APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1988, p. 3187–3190
0099-2240/88/123187-04$02.00/0

Vol. 54, No. 12

Interaction of Ammonia Monooxygenase from *Nitrosomonas europaea* with Alkanes, Alkenes, and Alkynes

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Received 6 July 1988/Accepted 27 September 1988

Ammonia monooxygenase of *Nitrosomonas europaea* catalyzes the oxidation of alkanes (up to C_8) to alcohols and alkenes (up to C_5) to epoxides and alcohols in the presence of ammonium ions. Straight-chain, N-terminal alkynes (up to C_{10}) all exhibited a time-dependent inhibition of ammonia oxidation without effects on hydrazine oxidation.

Ammonia monooxygenase is a membrane-bound enzyme that catalyzes the oxidation of ammonia to hydroxylamine in the nitrifying bacterium, *Nitrosomonas europaea* (9, 21). Whole cells of *N. europaea* are also capable of oxidizing various hydrocarbon compounds, including methane (9, 14), methanol (22), ethylene (11), propylene (4), benzene (8), phenol (8), cyclohexane (4), bromocarbons (10), and CO (15). Ammonia monooxygenase is thought to be the enzyme responsible for these oxidations (8–11). In the present study, we have investigated the oxidation of a range of straight-chain alkanes and alkenes by *N. europaea*. The aim of this study was to determine some of the limitations of the hydrocarbon substrate range of this enzyme. This approach would then allow for a more comprehensive comparison to be made between ammonia monooxygenase and the two forms of methane monooxygenase found in methanotrophic bacteria which can, in part, be distinguished on the basis of the limitations of their respective hydrocarbon substrate ranges (1–3).

N. europaea (ATCC 19178) was grown in 1 liter of medium in 2-liter shake flasks at room temperature (ca. 23°C) in medium as described previously (9). The ammonia-dependent O_2 uptake activity of these cultures (30°C, 10 mM NH_4^+) at the time of harvest (3 days) was consistently within the range of 150 to 190 μmol of O_2 consumed per h per mg of protein. Harvested cells were suspended in 50 mM potassium phosphate buffer, pH 7.7, containing 2 mM $MgCl_2$. The cells were centrifuged again (16,000 × *g* for 10 min), and the sedimented cells were suspended in buffer as above at approximately 0.07 g of wet weight per ml and used within 2 h of harvesting.

Incubations were conducted in stoppered 9.4-ml serum vials which contained 0.9 ml of buffer (as above, with 10 mM NH_4Cl). Gaseous hydrocarbons (up to 7 ml) were added to the vials as an overpressure, and liquid hydrocarbons (up to 30 μl) were added directly to the medium. Reactions were initiated by the addition of 0.1 ml of cell suspension to the incubation vial and proceeded with shaking of the vials (75 oscillations per min). After 1 h, the reaction was stopped by the addition of thiourea (EM Science, Gibbstown, N.J.) to a final concentration of 50 μM. Samples (5 μl) for product analysis were then drawn directly from the reaction medium. The concentration of each hydrocarbon which supported the highest level of product formation with 10 mM NH_4^+ was determined. This concentration of hydrocarbon was then used in the experiments described in Tables 1 and 2. Day-to-day variation in the amount of a given product formed under comparable conditions was within 10% of the values reported in Tables 1 and 2.

All products were identified and quantified by using a gas chromatograph (Shimadzu 8A) fitted with flame ionization detectors. C_1 through C_7 products were separated by using a Porapak Q column (Waters Associates, Inc., Millford, Mass.) as described previously (2). A second column, packed with 10% SP2100 on 80/100-mesh Supelcoport (Supelco, Bellefonte, Calif.), was used to separate C_8 products and compounds which coeluted on the Porapak Q column. Concentrations of products were determined by using peak area calibrations generated with aqueous solutions of the pure compound. O_2 measurements were made by using a Clark-type O_2 electrode (Hansatech, King's Lynn, Norfolk, U.K.) operated at 30°C. Protein concentrations of whole-cell suspensions were determined by using the Biuret assay (5) after solubilization in 3 M NaOH for 30 min at 60°C.

