Probing the Chemical Steps of Nitroalkane Oxidation Catalyzed by 2-Nitropropane Dioxygenase with Solvent Viscosity, pH, and Substrate Kinetic Isotope Effects†

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ABSTRACT: Among the enzymes that catalyze the oxidative denitrification of nitroalkanes to carbonyl compounds, 2-nitropropane dioxygenase is the only one known to effectively utilize both the neutral and anionic (nitronate) forms of the substrate. A recent study has established that the catalytic pathway is common to both types of substrates, except for the initial removal of a proton from the α carbon of the neutral substrates [Francis, K., Russell, B., and Gadda, G. (2005) *J. Biol. Chem. 280*, 5195−5204]. In the present study, the mechanistic properties of the enzyme have been investigated with solvent viscosity, pH, and kinetic isotope effects. With nitroethane or ethylnitronate, the k_{cat}/K_m and k_{cat} values were independent of solvent viscosity, consistent with the substrate and product binding to the enzyme in rapid equilibrium. The abstraction of the proton from the α carbon of neutral substrates was investigated by measuring the pH dependence of the $^{D}(k_{cat}/K_{NE})$ value with 1,1-[$^2H_2$]-nitroethane. The formation of the enzyme-bound flavosemiquinone formed during catalysis was examined by determining the pH dependence of the k_{cat}/K_m values with ethylnitronate and nitroethane and the inhibition by *m*-nitrobenzoate. Finally, α-secondary kinetic isotope effects with 1-[^{2}H]-ethylnitronate were used to propose a non-oxidative tautomerization pathway, in which the enzyme catalyzes the interconversion of nitroalkanes between their anionic and neutral forms. The data presented suggest that enzymatic turnover of 2-nitropropane dioxygenase with neutral substrates is limited by the cleavage of the substrate CH bond at low pH, whereas that with anionic substrates is limited by the non-oxidative tautomerization of ethylnitroante to nitroethane at high pH.

2-Nitropropane dioxygenase (E.C. 1.13.11.32) from *Neurospora crassa* is a flavin mononucleotide (FMN)-dependent enzyme that catalyzes the oxidative denitrification of nitroalkanes to their corresponding carbonyl compounds and nitrite (*1*).[1] Among the enzymes that are known to oxidize nitroalkanes, which comprise nitroalkane oxidase, horseradish peroxidase, D-amino acid oxidase, glucose oxidase, and propionate-3-nitronate oxidase (*2*−*6*), 2-nitropropane dioxygenase is unique in that it can effectively utilize both the neutral and anionic nitronate forms of the substrate (Scheme 1) (*7*). The study of the biochemical and mechanistic properties of 2-nitropropane dioxygenase is of considerable interest for both applied and fundamental reasons. In industrial applications, nitroalkanes are widely used as synthetic intermediates (*8*, *9*); however, many are anticipated to be toxic or carcinogenic (*10*−*15*). The enzymatic oxidation of nitroalkanes into less toxic species can therefore be exploited in bioremediation applications. From a fundamental standpoint, 2-nitropropane dioxygenase is the only reported flavin-dependent enzyme in which a transient anionic flavosemiquinone species has been observed in catalysis (*1*). Consequently, the enzyme serves well as a model system to understand the reactivity of anionic flavosemiquinones in the enzymatic catalysis of flavoproteins.

The crystal structure of 2-nitropropane dioxygenase from *Pseudomonas aeruginosa* has been recently solved both for the free enzyme and the enzyme in complex with 2-nitropropane (*16*). A conserved histidine is located in the active site of the bacterial enzyme near the α carbon of 2-nitropropane and has been proposed to act as the catalytic base for the oxidation of neutral nitroalkanes. In the enzyme from

