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Pathway of *n*-Alkane Oxidation in *Cladosporium resinae*

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Pathways of initial oxidation of *n*-alkanes were examined in two strains of *Cladosporium resinae*. Cells grow on dodecane and hexadecane and their primary alcohol and monoic acid derivatives. The homologous aldehydes do not support growth but are oxidized by intact cells and by cell-free preparations. Hexane and its derivatives support little or no growth, but cell extracts oxidize hexane, hexanol, and hexanal. Alkane oxidation by extracts is stimulated by reduced nicotinamide adenine dinucleotide (phosphate). Alcohol and aldehyde oxidation are stimulated by nicotinamide adenine dinucleotide (phosphate), and reduced coenzymes accumulate in the presence of cyanide or azide. Extracts supplied with ^{14}C-hexadecane convert it to the alcohol, aldehyde, and acid. Therefore, the major pathway for initial oxidation of *n*-alkanes is via the primary alcohol, aldehyde, and monoic acid, and the system can act on short-, intermediate-, and long-chain substrates. Thus, filamentous fungi appear to oxidize *n*-alkanes by pathways similar to those used by bacteria and yeasts.

Cladosporium (*Amorphotheca*) *resinae* is one of the most prominent hydrocarbon-utilizing fungi. Parbery (18), who reviewed the organism's biology and some applied aspects, indicated that knowledge of metabolic pathways was lacking. This is true of filamentous fungi in general, for studies of hydrocarbon-using bacteria and yeasts far exceed those of fungi (10).

Two strains of *C. resinae* under investigation in our laboratory grow on a variety of hydrocarbons as sole carbon source, but growth is best on *n*-alkanes of intermediate chain length (2a). Alkane-oxidizing systems are constitutive (J. D. Walker and J. J. Cooney, Can. J. Microbiol., in press). Cells and cell-free preparations oxidize alkanes and the homologous primary alcohols and aldehydes, suggesting that they may be intermediates in oxidation of *n*-alkanes as they are for a number of bacteria and yeasts (Walker and Cooney, in press). Therefore, we investigated the pathway for initial oxidation of *n*-alkanes in two strains of *C. resinae*.

MATERIALS AND METHODS

C. resinae ATCC 22711 and 22712 were grown in shaken flasks. Cell-free extracts were prepared from actively growing cultures by using a French pressure cell. Crude extracts were centrifuged at 10,000 × *g*, which removed debris and mitochondria. The supernatant fraction was used without further purification as a source of enzymes. Reaction rates were determined polarographically by using an oxygen probe. Details of these methods have been published elsewhere (Cofone et al., in press; Walker and Cooney, in press). Protein was estimated by the method of Lowry et al. (15) by using bovine serum albumin as standard.

Effects of temperature on oxygen uptake were determined with the oxygen probe and a model 5214 Haake constant temperature circulator, correcting for total dissolved oxygen at each temperature (21). Citrate-phosphate buffer was used throughout, adjusting the pH as required (3).

Accumulation of reduced nicotinamide adenine dinucleotide (NADH) was measured at 340 nm by using the extinction coefficient of 6.22×10^6 cm^2 per mol (4).

In some experiments ^{14}C-hexadecane was supplied and the radioactivity associated with suspected products was determined. Unlabeled suspected product was added to trap radioactivity in a large pool of unlabeled material. To a 125-ml flask was added 37 ml of a cell-free preparation containing 0.25 mg of protein/ml in buffer at pH 5.0; 250 μmol of NADH; 500 mg of hexadecanol, hexadecanal, or hexadecanoic acid emulsified in 10 ml of buffer; and 3 ml of buffer into which had been emulsified 600 μliters of *n*-hexadecane-*1*-^{14}*C* (sp act, 1 μCi/600 μliters of hexadecane). Duplicate samples were withdrawn immediately for counting. The reaction mixture was stirred for 1 h at