All straight-chain hydrocarbons (up to C_8) were oxidized by suspensions of whole cells of *N. europaea* to give hydroxylated products (Table 1). With an increase in carbon number from C_1 to C_4, the amount of oxidation product detected after 1 h also increased. The levels of oxidation products were markedly lower with increasing carbon numbers above C_4. In addition, progressively higher quantities of hydrocarbon were required to obtain maximal product formation. With hydrocarbons larger than ethane, both 1-ol and 2-ol oxidation products were observed. The ratio of 1-ol to 2-ol products varied with increasing hydrocarbon chain length (Table 1). No 3-pentanol or 3- or 4-octanol was detected. (3-Hexanol and 3- or 4-heptanol would not have been separated from the 2-ol products in our analyses.)

Lower levels of hydrocarbon oxidation were achieved with a lower concentration of ammonium ions (Table 1). This reflects the need for concurrent ammonia oxidation to supply the reductant required for monooxygenase-catalyzed hydrocarbon oxidations (9). In the absence of ammonium ions, the reductant is provided by endogenous respiration (8–11). Also in agreement with previous studies is the observation that specific inhibitors of ammonia oxidation, such as thiourea and acetylene, inhibited the oxidation of hydrocarbons.

Straight-chain N-terminal alkenes (up to C_5) were also oxidized by *N. europaea*. All alkenes tested gave rise to either epoxide or unsaturated alcohol products (Table 2). Some oxidation of 1-pentene may not have been catalyzed by ammonia monooxygenase, as indicated by the limited

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TABLE 1. Oxidation of *n*-alkynes by whole cells of *N. europaea*

Substrate (state)[a]	Amt of substrate (μmol) per vial[b]	Product	μmol of product formed (per mg of cell protein) after 1 h of incubation with:				
			10 mM NH_4^+	1 mM NH_4^+	No reductant	10 mM NH_4^+ plus 8 μmol of C_2H_2	10 mM NH_4^+ plus 50 μM thiourea
Methane (g)	290	Methanol	1.33	0.76	0.22	0.04	0.01
Ethane (g)	30	Ethanol	1.96	0.77	0.23	0	0
Propane (g)	70	Propan-1-ol	0.45				
		Propan-2-ol	2.94				
		Both	3.39	0.95	0.28	0	0
Butane (g)	70	Butan-1-ol	0.77				
		Butan-2-ol	2.70				
		Both	3.47	1.19	0.38	0	0
Pentane (l)	50	Pentan-1-ol	2.0				
		Pentan-2-ol	0.70				
		Both	2.70	1.00	0.38	0	0
Hexane (l)	75	Hexan-1-ol	1.39				
		Hexan-2-ol	0.35				
		Both	1.74	0.49	0.30	0	0
Heptane (l)	100	Heptan-1-ol	0.16				
		Heptan-2-ol	0.17				
		Both	0.33	0.14	0.05	0	0
Octane (l)	150	Octan-1-ol	0.04	0.04	0.01	0	0
		Octan-2-ol	ND[c]				

[a] g, Gas; l, liquid.
[b] Amount of each hydrocarbon which supported the formation of the greatest amount of product.
[c] ND, No product detected.

effects of acetylene and thiourea on the oxidation rate. Interestingly, both *cis*- and *trans*-2-butene were considerably better substrates than 1-butene when all three compounds were tested at the same gas-phase concentration (Table 2). The oxidation of both internal alkenes gave rise mainly to a single hydroxylated oxidation product which retained its original diastereomeric configuration. However, the *cis* configuration was clearly preferred over the *trans* for epoxide formation. The oxidation of all alkenes (apart from 1-pentene) was affected by the concentration of ammonium ions and the presence of ammonia oxidation inhibitors in the same way as alkane oxidations.

Ammonia oxidation in *N. europaea* is selectively and irreversibly inhibited in a time-dependent fashion by low concentrations of acetylene (12, 13). The kinetic evidence suggests that acetylene acts as a suicide inactivator of ammonia monooxygenase. Having demonstrated that whole cells of *N. europaea* were capable of oxidizing alkanes up to C_8, we were interested in determining whether the differences in the amounts of alkanes oxidized were reflected in the rates at which the corresponding *n*-alkynes inactivated ammonia oxidation. All *n*-alkynes tested (up to C_{10}) inhibited O_2 consumption in a time-dependent fashion (Fig. 1). With hydrazine (600 μM) as the electron donor instead of ammonia, addition of an *n*-alkyne (C_2 through C_{10}; 40 μM) resulted in no more than a 10% inhibition of the rate of O_2 uptake (data not shown). Because hydrazine oxidation does not require ammonia monooxygenase (9), the effects of *n*-alkynes appeared to be specific for ammonia oxidation. The higher alkynes (C_6 and above) supported more rapid inactivations than the lower alkynes. Our data suggest that the sensitivity to alkyne inhibition is inversely related to the apparent affinity of ammonia monooxygenase for the corresponding *n*-alkane as a substrate. Apparently, interaction with the inhibitory triple bond occurs with relative infrequency when the bond is in a molecule the analogous alkane of which is oxidized rapidly. On the other hand, the triple bond appears to be a more reactive site when it is present in a molecule the corresponding alkane of which is oxidized slowly.