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[1] As pointed out by one of the reviewers, the mechanistic data presented in this and a previous study (*1*) suggest that 2-nitropropane dioxygenase may be a misnomer for the nitroalkane-oxidizing enzyme from *N. crassa*, because the reaction mechanism is not a dioxygenase reaction and the enzyme is not the most specific for 2-nitropropane (*1*). In contrast, the available evidence suggest that the enzyme, which has broad substrate specificity (*1*), catalyzes a monooxygenase reaction in which a single oxygen atom from molecular oxygen is incorporated into the organic product of the reaction, suggesting that the enzyme may be properly named as nitroalkane monooxygenase. However, as pointed out by the same reviewer, it is not even proven to be a monooxygenase, because this would require isotopic-labeling experiments showing that the aldehyde oxygen atom incorporated in the product of the reaction originates from molecular oxygen, which might not be conclusive because of the expected rapid exchange of the aldehyde oxygen with the solvent as a result of hydration−dehydration equilibrium. For these reasons, we prefer to refer to the enzyme with its official IUBMB name, 2-nitropropane dioxygenase.

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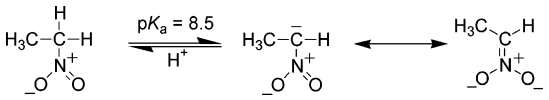
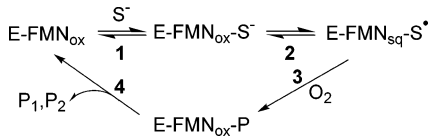
Scheme 1 Ionization of Nitroethane (Left) To Yield Ethylnitronate (Right) in Solution

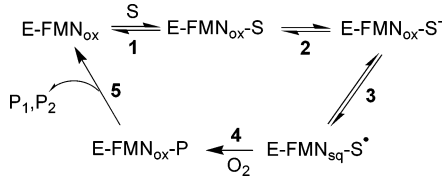


Scheme 2: Minimal Steady-State Kinetic Mechanism with Nitronates as the Substrate[a]



[a] E, enzyme; FMN_{ox}, oxidized flavin; FMN_{sq}, flavin semiquinone; S^-, anionic form of nitroalkane; $S^{\cdot}$, radical form of nitroalkane; P_1 and P_2, organic product of the reaction and nitrite.

Scheme 3: Minimal Steady-State Kinetic Mechanism with Nitroalkanes as the Substrate[a]



[a] E, enzyme; FMN_{ox}, oxidized flavin; FMN_{sq}, flavin semiquinone; S, neutral form of nitroalkane; S^-, anionic form of nitroalkane; $S^{\cdot}$, radical form of nitroalkane; P_1 and P_2, organic product of the reaction and nitrite.

N. crassa, this conserved residue is His196, which likely plays a similar role in the yeast enzyme.

The steady-state kinetic mechanism of 2-nitropropane dioxygenase has been recently elucidated with both the neutral and anionic forms of nitroethane, nitrobutane, nitrohexane, and 2-nitropropane (*1*). With nitronates as the substrate (Scheme 2), the formation of the enzyme−substrate complex (**1**) is followed by the transfer of a single electron from the organic substrate to the enzyme-bound flavin (**2**), yielding an anionic flavosemiquinone species ($E-FMN_{sq}-S^{\cdot}$). Molecular oxygen then reacts with the $E-FMN_{sq}-S^{\cdot}$ complex to oxidize the flavosemiquinone (**3**), resulting in the formation of the product of the reaction, which is subsequently released from the enzyme (**4**). Catalysis with nitroalkane substrates (Scheme 3) is initiated by an enzyme-catalyzed proton abstraction from the substrate α carbon (**2**) to yield an enzyme-bound alkyl nitronate. This species is then oxidized through the same pathway described above. In the present study, solvent viscosity, pH, and deuterium kinetic isotope effects with neutral and anionic nitroethane (ethylnitronate) as the substrate have been used to gain further insights into the mechanism of nitroalkane oxidation catalyzed by 2-nitropropane dioxygenase and the kinetic steps that limit enzymatic turnover with neutral and anionic substrates. A non-oxidative tautomerization pathway, in which the enzyme converts the substrate from its anionic to neutral forms, is proposed on the basis of measurements of α-secondary kinetic isotope effects at saturating oxygen concentrations.