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30 C. It was then acidified to pH 2 and extracted twice with diethyl ether, which extracted 96 to 99% of the hydrocarbons. The aqueous phase was concentrated by evaporation and counted. Hexadecanoic acid was crystallized directly from ether. The ether phases from reaction mixtures containing hexadecanol or hexadecanal were evaporated to dryness, and the respective 3,5-dinitrobenzoyl chloride and 2,4-dinitrophenylhydrazone derivatives were prepared as described by Shriner et al. (20). The acid and the derivatives were chromatographed on thin-layer plates coated with kieselguhr G. Plates with the alcohol derivative were developed with ethanol, plates with the aldehyde derivative were developed with benzene:ethanol:chloroform (2:1:1, vol/vol/vol), and those with the acid were developed with benzene:methanol:acetic acid (45:8:4, vol/vol/vol). Plates were examined under ultraviolet light or, for the acid, by spraying with 0.2% bromocresol green in 1-butanol. Products were identified by co-chromatography with the acid or with synthetic derivatives. Samples were eluted from the adsorbent with methanol. Each was added to a vial containing 15 ml of scintillation fluid (6 g of 2,5-diphenyloxazole, 412 ml of methyl cellosolve, and 588 ml of toluene) and counted in a liquid scintillation detector. Quenching was corrected by using an internal standard (^{14}C-sucrose, 1,100 dpm per 100 μliters) according to the method of Wang and Willis (24). In several experiments 87 to 90% of the total radioactivity supplied was recovered. Only 1 to 7% remained in the aqueous fraction, indicating that little of the hydrocarbon was converted to water-soluble products and that the unlabeled suspected product trapped the bulk of the radioactive material.

Hexane, dodecane, and hexadecane were 99% mole purity and were obtained from Phillips Petroleum, Bartlesville, Okla. Manufacturer's analyses indicated that contaminants were other close boiling paraffins and isoparaffins and that oxygenated derivatives were not detected. Hexanol (97% mole purity), dodecanol (98%), hexadecanol (97%), hexanoic acid (99%), dodecanoic acid (98%), and hexadecanoic acid (93%) were from Matheson, Coleman, and Bell. Piperonyl butoxide was purchased from K & K Labs, Plainview, N.Y. Hexanal ("99+% pure"), dodecanal (99+%), and hexadecanal sodium bisulfite addition compound (99%) were from Aldrich Chemical Co., Milwaukee, Wis. Hexadecanal was prepared by boiling the sodium bisulfite addition compound in 0.1 N HCl until the solution almost cleared. After the solution had cooled to ambient temperature, the aldehyde was extracted into chloroform and crystallized from chloroform. *n*-Hexadecane-*1*-^{14}*C* was obtained from Amersham Searle, Arlington Heights, Ill., and nicotinamide adenine dinucleotide (NAD), NAD phosphate (NADP), NADH, and reduced NADP (NADPH) were from Sigma Chemical Co., St. Louis, Mo.

RESULTS AND DISCUSSION

Each strain grew faster on glucose or glutamic acid than on *n*-alkanes or their oxygenated derivatives (Table 1). Hexadecanol and hexadecanoic acid were the best alkane-related substrates examined: the alcohol supported five times more growth of strain 22712 than the sugar or the amino acid, and hexadecanoic acid supported more growth of both strains, although growth was significantly slower than on the sugar or the amino acid. Little or no growth was noted on hexane or its derivatives (Table 1). These compounds inhibit endogenous respiration of whole cells, although they are oxidized by cell-free preparations (Walker and Cooney, in press). These and other observations led to the conclusion that hexane is toxic to cell membranes (Walker and Cooney, in press). Therefore, *C. resinae* can grow on some of the compounds proposed as intermediates in *n*-alkane oxidation, and those which do not support growth can be oxidized by cells or cell-free preparations.

TABLE 1. *Growth of C. resinae in shaken flasks on glucose, glutamic acid, and hydrocarbons*

Substrate	Stationary phase values for			
	Strain 22711		Strain 22712	
	Days to reach stationary phase	mg dried cells per ml	Days to reach stationary phase	mg dried cells per ml
Glucose	4	4.2	4	3.7
Glutamic acid	6	4.0	6	3.9
Hexane	32	0.05	NG[a]	NG
Hexanol, hexanal, hexanoic acid	NG[a]	NG	NG	NG
Dodecane	36	0.3	36	0.3
Dodecanol	32	0.4	36	0.2
Dodecanal	NG	NG	NG	NG
Dodecanoic acid	36	0.4	NG	NG
Hexadecane	32	0.5	32	0.8
Hexadecanol	36	1.0	36	22.0
Hexadecanal	NG	NG	NG	NG
Hexadecanoic acid	20	15.4	24	21.0

[a] No evidence of growth after 60 days of incubation.