Whole cells of *N. europaea* can oxidize a wide range of simple hydrocarbons (4, 8–11, 14). These oxidations all appear to be catalyzed by ammonia monooxygenase for the following reasons. (i) The reaction products are compatible with a monooxygenase-catalyzed reaction (e.g., insertion of an O atom in a C–H or a C=C bond). (ii) The oxidations are inhibited by thiourea (7) and acetylene (12), specific inhibitors of ammonia oxidation. (iii) The amount of hydrocarbon oxidized depends on the ammonium ion concentration. Ammonia oxidation generates hydroxylamine, which in turn must be oxidized to resupply the reductant required for further monooxygenase activity (9). An alternative hydrocarbon substrate which competes with ammonia will therefore lower the rate of monooxygenase activity through indirect effects on the rate of supply of reductant to the enzyme. This explains the apparently contradictory relationship in which high ammonium concentrations (10 mM) support hydrocarbon oxidations better than lower, subsaturating concentrations (1 mM; K_m = 1.2 mM [9]). The dynamic nature of the interrelationship between competing substrates and the rate of supply of reductant to the monooxygenase also dictates that the relative rates of consumption of each substrate will not remain constant with time and will be dependent on both relative concentrations and affinities. This effect has been shown previously in studies of methane (9) and phenol oxidation (8). For this reason, the

TABLE 2. Oxidation of alkenes by whole cells of *N. europaea*

Substrate (state)[a]	Amt of substrate (μmol) per vial[b]	Products	μmol of product formed (per mg of cell protein) after 1 h of incubation with:				
			10 mM NH_4^+	1 mM NH_4^+	No reductant	10 mM NH_4^+ plus 8 μmol of C_2H_2	10 mM NH_4^+ plus 50 μM thiourea
Ethylene (g)	30	Ethylene oxide	1.54	0.83	0.37	0.01	0.02
Propylene (g)	70	Propylene oxide	1.48				
		2-Propene-1-ol	0.50				
		Both	1.98	1.30	0.45	0.06	0
1-Butene (g)	150	1,2-Epoxybutane	0.48				
		3-Butene-1-ol	0.06				
		3-Butene-2-ol	0.43				
		All three	0.97	0.54	0.45	0.06	0
1-Pentene (l)	170	1,2-Epoxypentane	0.19				
		4-Pentene-1-ol	0.18				
		4-Pentene-2-ol	0.29				
		All three	0.66	0.38	0.19	0.17	0.06
2-Butene (*cis*) (g)	150	*cis* 2,3-Epoxybutane	0.48				
		cis 2-Butene-1-ol	1.84				
		Both	2.32	1.96	0.93	0.08	0
2-Butene (*trans*) (g)	150	*trans* 2,3-Epoxybutane	0.10				
		trans 2-Butene-1-ol	2.15				
		Both	2.25	2.07	0.80	0.08	0

[a] g, Gas; l, liquid.
[b] Amount of each hydrocarbon which supported the formation of the greatest amount of product.

values presented in Tables 1 and 2 should not be regarded as either V_{max} rates or steady-state rates. Obviously, the choice of reductant and the incubation period will affect the amount of oxidation product formed (9, 11). However, many other factors will also influence the rate of oxidation of a particular hydrocarbon. These include the solubility of the substrate and the affinity of ammonia monooxygenase for the substrate. Furthermore, functional group selectivity (e.g., C–C versus C=C) and positional preference (e.g., C_1 versus C_2) by ammonia monooxygenase will also affect the identities and quantities of products formed.