MATERIALS AND METHODS

Materials. 2-Nitropropane dioxygenase was obtained through the expression and purification protocols described previously (*1*). Nitroethane, 1,1-[$^2H_2$]-nitroethane, and *m*-nitrobenzoic acid were from Sigma-Aldrich. All other reagents were of the highest purity commercially available.

Methods. Enzyme activity was measured with the method of initial rates (*17*) in air-saturated buffer containing 1% ethanol, by monitoring the rate of oxygen consumption with a computer interfaced Oxy-32 oxygen-monitoring system (Hansatech Instrument Ltd.), equipped with a water bath thermostated at 30 °C. The enzyme concentration was expressed per enzyme-bound FMN content, using an $\epsilon_{444\ nm}$ value of 11 850 M^{-1} cm^{-1} (*1*), and ranged from 0.02 to 3.9 μM. Stock solutions of nitroethane were prepared in 100% ethanol. The nitronate form of the substrate was prepared through a reaction of the nitroalkane with a 1.2 molar excess of potassium hydroxide for 24 h at room temperature. To minimize changes in the ionization state of the nitroalkane, enzymatic assays were initiated by the addition of the substrate to the assay reaction mixture.[2] When both the organic substrate and oxygen were varied, the assay mixtures were equilibrated with the appropriate O_2/N_2 gas mixture by bubbling the gas for at least 10 min, before the reaction was started with the addition of the enzyme and the organic substrate. Substrate concentrations ranged from 0.5 to 10 mM. When the pH was varied, 50 mM sodium pyrophosphate was used as a buffer between pH 5.5 and 10. Deuterium substrate kinetic isotope effects were determined using 1,1-[$^2H_2$]-nitroethane in air-saturated buffers or 1-[^2H]-ethylnitronate at varying concentrations of both the organic substrate and oxygen. Activity assays were carried out by alternating substrate isoptomers, and $^D(k_{cat}/K_m)$ values were calculated from the ratio of the kinetic parameter obtained with the unlabeled substrate to that with the labeled substrate. The effect of solvent viscosity on the kinetic parameters of the enzyme was determined in 50 mM sodium pyrophosphate at pH 10 with nitroethane and at pH 6.5 with ethylnitronate using glycerol as viscosigen. The relative viscosities at 30 °C were calculated from the values at 20 °C reported by Lide (*18*).

The reductive half-reaction of 2-nitropropane dioxygenase with ethylnitronate as the substrate was monitored using a Hi-Tech SF-61 stopped-flow spectrophotometer thermostated at 30 °C. The rate of flavin reduction was measured by monitoring the increase in absorbance at 370 nm that results from anaerobic mixing of the enzyme with the substrate at pH 8.5. A ∼28 μM solution of 2-nitropropane dioxygenase was loaded into a tonometer and was made anaerobic by a 25 cycle treatment of vacuuming and flushing with oxygen-free argon (pretreated with an oxygen-scrubbing cartridge, Agilent, Palo Alto, CA). The anaerobic enzyme solution was then mounted onto the stopped-flow instrument, which had been pretreated with an oxygen-scrubbing system composed of 5 mM glucose and 450 units of glucose oxidase. Ethylnitronate was prepared in water and was made anaerobic by flushing with oxygen-free argon for at least 20 min before mounting onto the stopped-flow spectrophotometer. 2-Ni-

[2] The second-order rate constant for deprotonation of nitroethane has a value of 6 M^{-1} s^{-1} (*23*), ensuring that in assays initiated with the neutral nitroalkane a negligible amount of anionic substrate is present during the time required to determine initial rates (typically ∼30 s). Similarly, the second-order rate constant for protonation of ethylnitronate is 15 M^{-1} s^{-1} (*31*), ensuring a negligible amount of neutral substrate in assays initiated with fully unprotonated alkyl nitronates.

tropropane dioxygenase was mixed anaerobically with an equal volume of substrate to give a reaction mixture containing ~14 μM enzyme and 2−50 mM ethylnitronate. For each concentration of substrate, traces were recorded in triplicate and the average value is reported (measurements typically differed by ≤5%).