Preliminary experiments with cell-free extracts showed that maximum oxygen consumption was obtained when 8 to 16 μliters of hydrocarbon were emulsified per ml of cell extract, so 12 μliters per ml were used throughout; solid hydrocarbons were used at 12 μliters per ml. Boiled extracts could not be substituted for native enzymes. Oxygen consumption was linear with respect to protein concentration over a range of 0.1 to 0.4 mg per ml, and extracts were routinely adjusted to contain 0.25 mg per ml. At concentrations as high as 0.05 M, glutathione had no effect on hexadecane oxidation. Addition of a surfactant (0.1 ml of Tween 80 or

0.1 ml of sodium deoxycholate) did not affect oxygen consumption on hexadecane, indicating that hydrocarbon emulsions provided adequate surface area for enzyme-substrate contact. Hexadecane oxidation was measured at 5-degree intervals from 20 to 50 C. Maximum activity was observed at 45 C and no activity was noted at 50 C. The optimal growth temperature for these organisms is 30 C and they do not grow at 35 C or above; 30 C was used in subsequent experiments with all substrates. The optimal pH for hexadecane oxidation was 4.5, which was used for all alkanes. Oxygen uptake on 6-, 12-, or 16-carbon alkanes was stimulated by NADH or NADPH, but oxidized forms of the coenzymes did not stimulate (Table 2).

When extracts were supplied with ^{14}C-hexadecane in the presence of excess 1-hexadecanol, 94% of the radioactivity extracted into ether was associated with the dinitrobenzoyl derivative prepared from hexadecanol. Of the radioactivity supplied, 83% was recovered as the hexadecanol derivative, indicating that the primary alcohol is a major intermediate in *n*-hexadecane oxidation.

Some pathways proposed for oxidation of alkanes are summarized in Fig. 1. Lack of stimulation by NAD or NADP suggests that the alkene is not an intermediate. Moreover, dehydrogenation is energetically unfavorable (16), and in bacterial (5, 17) and yeast (8, 9) systems alkanes and alkenes yield different products. Alkane hydroxylation systems involving mixed function oxidases and cytochrome P-450 occur in bacteria (1, 7), liver microsomes (M. J. Coon, H. W. Strobel, A. P. Autor, J. Heidema, and W. Duppel, Biochem. J., **125**:2, 1971; 19), and yeast (11). Piperonyl butoxide has been reported to inhibit cytochrome P-450 specifically (6), but at concentrations as high as 10 mg per ml it did not inhibit hexadecane oxidation by *C. resinae* extracts. Thus, indirect evidence suggests that alkane oxidation does not involve alkene formation and that if it proceeds via a mixed-function oxidase, cytochrome P-450 is not involved. Initial oxidation could also involve a hydroperoxide (Fig. 1), but evidence is lacking at present.

Oxidation of hexadecanol was optimum at pH 6. Oxygen consumption was stimulated by NAD and NADP (Table 2). With NADH or NADPH, Q_{O_2} values were less than the hexadecanol control, suggesting that hexadecanol inhibits NAD(P)H oxidase. Involvement of oxidized coenzymes was further demonstrated by accumulation of NADH in the presence of electron transport inhibitors (Table 3). When extracts were incubated for an hour in the presence of ^{14}C-hexadecane and an excess of hexadecanal, 80% of the radioactivity supplied

TABLE 2. *Effects of coenzymes on oxidation of alkanes, alcohols, and acids by cell-free preparations of C. resinae ATCC 22711*

Carbon length of substrate	Coenzyme	$Q^a_{O_2}$ on:		
		Alkane	Primary alcohol	Aldehyde
C-6		10	96	25
C-6	NAD		111	45
C-6	NADH	24		
C-12		12	106	38
C-12	NAD		128	53
C-12	NADH	29		
C-16		12	25	26
C-16	NAD	12	67	55
C-16	NADP	12	67	54
C-16	NADH	24	−12	17
C-16	NADPH	21	−13	18

