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Kinetic Mechanism and Substrate Specificity of Nitroalkane Oxidase

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Nitroalkane oxidase from *Fusarium oxysporum* catalyzes the oxidation of nitroalkanes to aldehydes, transferring the electrons to oxygen to form hydrogen peroxide. The steady-state kinetic patterns have been determined with nitroethane, 1-nitropropane, and 1-nitropentane as substrates. In all three cases, the data fit best to a ping pong kinetic mechanism. The pH dependences of the V/K values for 1-nitropentane and phenylnitromethane show that an amino acid residue on the enzyme with a pK_a value of 6.7 must be unprotonated for activity with both substrates. A second group must be protonated for activity. The pK_a value of this group matches the pK_a values of the nitroalkanes, 9.3 with nitropropane and 6.7 with phenylnitromethane, establishing that the nitroalkane must be in the neutral rather than the anionic form for catalysis.

Relatively little is known about biological systems for metabolizing nitroalkanes (1). Little (2) reported in 1951 that *Neurospora crassa* extracts were able to catalyze the oxidation of nitroethane to acetaldehyde and nitrite, using molecular oxygen. Several strains of *Streptomyces* have been reported to catalyze the oxidation of 2-nitropropane to acetone and nitrite (3). *Aspergillus flavus* has been reported to contain an enzyme able to form nitrite from β-nitropropionic acid (4). In addition, unidentified bacteria from the ruminal fluid of cattle are reported to be able to carry out the degradation of 3-nitropropanol, a component of a miserotoxin from milkvetch (5). In 1978 Kido et al. (6) reported the initial characterization of a nitroalkane oxidase from *Fusarium oxysporum.* This enzyme catalyzed the oxidation of primary and secondary nitroalkanes by molecular oxygen to yield the respective aldehyde or ketone, nitrite, and hydrogen peroxide. The enzyme was colorless, although it required added FAD or FMN for activity. No mechanistic studies of this enzyme have as yet been reported. We describe here initial studies of the kinetic mechanism and substrate specificity of this novel enzyme.

EXPERIMENTAL PROCEDURES

Aldehyde dehydrogenase from bakers yeast was from Sigma Chemical Company. Phenylnitromethane was synthesized using the method of Kornblum et al. (7). Nitroalkane oxidase was purified from *F. oxysporum* (ATCC 659) cells grown and induced with nitroethane essentially as described by Kido et al. (6).

Protein concentrations were determined by the method of Bradford (8) using bovine serum albumin as the standard. For analyzing the steady-state kinetics with nitroethane, 1-nitropropane, and 1-nitropentane, a coupled assay with aldehyde dehydrogenase was developed, following the increase in absorbance at 340 nm at 30 °C. Final concentrations of 10 μM FAD, 5 mM DTT, 1 mM NAD, 0.02 units of aldehyde dehydrogenase, and 0.2 M Tris-Cl, pH 8.0, were used in a volume of 1 ml. In order to prevent a prolonged lag time in the above reaction, it was necessary to preincubate nitroalkane oxidase with 50 μM FAD and 25 mM DTT at room temperature for 15 minutes. Stock substrate solutions were made in the appropriate buffer prior to use.

When both nitroalkane and oxygen were to be varied, the aldehyde dehydrogenase coupled assay was used on an Applied Photophysics Limited Model DX.17MV stopped-flow spectrofluorometer interfaced with an Acorn Archimedes 420/1 computer. The oxygen was removed from the enzyme reaction mixture described above by alternately

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TABLE 1
Steady-State Kinetic Parameters for Nitroalkane Oxidase[a]

Substrate	K_a (mM)	V/K_a (mM^{-1} min^{-1})	K_{O_2} (μM)	V/K_{O_2} (mM^{-1} min^{-1})	V (min^{-1})
Nitroethane	2.7 ± 0.10	11.3 ± 0.22	20.3 ± 1.7	1500 ± 100	30.4 ± 0.7
1-nitropropane	0.117 ± 0.008	453 ± 23	41.2 ± 3.3	1290 ± 80	53.1 ± 1.5
1-nitropentane	0.0060 ± 0.0010	6060 ± 890	26.5 ± 2.1	1390 ± 90	36.9 ± 1.0
Phenylnitromethane[b]	0.010 ± 0.003	1600 ± 450	ND[c]	ND[c]	16.0 ± 1.3

[a] pH 8.0, 30°C.
[b] pH 7.0, 30°C.
[c] Not determined.

filling and evacuating the vessel containing the reaction mixture with purified nitrogen obtained by passage through a column of BASF catalyst heated to 120 °C. Different oxygen concentrations were bubbled through the substrate solutions for at least 10 minutes to obtain final oxygen concentrations of 0.03, 0.06, 0.12, and 0.6 mM. The concentrations of the nitroalkanes varied from 20 μM to 5 mM. The stopped-flow apparatus was thermostated at 30 °C and the reaction was monitored for 50 - 200 seconds after mixing. A linear least squares fit was made to the straight portion of the curve and used to calculate the rate. Initial rates were determined in triplicate.