Data Analysis. Steady-state kinetic data were fit with KaleidaGraph software (Synergy Software, Reading, PA) or Enzfitter software (Biosoft, Cambridge, U.K.). Kinetic parameters determined under atmospheric oxygen were obtained by fitting the data to the Michaelis−Menten equation for one substrate. When the saturation of 2-nitropropane dioxygenase with the organic substrate was not attained, the data were fit with eq 1

$$\frac{v}{e} = \frac{k_{cat}}{K_a}A \tag{1}$$

where k_{cat}/K_a is the second-order rate constant for the reaction with the organic substrate, A is the concentration of the organic substrate, and e is the concentration of the enzyme. When initial rates were determined by varying the concentrations of both the nitroalkane substrate and oxygen, the kinetic data were fit to eq 2

$$\frac{v}{e} = \frac{k_{cat}AB}{K_aB + K_bA + AB + K_{ia}K_b} \tag{2}$$

which describes a sequential steady-state kinetic mechanism. The dissociation constant (K_{is}) for the inhibition of 2-nitropropane dioxygenase by *m*-nitrobenzoate with respect to ethylnitronate as the substrate was determined by fitting the data with eq 3

$$\frac{v}{e} = \frac{k_{cat}A}{K_a\left[1 + \left(\frac{I}{K_{is}}\right)\right] + A} \tag{3}$$

which describes a competitive inhibition pattern, where I is the concentration of the inhibitor. The pH dependence of the steady-state kinetic parameters was determined by fitting initial rate data to eq 4

$$\log Y = \log\left(\frac{C}{1 + \frac{10^{-pH}}{10^{-pK_{a1}}} + \frac{10^{-pK_{a2}}}{10^{-pH}}}\right) \tag{4}$$

which describes a bell-shaped curve with a slope of +1 at low pH and a slope of −1 at high pH, where C is the pH-independent value of the kinetic parameter of interest. The pH dependence of the $^{D}(k_{cat}/K_m)$ values were determined by fitting the data with eq 5

$$\log Y = \log\frac{Y_L + Y_H\left(\frac{10^{-pK_a}}{10^{-pH}}\right)}{1 + \left(\frac{10^{-pK_a}}{10^{-pH}}\right)} \tag{5}$$

which describes a curve with plateau regions at both high and low pH. Y_L and Y_H are the limiting values at low and high pH, respectively, and K_a is the dissociation constant for the ionizable groups. The pH dependence of *m*-nitrobenzoate inhibition with respect to ethylnitronate as the substrate was determined by fitting the data with eq 6

$$\log Y = \log\left(\frac{C}{1 + \frac{10^{-pK_a}}{10^{-pH}}}\right) \tag{6}$$

which describes a curve with a slope of −1 and a plateau region at low pH. The viscosity effects on the kinetic parameters of the enzyme with nitroethane or ethylnitronate as the substrate were fit to eq 7

$$\frac{k_o}{k_\eta} = S(\eta_{rel} - 1) + 1 \tag{7}$$

where $(k)_o$ and $(k)_\eta$ are the kinetic parameters of interest determined in the absence and presence of viscosigen, respectively, S is the degree of viscosity dependence, and η_{rel} is the relative viscosity. Stopped-flow traces were fit with eq 8

$$A_{total} = A_te^{-k_{obs}t} + A \tag{8}$$

which describes a single-exponential process, where k_{obs} is the first-order rate constant for flavin reduction, t is time, A_t is the absorbance at time t, and A is the final absorbance. Pre-steady-state kinetic parameters were determined using eq 9

$$k_{obs} = \frac{k_{red}S}{K_d + S} \tag{9}$$

where k_{obs} is the observed rate of flavin reduction, k_{red} is the limiting rate of flavin reduction at saturating substrate concentrations, K_d is the dissociation constant, and S is the concentration of the substrate.