[a] Each reaction mixture contained 0.25 mg of protein and 12 μliters of liquid substrate or 12 μg of solid substrate per ml suspended as an emulsion in citrate-phosphate buffer which was adjusted to pH 4.5 for alkanes, to 6.0 for alcohols, and to 5.5 for aldehydes. Cofactors were added at 5 μmol per ml. Reactions were incubated at 30 C. Q_{O_2} values have been corrected for endogenous and coenzyme controls and are expressed as microliters of O_{O_2} consumed per hour per milligram of soluble protein.

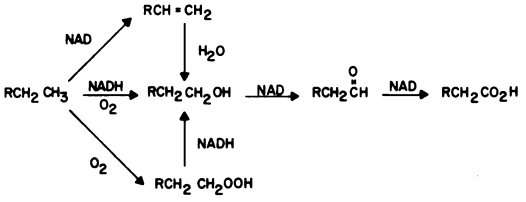

FIG. 1. *Pathways proposed for monoterminal oxidation of alkanes.*

TABLE 3. *Effect of electron transport inhibitors on hexadecanol and hexadecanal oxidation*

Substrate[a]	Inhibitor[a]	nmol of NADH per mg of protein per h
Hexadecanol		10
Hexadecanol	NaN_3	63
Hexadecanol	KCN	70
Hexadecanal		21
Hexadecanal	NaN_3	103
Hexadecanal	KCN	103

[a] Conditions were as indicated for Table 2. Each reaction mixture contained 5 μmol of NAD per ml. NaN_3 was present at 10 mM and KCN was present at 1 mM.

was recovered in the dinitrophenylhydrazine derivative prepared from hexadecanal. Thus, *C. resinae* oxidizes primary alcohols to the homologous aldehyde via an NAD(P)-linked dehydrogenase. Hexanol and dodecanol oxidation were also stimulated by NAD (Table 2), indicating that the system is not specific for long-chain alcohols. *Pseudomonas aeruginosa* has two alcohol dehydrogenases, one of which is constitutive, requires NADP, and is dependent on substrate chain length. The second is induced; it does not require NAD or NADP and it may be chain-length regulated (22, 23). The inducible enzyme was suggested as the participant in alcohol dissimilation. The yeast *Candida tropicalis* has a soluble and a particulate alcohol dehydrogenase. The soluble enzyme is induced by growth on *n*-alkane, it is NAD-linked, and it is active on C-2 through C-16 alcohols (12–14). These observations suggest that the alcohol dehydrogenase activity of *C. resinae* should be examined further.

Oxygen uptake on hexadecanal was optimum at pH 5.5. Hexadecanal oxidation was stimulated by NAD and NADP, and, with reduced forms of the coenzymes, consumption was less than in the control (Table 2). NADH accumulated in the presence of cyanide or azide (Table 3). Hexanal and dodecanal were also oxidized (Table 2). By using ^{14}C-hexadecane and an excess of hexadecanoic acid, 66% of the radioactivity supplied was recovered in the hexadecanoic acid. Therefore, aldehydes are oxidized to the homologous acid by an NAD(P)-dependent system which is active on short-, intermediate-, and long-chain compounds.

The data support the conclusion that the initial oxidation of *n*-alkanes by *C. resinae* involves conversion to the homologous primary alcohol, aldehyde, and monoic acid, as indicated in Fig. 1. Since cellular fatty acids show little correlation with *n*-alkane growth substrate, the fatty acid is oxidized via β-oxidation rather than being incorporated directly into cell lipids (2). It is not evident why the 12- and 16-carbon aldehydes do not support growth, because they are readily oxidized and because the 12- and 16-carbon acids do support growth.

Other pathways may also operate in this fungus, such as diterminal oxidation yielding the dioic acid or subterminal oxidation yielding alkenes, secondary alcohols, and ketones. But monoterminal oxidation appears to be the major pathway, which is consistent with mechanisms of *n*-alkane oxidation demonstrated for a number of bacteria and yeasts.

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