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n-Alkane Oxidation Enzymes of a Pseudomonad[1]

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A nicotinamide adenine dinucleotide (NAD)-dependent n-alkane dehydrogenase and an NAD phosphate (reduced form)-dependent alkane hydroxylase have been purified from cell-free extracts of *Pseudomonas* sp. strain 196Aa grown anaerobically on n-alkane. The n-alkane dehydrogenase (fraction R-3), obtained as a single peak from Bio-Gel P-60, showed an overall 135-fold purification and was demonstrated by infrared spectroscopy and gas chromatography to convert n-decane to 1-decene. The alkene hydroxylase activity in the S-3 fraction, purified 167 times from diethylaminoethyl-cellulose, was shown by the same methodology to convert decene to decanol. Commercial ferredoxin has been shown to increase the alkane dehydrogenase activity. An NAD-, flavine adenine dinucleotide-, and iron-dependent alcohol dehydrogenase was demonstrated in the R-3 fraction. A mechanism for the anaerobic conversion of n-alkane to fatty acid has been proposed.

Various bacteria dehydrogenate n-alkanes to alkenes. Senez and Azoulay (6) reported that resting cells of *Psuedomonas aeruginosa* grown aerobically reduce pyocyanin under anaerobic conditions and contain a nicotinamide adenine dinucleotide (NAD)-dependent dehydrogenase, which is specifically inhibited by mercaptoethanol and dehydrogenates both 1-heptanol and heptyllic aldehyde. Chouteau et al. (3) identified 1-heptene formation from n-heptane by *P. aeruginosa* (Sol 20). Azoulay et al. (2) studied the enzymes from this organism grown aerobically on n-heptane and demonstrated a requirement for Fe^{2+} and O_2 in the system.

Wagner et al. (9) were able to isolate the identify 1-hexadecene by various analytical means from cultures of *Micrococcus certificans, Mycobacterium phlei, Nocardia* sp., *Pseudomonas* sp., or *Rhodotorula* sp. grown on alkene-free n-hexadecane. Iizuka et al. (4) identified 1-decene derived from n-decane using cell-free extracts of *Canadida rugosa*, which contained an NAD-dependent dehydrogenase, and presented evidence that primary alcohol formation involved the addition of water rather than molecular oxygen to the double bond of 1-decene. Abbot et al. (1) have shown the formation of mono-alkenes, which were formed by *Nocardia* grown on hexadenane.

Traxler and Bernard (8) observed that *Pseudomonas* sp. strain 196Aa grew very poorly on n-alkanes if provided forced aeration or aeration by vigorous shaking, but would grow well in static aerobic cultures or in the anaerobic incubator if nitrate was present as the electron acceptor.

This report describes the initial oxidative products fromed from n-alkanes by cell-free preparations of this pseudomonad. The respective enzymes have been partially purified, and the reaction products have been identified by gas chromatography and infrared spectroscopy.

MATERIALS AND METHODS

Psuedomonas sp. strain 196Aa was maintained at 4°C on Trypticase soy agar slants and transferred monthly. Culture purity was checked on each transfer by examining colony morphology and Gram reaction. Inocula were prepared by transfer of a loopful of cells from the Trypticase soy agar slant culture to a flask of mineral salts-hexadecane medium, which was incubated at 30°C without agitation. The inoculum (100 ml) was added to 10 liters of mineral salts-hydrocarbon medium incubated under deoxygenated nitrogen (8). Nitrogen flushing was started prior to inoculation while the medium temperature was still above 100°C.

The hydrocarbons used in these experiments were n-octane (99.7 mol% purity, Eastman Organic Chemicals), n-decane, and n-hexadecane (99 mol% purity [alkene-free], Matheson, Colemen and Bell, Inc.). Other components of the medium were of analytical or reagent grade.