The assay developed for phenylnitromethane oxidation followed the decrease in absorbance at 290 nm due to the conversion of phenylnitromethane to benzaldehyde. The difference in the extinction coefficients at this wavelength was determined to be dependent on the ionization state of the phenylnitromethane, with a limiting value of 15.0 $mM^{-1}cm^{-1}$ at high pH and a pK value for the extinction coefficient of 6.7. The final concentrations of nitroalkane oxidase, FAD, and DTT were the same as in the coupled assay.

When the pH was varied, the following buffers were used at a concentration of 0.2 M: MES, pH 5.5 - 6.5; MOPS, pH 6.5 - 7.5; HEPES, pH 7.5 - 8.5; AMPSO, pH 8.5 - 9.5; and glycine, pH 9.0 - 10.0. The buffers were adjusted to the necessary pH with KOH.

Kinetic data were fit to equations 1 - 4 of Cleland (9) using the KinetAsyst software (IntelliKinetics, State College, PA). When only one substrate was varied the data were fit to equation 1. When both substrates were varied, the data were fit to equations 2 and 3, where K_a is the K_m value for the nitroalkane and K_{O_2} is the K_m value for oxygen. The pH-rate profiles were fit to equation 4, where C is the pH-independent V/K value.

$$v = \frac{VA}{K_a + A} \tag{1}$$

$$v = \frac{VAO_2}{K_aO_2 + K_{O_2}A + AO_2} \tag{2}$$

$$v = \frac{VAO_2}{K_{ia}K_{O_2} + K_aO_2 + K_{O_2}A + AO_2} \tag{3}$$

$$\mathrm{Log}(V/K) = \log\left(\frac{C}{1 + \frac{[H]}{K_a} + \frac{K_b}{[H]}}\right) \tag{4}$$

RESULTS

Kinetics. Steady-state kinetic parameters were determined with nitroethane, 1-nitropropane, and 1-nitropentane as substrates. Both the nitroalkane and oxygen concentrations were varied at pH 8.0. Table 1 summarizes the kinetic data with all three substrates. The data for all three substrates fit best to equation 2, which describes a ping pong kinetic mechanism. Fitting the data to equation 3, which describes a sequential mechanism, did not improve the statistical quality of the fit and gave values for K_{ia} which were not significantly different from zero. 1-Nitropropane had the highest V_{max} value; however, nitroethane and 1-nitropentane were both

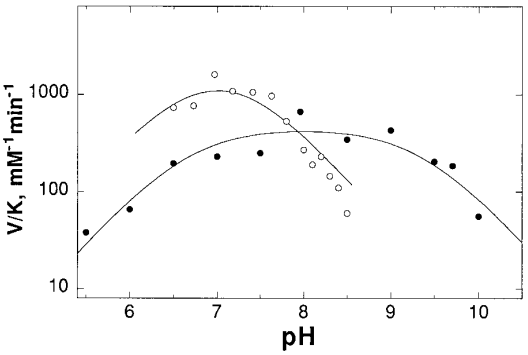


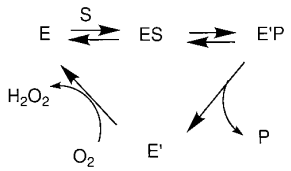
FIG. 1. pH Dependence of the V/K values for 1-nitropropane (●) and phenylnitromethane (○). The lines are from fits of the data to equation 4.

reasonably good substrates. The K_m values for the three substrates decreased with increasing length of the carbon chain. In contrast, the K_m values for oxygen for all three substrates were comparable. The V/K_{O_2} values did not differ significantly among all three substrates.