RESULTS

Reductive Half-Reaction with Ethylnitronate as the Substrate for 2-Nitropropane Dioxygenase. An anionic flavosemiquinone was previously observed during both static mixing and turnover reactions of 2-nitropropane dioxygenase with either ethylnitronate or nitroethane as the substrate and was proposed to react with oxygen to generate superoxide (*1*). An alternative possibility is that the flavosemiquinone and the resulting substrate radical react to generate a flavin N(5) adduct, similar to that seen in nitroalkane oxidase (*19*). To test for this possibility, the reductive half-reaction of 2-nitropropane dioxygenase with ethylnitronate as the substrate was monitored at pH 8.5 and 30 °C, because a flavin N(5) adduct with typical absorbance in the 320−360 nm region of the absorbance spectrum would be formed in the absence of oxygen. As shown in Figure 1A, anaerobic mixing of the enzyme with the substrate rapidly generated an anionic flavosemiquinone with peaks centered at 370 and 473 nm. A plot of k_{obs} versus the substrate concentration showed hyperbolic behavior with a limiting rate of flavin reduction (k_{red}) of ~380 s^{-1} and a K_d for ethylnitronate binding of ~40 mM. Under the same conditions, the k_{cat} value with ethylnitronate was ~70 s^{-1}, establishing the transient anionic flavosemiquinone as a catalytically relevant species. As

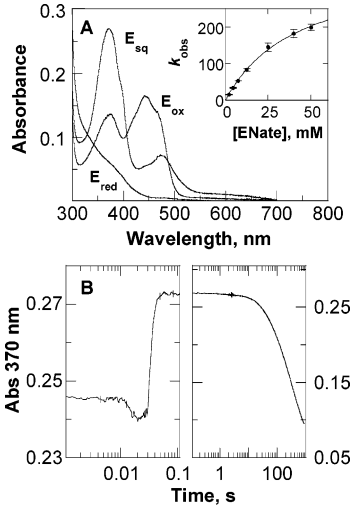


FIGURE 1: Reductive half-reaction of 2-nitropropane dioxygenase with ethylnitronate as the substrate. (A) UV−vis absorbance spectra of 2-nitropropane dioxygenase in its oxidized, semiquinone, and hydroquinone forms, observed in the stopped-flow spectrophotometer. An anaerobic solution of 2-nitropropane dioxygenase was mixed with either 50 mM sodium pyrophosphate (E_{ox}) or 100 mM ethylnitronate to give a final concentration of enzyme of ~14 μM and a substrate concentration of 50 mM. Spectra were recorded 1 s (E_{sq}) and 78 min (E_{red}) after mixing at pH 8.5 and 30 °C. (Inset) Rate of flavin reduction versus the concentration of ethylnitronate. 2-Nitropropane dioxygenase (14 μM) was mixed anaerobically with 4−100 mM ethylnitronate in 50 mM sodium pyrophosphate at pH 8.5 and 30 °C, and the absorbance changes at 370 nm were recorded over time. Data were fit with eq 9. (B) Representative trace showing the absorbance at 370 nm versus time, obtained with 50 mM ethylnitronate. Note the log scale on the time axis and the difference scales on the absorbance axis in the two panels.

shown in Figure 1B, the flavosemiquinone was slowly reduced to the hydroquinone form over >900 s, clearly indicating that this reaction does not occur in the normal catalytic pathway for ethylnitronate oxidation.[3] Thus, the oxidation of nitroalkanes catalyzed by 2-nitropropane dioxygenase occurs with a mechanism that is unique from that of nitroalkane oxidase.

K_m Values for Oxygen at pH 6 and 9. Recent steady-state kinetic studies of 2-nitropropane dioxygenase showed that the enzyme reacts with oxygen before the release of the organic product of the reaction, with K_m values for oxygen below 5 μM with either nitroethane or ethylnitronate at pH 8 and 30 °C (*1*). As a first step toward the investigation of the pH effects on the $^D(k_{cat}/K_m)$ values with nitroethane and ethylnitronate, the K_m values for oxygen with these substrates were determined here at pH 6 and 9, by measuring initial rates of the reaction at varying concentrations of both oxygen and the organic substrate. As summarized in Table 1, at both of these pH values, the K_m values for oxygen determined with nitroethane were at or below 10 μM, allowing for fairly good approximations of the k_{cat}/K_m and k_{cat} values with the neutral substrate to be obtained under atmospheric conditions in the pH range from 6 to 9.[4] In contrast, when ethylnitronate was used as the substrate, a K_m value for oxygen of ~45 μM was determined at pH 9 (Table 1). Consequently, while the $^D(k_{cat}/K_m)$ values with nitroethane could be determined at atmospheric oxygen, those with ethylnitronate had to be determined at varying concentrations of both the organic substrate and oxygen to avoid artifactual contributions arising from the lack of oxygen saturation on the enzyme.