The cells were harvested from the late log phase by centrifugation for 15 min at 3,000 × g in a Sorvall RC-2B at 0°C, washed twice with 0.02 M tris(hy-

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TABLE 1. *Specific activity of $NADH_2$ oxidase and alkane dehydrogenase in cell-free preparations*[a]

Fraction	NADH oxidase sp act	Alkane dehydrogenase			Fold purification
		Sp act	Yield	% Recovery	
Crude extract	2.7	0.37	262	100	
R-3 fraction	0.7	6.6	92.4	31.4	18.0
R-3 + ferredoxin (μg/ml)		11.2	156.8	60.0	30.0
S-3 fraction	3.8				
40% $(NH_4)_2SO_4$, ppt of R-3		16.2	120	45.8	43.8
Bio-Gel P-60 peak R-3		50.0	160	60.0	135.0

[a] Alkane dehydrogenase assay: Δ optical density at 340 nm; NAD^+, 1 μmol; $MgCl_2$, 10 μmol; *n*-decane, 0.04 ml; enzyme, 0.1 ml (3.7 mg/ml); buffer to 2.5 ml. NADH oxidase assay: Δ optical density at 340 nm; NADH, 1.5 μmol; enzyme, 0.1 ml; buffer to 2.5 ml.

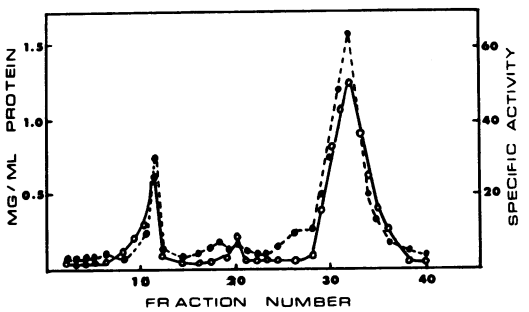

FIG. 1. *Column chromatogram of the alkane dehydrogenase in the R-3 fraction on Bio-Gel P-60 as described in the text. Symbols: protein* (●), *mg/ml; alkane dehydrogenase specific activity* (○).

droxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.8), and suspended in this buffer supplemented with 0.067 M dithioerythritol. The final cell concentration was 0.6 mg (dry weight)/ml.

Cell-free extracts were prepared by sonication of the above suspension in the Bronwill Biosonik III (60 kc at 10- to 15-s intervals) for a total of 3 min at 0 to 4°C. The disrupted cell suspensions were centrifuged at 6,780 × *g* for 15 min to remove cell debris. This yielded a supernatant, S-1, which was centrifuged at 15,900 × *g* for 30 min to give supernatant S-2 and pellet R-2. Supernatant S-2 was centrifuged at 41,300 × *g* for 1.5 h and gave fractions S-3 and R-3. All centrifugations were performed at 0°C. The protein concentration of each fraction was calculated from the absorbance at 260 and 280 nm by the method of Warburg and Christian (10).

Alkane dehydrogenase activity was precipitated from the Tris-hydrochloride buffer solution of fraction R-3 at 0 to 4°C by 40% ammonium sulfate saturation. The alkene hydroxylase was precipitated from fraction S-3 at 0 to 4°C with 60% ammonium sulfate saturation. The precipitates were collected by centrifugation at 10,000 × *g* for 10 min and resuspended in a minimal amount of 0.22 M Tris-hydrochloride buffer at pH 7.8. The extracts were dialyzed against buffer for 20 to 24 h at 0°C, with a change of buffer each 4 h.

The dialysate of the ammonium sulfate fraction R-3 was loaded onto a Bio-Gel P-60 column, washed, and equilibrated with 0.02 M Tris-hydrochloride buffer (pH 7.8) containing 0.01% MgCl. The sample was eluted with 0.5 M Tris-hydrochloride buffer (pH 7.8) under 2 lb/in² of pressure. Fractions were collected in 3-ml portions in an automatic fraction collector. The protein concentration and the alkane dehydrogenase and aldehyde dehydrogenase activities were determined in each fraction.

The dialyzed fraction S-3 precipitated with ammonium sulfate was loaded onto a diethylaminoethylcellulose column, equilibrated, and washed with 200 ml of 0.02 M Tris-hydrochloride buffer at pH 7.8 containing 0.01% MgCl.

Enzymes were assayed spectrophotometrically by measuring the change in absorbance of pyridine nucleotides at 340 nm in a Hitachi-Perkin-Elmer UV-VIS model 139 spectrophotometer equipped with a Sargent model SR recorder (5). One unit of enzyme was defined as the quantity of enzyme required to cause a change in optical density of 0.01 U/min. Specific activity was expressed as units of enzyme per milligram of protein.