pH Dependence. The effects of pH on the V/K values for both 1-nitropropane and phenylnitromethane were determined. Nitroalkane oxidase and aldehyde dehydrogenase were sufficiently stable between pH 5.5 and 10.5 to use the aldehyde dehydrogenase coupled assay over this range. The pH dependence of the V/K value for 1-nitropropane fit best to equation 4 (Figure 1), consistent with the involvement of two ionizable groups. One group with an apparent pK_a of 6.7 $\pm$ 0.16 must be deprotonated for activity and one with an apparent pK_a of 9.3 $\pm$ 0.18 must be protonated. This upper pK_a value is close to the pK_a value of the nitropropane α proton of 8.98 (10). In order to test whether the higher pK_a value was indeed due to the substrate, the pH dependence of the V/K value for phenylnitromethane, with a pK_a of 6.8, was determined. The V/K values for phenylnitromethane similarly fit best to equation 4, indicating the involvement of two ionizable groups (Figure 1). However, the two pK_a values were too close together to resolve, having an average value of 6.7 $\pm$ 0.065. This is the expected result if a group on the enzyme surface with a pK_a value of 6.7 must be deprotonated and phenylnitromethane with a pK_a value of 6.8 must be protonated. These results are thus consistent with the neutral form of the nitroalkane being the active form of the substrate.

DISCUSSION

Nitroalkane oxidase from *F. oxysporum* is the only enzyme described to date which appears to carry out a simple oxidase reaction on nitroalkanes as its physiological role. The flavoproteins D-amino acid oxidase (11,12) and glucose oxidase (13) and the heme protein horseradish peroxidase (14) have been reported to catalyze the oxidation of nitroalkanes, but these are clearly not the physiological reactions. In contrast, the ability of nitroethane to induce nitroalkane oxidase production by *F. oxysporum* (6) is strong evidence that the physiological role of this enzyme is nitroalkane oxidation. While the oxidative denitrification of nitroalkanes by horseradish peroxidase and glucose oxidase appears to be a dioxygenase reaction involving radical intermediates, the reaction catalyzed by D-amino acid oxidase is an oxidase reaction similar to that catalyzed by nitroalkane oxidase. However, nitroalkane oxidation by D-amino acid oxidase requires the nitroalkane anion (12). In contrast, the data presented here establish that nitroalkane oxidase requires the neutral nitroalkane as substrate. This conclusion is based upon the identity of the upper pK_a value seen in the V/K pH profile with the pK_a values of 1-nitropropane and phenylnitromethane. The shift of three pH units in this pK_a value, from



SCHEME 1

9.3 to 6.7, is much too large to be attributed to a kinetic complexity such as a high commitment to catalysis, which can perturb pK_a values determined from kinetics (15). Indeed, the lack of a change in the lower pK_a value of 6.7 suggests that nitropropane and phenylnitromethane are not sticky substrates, so that their K_m values approximate their K_d values. A reasonable role for the residue with the pK_a value of 6.7 is to act as the base for abstracting the substrate α-proton.

The steady state kinetic data described here are consistent with a ping pong kinetic mechanism for nitroalkane oxidase (Scheme 1). In such a mechanism the nitroalkane would first be oxidized to the aldehyde P. After release of the aldehyde to solution, the reduced enzyme E′ would then react with oxygen to form hydrogen peroxide. However, it can be difficult to distinguish ping pong and sequential mechanisms based solely on initial velocity patterns if the K_{ia} term in equation 3 is small (15-17). In addition, many flavoprotein oxidases exhibit ping pong kinetic patterns but do not utilize such a mechanism (18). Instead, oxygen reacts with the enzyme after substrate oxidation has occurred but before product release. Irreversibility of the reductive half reaction can generate apparently ping pong kinetics. A sensitive test for whether the second substrate in a multiple substrate reaction reacts with an enzyme species which still has product or substrate bound is to determine the effect of changing the first substrate upon the V/K value for the second substrate (16,17). In the mechanism of Scheme 1, oxygen reacts with the same enzyme species no matter which nitroalkane is used as substrate. Since the V/K_{O_2} value is the apparent second order rate constant for the reaction of oxygen with the enzyme, its value will be unaffected by changing the nitroalkane if Scheme 1 is correct. The data in Table 1 show that the V/K value for oxygen is unaffected when the nitroalkane is changed, despite a change in the value of the V/K value for the nitroalkane of almost three orders of magnitude. The most straightforward explanation for the lack of a change in the V/K_{O_2} value and for the parallel line kinetics is a kinetic mechanism in which the nitroalkane is first oxidized to the aldehyde, which then dissociates from the enzyme. The reduced enzyme would then react with O_2 to form hydrogen peroxide.

While the range of substrates characterized here is not extensive, the data are consistent with a hydrophobic binding site for nitroalkanes in the enzyme. Thus, the K_m value for the nitroalkane decreases 23-fold on going from nitroethane to 1-nitropropane, and another 19-fold for 1-nitropentane. The ability to use phenylnitromethane as a substrate is further evidence for a hydrophobic binding site.

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