This work further defines the similarities which exist between ammonia monooxygenase and methane monooxygenase from methanotrophs such as *Methylococcus capsu-*

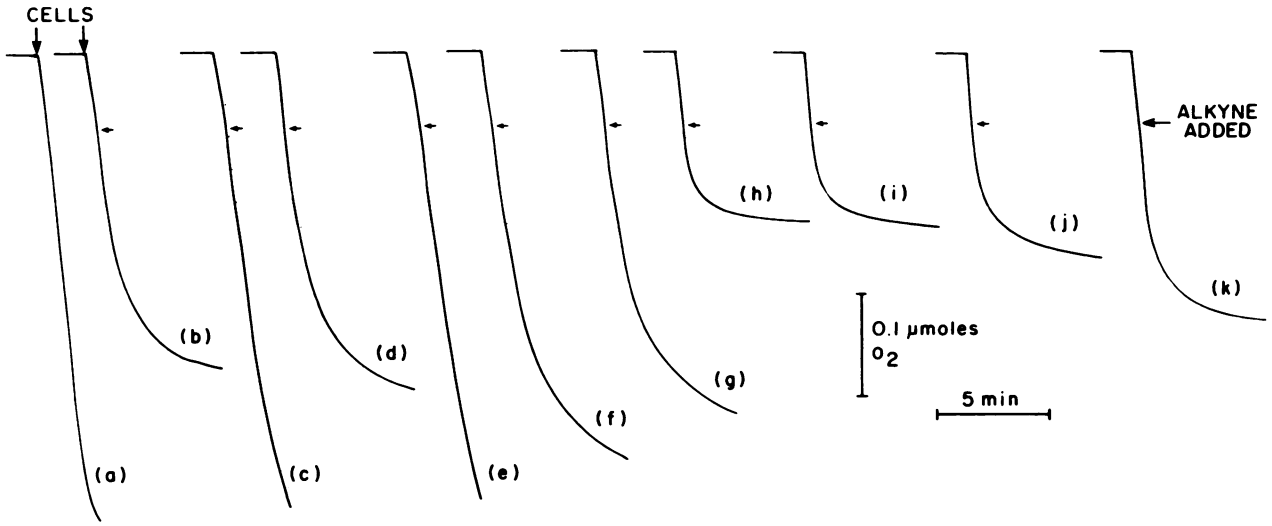

FIG. 1. Inhibition of ammonia-dependent O_2 uptake in whole cells of *N. europaea* by *n*-alkynes. Traces from a series of experiments conducted in an oxygen electrode are shown. In each experiment, 2 ml of 50 mM sodium phosphate buffer, pH 7.7, plus 2 mM $MgCl_2$ was added to the electrode chamber at 30°C. NH_4Cl was added to an initial concentration of 10 mM. The reaction was initiated by the addition of whole cells to a final concentration of 0.05 mg of protein per ml. After a steady rate of O_2 uptake was established, individual *n*-alkynes were added. a, No addition; b, acetylene; c and d, propyne; e and f, 1-butyne; g, 1-pentyne; h, 1-hexyne; i, 1-heptyne; j, 1-octyne; k, 1-decyne. In all cases, alkynes were added to a final concentration of 40 μM, except for traces (d) and (f) which represent concentrations of 300 μM. Alkynes for traces b to f were added from saturated aqueous solutions at 23°C. Alkynes for traces g to k were added from 10 mM stock solutions in dimethylsulfoxide.

latus (Bath) and *Methylosinus trichosporium* OB3b (1–3). Two forms of methane monooxygenase are known, a soluble NADH-linked form (1, 6) and a putatively copper-containing membrane-bound form (16, 17). Like ammonia monooxygenase, both enzymes oxidize ammonia and hydrocarbons other than methane (1). Ammonia monooxygenase is similar to membrane-bound methane monooxygenase in cellular location (17, 21), inhibitor profile (7, 20), molecular weight of the [^{14}C]acetylene-binding component (12, 18), and the putative involvement of copper (17, 19). However, their hydrocarbon substrate ranges are notably different. While it has been reported that the particulate methane monooxygenase is unable to oxidize aromatic compounds or alkanes larger than pentane (2), whole cells of *N. europaea* can oxidize benzene, phenol, and cyclohexane (4, 8) and alkanes up to octane (Table 1). These reactions distinguish ammonia monooxygenase from the particulate methane monooxygenase. In fact, the substrate range of ammonia monooxygenase would appear to be more similar to that of the soluble methane monooxygenase, which will oxidize alkanes up to C_{16}, alkenes, halocarbons, and aromatic and heterocyclic compounds (1, 3). The inhibition of ammonia monooxygenase by 1-decyne (Fig. 1) indicates that this enzyme can also interact with hydrocarbons larger than octane.

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