Reactions between partially purified enzyme preparations and the substrate were carried out anaerobically in Thunberg tubes. The alkane dehydrogenase system contained 0.1 ml of fraction R-3 (3.7 mg of protein per ml), 0.04 ml of *n*-decane, 1 μg of ferredoxin, 10 μmol of $MgCl_2$, 1 μmol of NAD, and 0.22 M Tris-hydrochloride buffer (pH 7.8) containing 0.067 M dithioerythritol to a volume of 2.5 ml. The reaction mixture for the alkene-hydroxylating system contained 0.1 ml of fraction S-3 (25.5 mg of protein per ml), 0.04 ml of 1-decane, 3.0 μmol of NAD phosphate, reduced form, and 0.22 M Tris-hydrochloride buffer, pH 7.8, containing 0.067 M dithioerythritol to a total volume of 2.5 ml. Immediately after incubation, the reaction mixture was extracted three times with an equal volume of diethyl ether. The ether extracts were pooled from each sample, dried over anhydrous Na_2SO_4, and evaporated under vacuum. The residue was reconstituted in carbon tetrachloride or carbon disulfide, or left untreated.

Gas chromatographic analyses were performed in a Perkin-Elmer model 154 gas chromatograph equipped with a thermal conductivity detector, and a glass column (0.25 by 41 inches [ca. 0.63 by 10.2 cm]) packed with 10% tergitol (NPX) on 80/100 Chromosorb P AW. Samples and authentic standards

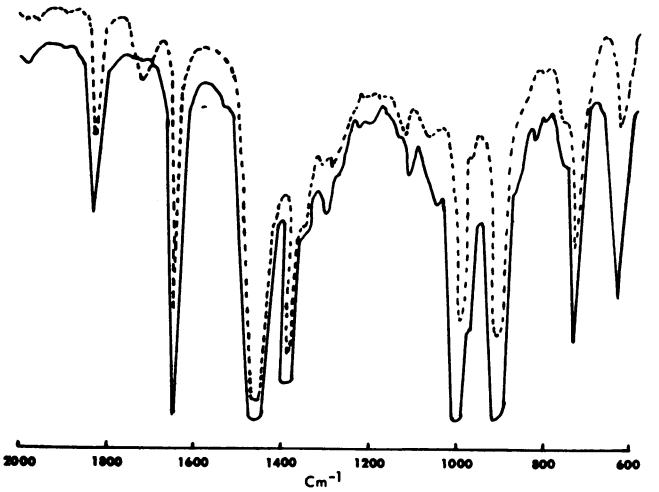

FIG. 2. *Infrared spectrum of 1-decene produced from n-decane by the R-3 fraction. Symbols: standard 1-decene (——); reaction product (- - -).*

TABLE 2. *Specific activity of alkene-hydroxylating system in cell-free preparations*[a]

Fraction	U/ml	Protein (mg/ml)	Sp act
Crude extract	40	35.5	1.2
S-3	350	25.5	14.0
60% $(NH_4)_2SO_4$, ppt of S-3	160	25.0	6.4
DEAE-cellulose S-3	100	0.6	166.7

[a] Assay: Δ optical density at 340 nm; NADPH, 3.0 μmol; 1-decene, 0.04 ml (25.5 mg/ml); enzyme, 0.1 ml; buffer to 2.5 ml. DEAE,Diethylaminoethyl.

TABLE 3. *Specific activity of alcohol dehydrogenase in cell-free enzyme preparation*[a]

Fraction	U/ml	Protein (mg/ml)	Sp act	Fold purification
Crude extract	30	35.5	0.8	
S-3	40	25.0	1.2	2.0
60% saturation	60	2.5	30.0	35.0

[a] Assay: Δ optical density at 340 nm; NAD^+, 1 μmol; $FeSO_4$, 0.27 μmol; flavine adenine dinucleotide, 0.2 μmol; decanol, 0.04 ml; enzyme, 0.1 ml (25.5 mg/ml); buffer to 2.5 ml.

were injected in 1- to 2-μl quantities. The temperature was isothermal at 75°C for the resolution of alkane and alkene and at 172°C for the resolution of the alkene, alcohol, aldehyde, and fatty acid.

Infrared analyses were performed in a Beckman IR-10. Spectra were obtained in KBr cells with a slit width of 0.094 mm. The infrared spectra of the samples were compared with authentic standards (7).

