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# Solvent isotope and viscosity effects on the steady-state kinetics of the flavoprotein nitroalkane oxidase

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ABSTRACT

**The flavoprotein nitroalkane oxidase catalyzes the oxidative denitrification of a broad range of primary and secondary nitroalkanes to yield the respective aldehydes or ketones, hydrogen peroxide and nitrite. With nitroethane as substrate the $^{D2O}(k_{cat}/K_M)$ value is 0.6 and the $^{D2O}k_{cat}$ value is 2.4. The $k_{cat}$ proton inventory is consistent with a single exchangeable proton in flight, while the $k_{cat}/K_M$ is consistent with either a single proton in flight in the transition state or a medium effect. Increasing the solvent viscosity did not affect the $k_{cat}$ or $k_{cat}/K_M$ value significantly, establishing that nitroethane binding is at equilibrium and that product release does not limit $k_{cat}$.**
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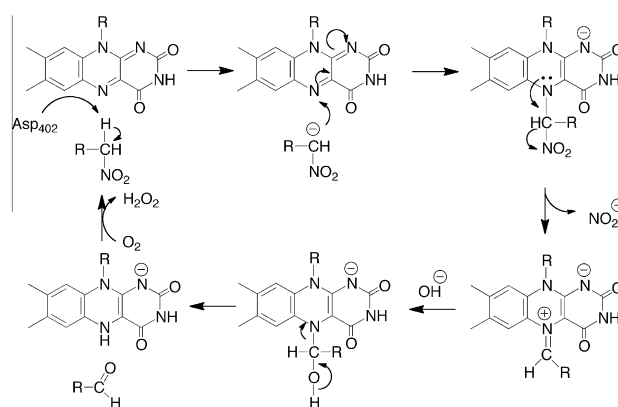
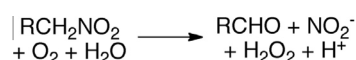
Nitroalkane oxidases are flavoproteins that catalyze the oxidation of primary and secondary nitroalkanes to the corresponding aldehydes or ketones (Scheme 1). Nitroalkane oxidase activity was first identified in the soil fungus *Fusarium oxysporum*[1], but has since been identified in other fungi [2]. Extensive mechanistic and structural studies of the enzyme from *F. oxysporum* have been carried out to date, including determination of structures of reaction intermediates [3] and mutagenesis of active site residues [3–6]. These studies have provided support for the mechanism shown in Scheme 2. Here, the neutral nitroalkane is the active form of the substrate in solution. The active site residue Asp402 acts as an active site base to form the anionic nitroalkane; this is supported both by the effects of mutating this residue [4,7] and by structures of nitroalkanes bound to mutant enzymes with low catalytic activity[3,8]. The nitroalkane anion then attacks the FAD cofactor at the N5 position to form an adduct that eliminates nitrite to form a cationic imine; the latter species has been trapped and its structure determined [3,9]. Finally, attack of hydroxide on the imine leads to formation of the aldehyde product and reduced flavin. The reoxidation of the flavin by molecular oxygen forms hydrogen peroxide in a reaction typical of flavoprotein oxidases [10,11].




Scheme 2.




Scheme 1.

The first step in the nitroalkane oxidase reaction is the removal of a proton from a carbon of the substrate. The corresponding non-enzymatic ionization of nitroalkanes has served as a model

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for proton abstraction from carbon because the relatively low $pK_a$ of the alpha carbon makes the non-enzymatic reaction readily accessible [12,13]. When nitroethane is the substrate for nitroalkane oxidase, the proton abstraction from the substrate by Asp402 is rate-limiting for the reductive half-reaction [14,15]. This has allowed a direct comparison between the reaction in solution and the reaction catalyzed by the enzyme [16], providing evidence for the contributions of substrate desolvation and quantum mechanical tunneling to the billion-fold rate enhancement by the enzyme [8,17].

In the present manuscript we describe the effects of the solvent on the steady-state kinetic parameters of nitroalkane oxidase. The effect of $D_2O$ on the kinetics provides insight into the role of transfer of exchangeable protons during the reaction [18], while the effect of solvent viscosity provides insight into the contribution of diffusive steps to the kinetics [19].

## 1. Experimental procedures

### 1.1. Materials

Nitroethane was from Sigma–Aldrich. Deuterium oxide (99.9%) was from Cambridge Isotope Laboratories, Inc. Nitroalkane oxidase was purified from *F. oxysporum* (ATCC 695) as described by Gadda and Fitzpatrick [20,21] and stored at −70 °C. All other reagents were of the highest purity commercially available.

### 1.2. Enzyme assays

Enzyme activity was measured in the presence of 0.5 mM FAD in air-saturated buffer by monitoring the rate of oxygen consumption with a computer-interfaced Hansatech oxygen monitoring system thermostatted at 30 °C as described previously [15]. Assays were started by adding 10 μl of enzyme in aqueous buffer and 10 μl of nitroethane in dimethyl formamide or ethanol (to prevent ionization of the substrate) to a total volume of 1 ml. For solvent isotope effect analyses, the buffer components were prepared directly in $D_2O$ and the pD was adjusted with NaOD, KOD, or DCl. pD values were determined by adding 0.4 to the pH electrode reading [18]. Potassium phosphate (50 mM) was used as the buffer over the pD range 7–8, and 50 mM sodium pyrophosphate was used between pD 8 and 10.2. For proton inventories, the mole fraction of $D_2O$, $n$, was determined by combining the appropriate volumes of buffer in $D_2O$ and $H_2O$ [18]. For solvent viscosity studies, the relative $k_{cat}$ and $k_{cat}/K_M$ values at each viscosity were measured in 50 mM sodium pyrophosphate, pH 8.5, containing varying concentrations of glycerol as the viscosigen.

