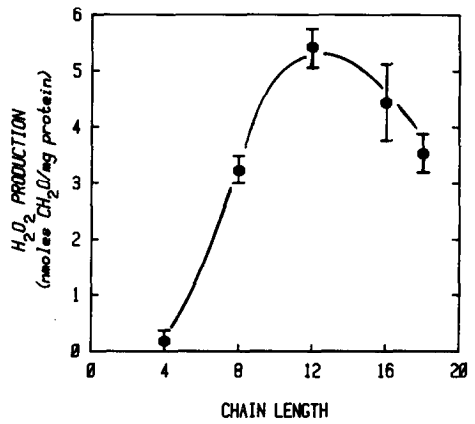

Fig. 3. Chain length specificity of acyl-CoA oxidase activity for acyl-CoA substrates. Formaldehyde production was measured in the presence of 20 μM antimycin A, 0.02% Triton X-100, 200 μg·ml^{-1} BSA and 300 μM acyl-CoA substrate. Activities reported in the figure have been corrected for basal activity occurring in the absence of added acyl-CoA. Protein concentration was 1.2 mg·ml^{-1}. Error bars represent the standard deviation of 5 determinations.

To our knowledge, fatty acyl-CoA oxidase activity in other organisms has always been reported as occurring in close association with peroxisomes and glyoxysomes. Interestingly, Forney et al. (2) have demonstrated the presence of ~0.1 μ diameter microbodies in the periplasmic space of P. chrysosporium mycelia. The microbodies were heavily stained by 3,3'-diaminobenzidine, a commonly used stain for peroxisomes (10). The appearance of these microbodies was correlated with the lignolytic activity of the cultures, and with the ability of mycelia to produce H_2O_2. It is possible, therefore, that the acyl-CoA oxidase activity which we report in this communication may be localized in these periplasmic microbodies, which Forney et al. (2) postulated to be the site of extracellular H_2O_2 production by P. chrysosporium.

A wide variety of other enzyme systems are known to produce H_2O_2 during normal metabolism, including several which have already been described in wood-degrading fungi (19-21). Oxidases for a variety of substrates are known to be present in many peroxisomes (10), making difficult any absolute determination of the role fatty acyl-CoA oxidase plays in lignin biodegradation. Further experiments are in progress in an effort to isolate peroxisomes from P. chrysosporium and to survey the organism for other active H_2O_2-producing enzymatic systems.

ACKNOWLEDGMENT

We wish to thank Dr. R. E. MacDonald for valuable scientific discussions and Lee Dexter for expert technical assistance.

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