RESULTS AND DISCUSSION

Crude extracts of the alkane-grown cells contained NADH oxidase (Table 1), which interfered with the measurement of the alkane dehydrogenase activity. NADH oxidase and alkane dehydrogenase could be separated by centrifugation at 41,000 × *g* for 1.5 h. After centrifugation, the NADH oxidase activity was present in the supernatant fraction S-3, whereas the alkane dehydrogenase was in the R-3 fraction. Separation of these two enzymes increased the specific activity of the alkane dehydrogenase from 0.37 to 6.6, which resulted in an 18-fold purification. If ferredoxin (1 μg/ml) was added to the R-3 fraction, there was a further increase in specific activity of the alkane dehydrogenase to 11.2. The solubilized alkane dehydrogenase was precipitated by 40% saturated $(NH_4)_2SO_4$. When dissolved and dialyzed, it had increased specific activity and 43.8-fold purification. When the dialysate was subjected to chromatography on a Bio-Gel P-60 column, 135-fold purification was achieved (Table 1). This material exhibits a major and minor peak (Fig. 1). The major peak, located between fractions 28 and 36, contained the main proportion of the dehydrogenase.

After reaction of fraction R-3 with *n*-decane, the hydrocarbon was extracted, and the infrared spectrum was obtained and compared with a standard of 1-decene (Fig. 2). Significant correlations occur at 1,825, 1,645, 990, and 910 cm^{-1}, all indicative of 1-alkene absorption. A gas chromatogram of *n*-decane and 1-decene gave the same peak geometry and retention times as the reaction mixture from the Thunberg tube. A gas chromatogram of the substrate *n*-decane did not show the 1-decane shoulder, which substantiated the purity of the *n*-decane used in these experiments. These data indicate

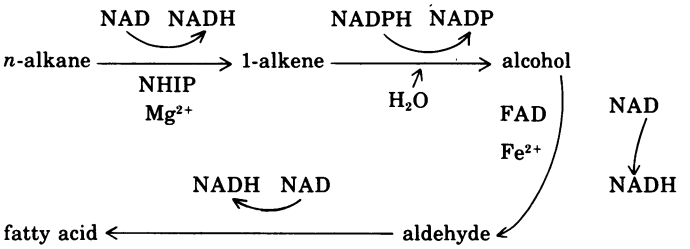

FIG. 3. *Scheme for anaerobic conversion of an n-alkane to fatty acid. NHIP, Non-heme iron protein.*

TABLE 4. *Specific activity of aldehyde dehydrogenase in the cell-free enzyme preparation*[a]

Fraction	U/ml	Protein (mg/ml)	Sp act	Fold purification
Crude extract	35	35.5	1.0	
S-3	25	25.0	1.0	
R-3	75	3.7	20.0	20.0

[a] Assay: Δ optical density at 340 nm; NAD^+, 1 μmol; decanol, 0.04 ml; enzyme, 0.1 ml (3.7 mg/ml); buffer to 2.5 ml.

that the R-3 fraction converted *n*-decane to decene.

After separation from the alkane dehydrogenase by centrifugation, the specific activity of the alkene hydroxylating enzyme was increased from 1.2 to 14 (Table 2). The NADH oxidase did not interfere with the assay of this enzyme, which has a specific requirement for NADPH. When fraction S-3, precipitated with 60% ammonium sulfate, was subjected to diethylaminoethyl column chromatography, the specific activity was increased to 166.7 as compared to 1.16 in the crude extract. Thus, an overall 143-fold purification of the alkene-hydroxylating enzyme was achieved.

A gas chromatogram of the ether extract of the reaction mixture had the same peak geometry and retention times as a reference mixture of 1-decene and *n*-decanol. Analysis of the same extract by infrared spectrophotometry showed absorption at 3,600 cm^{-1}, which indicated the presence of a primary alcohol. These data indicated that fraction S-3 converted the 1-alkene to the corresponding primary alcohol.

The alcohol dehydrogenase activity was present in the S-3 fraction, and the aldehyde dehydrogenase activity was present in R-3 fraction. Table 3 shows that the specific activity of the alcohol dehydrogenase in the S-3 fraction was nearly twice as much as in the crude extract. Further purification by precipitation with ammonium sulfate at 60% saturation increased the enzyme specific activity to 30, giving a 35-fold overall purification. Table 4 shows the specific activity of the aldehyde dehydrogenase in the R-3 fraction. No attempt has been made to further purify either the alcohol dehydrogenase or the aldehyde dehydrogenase.

It is concluded that this organism has the enzymatic capability to convert an *n*-alkane anaerobically to the level of fatty acid via the scheme shown in Fig. 3.

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