### 1.3. Data analysis

Data were fit using KaleidaGraph (Synergy). The steady-state kinetic parameters of nitroalkane oxidase were determined by fitting the data to the Michaelis–Menten equation [22,23] or to Eq. (1) in cases where substrate inhibition was seen. The pH-dependences of steady state kinetic parameters were determined by fitting $k_{cat}$ and $k_{cat}/K_M$ values as a function of pH to Eqs. (2) and (3)[24]. Eq. (2) was used to fit data from pH-profiles that decreased with unit slope at low pH. Eq. (3) was used to fit data that decreased with unit slope at both low and high pH. K1 and K2 are the dissociation constants for ionization of groups that must be unprotonated and protonated for catalysis, respectively, and C is the pH-independent value of the kinetic parameter of interest. Solvent isotope effects were calculated by fitting the data to Eq. (4)[25], which describes separate isotope effects on $k_{cat}$ and $k_{cat}/K_M$ values, or Eq. (5), which includes the effect of substrate inhibition. $F_i$ is the fraction of deuterium label in the solvent, and $E_{VK}$, $E_V$ and $E_{Ki}$ are the isotope effects on the $k_{cat}/K_M$,$k_{cat}$ and $K_i$ values, respectively. To determine the effects of solvent viscosity on the steady-state kinetic parameters, the relative $k_{cat}$ and $k_{cat}/K_M$ values at each viscosity were fit to Eq. (6), where $Y_0$ is the $k_{cat}$ or $k_{cat}/K_M$ value in the absence of a viscosigen, $Y_\eta$ is the value at a specific viscosity, $\eta_{rel}$ is the viscosity relative to water and $m$ is the slope of the line indicating the degree of diffusion-limitation. The relative viscosities of solutions containing varying amounts of glycerol were determined from literature values [26].

$$v = \frac{k_{cat}A}{K_a + A + A^2/K_i} \quad (1)$$

$$\mathrm{Log}Y = \mathrm{Log}\frac{C}{1 + \frac{H}{K_1}} \quad (2)$$

$$\mathrm{Log}Y = \mathrm{Log}\frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}} \quad (3)$$

$$v = \frac{k_{cat}A}{K_a(1 + F_i(E_{VK} - 1)) + A(1 + F_i(E_v - 1))} \quad (4)$$

$$v = \frac{k_{cat}A}{K_a(1 + F_i(E_{VK} - 1)) + A(1 + F_i(E_v - 1)) + (A^2/K_i)(1 + F_i(E_{K_i} - 1))} \quad (5)$$

$$\frac{Y_0}{Y_\eta} = 1 + m\eta_{rel} \quad (6)$$

## 2. Results

### 2.1. Solvent isotope effects

Solvent isotope effects on the reaction catalyzed by nitroalkane oxidase were determined to probe the timing of steps involving solvent exchangeable protons. All assays were carried out in air-saturated buffers, which contain 230 μM oxygen at 30 °C; since the $K_M$ value for oxygen is 20 μM when nitroethane is the substrate for nitroalkane oxidase [27], both $k_{cat}$ and $k_{cat}/K_M$ values can readily be determined under these conditions. As a necessary prerequisite, the effects of $D_2O$ on the pH dependences of the $k_{cat}/K_M$ and $k_{cat}$ values with nitroethane as substrate were determined over the accessible pH range (Fig. 1). Nitroalkane oxidase shows substrate inhibition at high concentrations of nitroethane, with a value of 30 mM, 10-fold the $K_M$ value for the substrate [28]. The substrate inhibition was more pronounced in $D_2O$ (results not shown). In $H_2O$ the $k_{cat}/K_M$ pH profile is bell-shaped; Asp402, the active site base, is responsible for the lower $pK_a$ value, while the upper $pK_a$ is likely due to the flavin cofactor [4]. The $k_{cat}$ profile in $H_2O$ is also bell-shaped. The $pK_a$ values determined from the data in Fig. 1 (Table 1) agree with previously reported pH profiles for this enzyme [15]. When assays are carried out in $D_2O$, all the $pK_a$ values are shifted upward (Fig. 1, Table 1), as is expected from the known effects of $D_2O$ on $pK_a$ values [18]. In the $k_{cat}/K_M$ profile in $D_2O$ only a single $pK_a$ value for a protein group that must be unprotonated for catalysis is seen (Fig. 1A). The $pK_a$ value on the basic limb of the curve is shifted above pH 10 in $D_2O$; initial rates could not be measured at high enough values to define this $pK_a$ value due to the instability of the enzyme at high pH. In contrast, both $pK_a$ values are seen in the $k_{cat}$ profile in $D_2O$ (Fig. 1B). The solvent isotope effects on both the $k_{cat}/K_M$ and $k_{cat}$ values can be calculated from the pH-independent values of each kinetic parameter in $H_2O$ and $D_2O$. This gives a normal solvent

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