pH Dependence of the $^D(k_{cat}/K_m)$ Value with 1,1-[$^2H_2$]-Nitroethane. The effect of pH on the $^D(k_{cat}/K_{NE})$ value[5] with 1,1-[$^2H_2$]-nitroethane as the substrate for 2-nitropropane dioxygenase was determined in air-saturated buffer in the pH range from 5.5 to 10 at 30 °C. As shown in Figure 2A, the $^D(k_{cat}/K_{NE})$ value decreased from an upper limiting value of 7.4 ± 0.3 at low pH to a lower limiting value of 3.5 ± 0.1 at high pH, consistent with the isotope-sensitive step in which the neutral organic substrate is enzymatically deprotonated, being partially masked by other kinetic steps belonging to the second-order rate constant k_{cat}/K_m (*20*). Consistent with previous results showing the requirement for both an unprotonated and a protonated amino acid residue in the oxidation of neutral substrates catalyzed by 2-nitropropane dioxygenase (*1*), the pH profiles of the k_{cat}/K_{NE} values were bell-shaped with both the light and heavy substrates (Figure 3A). The pK_a values for the unprotonated and protonated groups required for catalysis were 7.2 ± 0.1 and ≥9.3[6] with nitroethane and 7.5 ± 0.1 and ≥9.3 with 1,1-[$^2H_2$]-nitroethane, respectively.

pH Dependence of the $^D(k_{cat}/K_m)$ Value with 1-[^2H]-Ethylnitronate. In a recent mechanistic study at atmospheric oxygen, a $^D(k_{cat}/K_{ENate})$ value of ~0.76 was determined with 1-[^2H]-ethylnitronate as the substrate for 2-nitropropane dioxygenase at pH 8 and 30 °C (*1*). Such an inverse kinetic isotope effect was assigned to the kinetic step in which superoxide reacts with the nitroalkane radical in the enzyme active site to form peroxynitroethane. In the present study, we have expanded that initial observation by measuring kinetic isotope effects at varying concentrations of both 1-[^2H]-ethylnitronate and oxygen as substrates for 2-nitropropane dioxygenase in the pH range from 6.5 to 9.5 at 30 °C. As shown in Figure 2B, the $^D(k_{cat}/K_{ENate})$ values with 1-[^2H]-ethylnitronate as the substrate decreased from an upper limiting value of 0.97 ± 0.04 at low pH to a lower limiting value of 0.63 ± 0.06 at high pH. The pK_a values seen in the

[3] The turnover number of 2-nitropropane dioxygenase with ethylnitronate as the substrate at pH 8.5 and 30 °C is 70 s^{-1}. For the hydroquinone to be a catalytically relevant species, it would have to form at least 63 000 times in 900 s, clearly establishing that the flavin hydroquinone species or a flavin N(5) adduct with similar spectroscopic properties is not formed during the reductive half-reaction of 2-nitropropane dioxygenase.

[4] The solubility of oxygen in aqueous solution at 30 °C is ~230 μM, a value which is 23 times larger than the upper limiting value of 10 μM, experimentally determined for the K_m value for oxygen with nitroethane as the substrate for the enzyme between pH 6 and 9. This ensures that in atmospheric oxygen 2-nitropropane dioxygenase is at least 96% saturated with oxygen when nitroethane is used as the substrate.

[5] Saturation of 2-nitropropane dioxygenase with nitroethane, 1,1-[$^2H_2$]-nitroethane, ethylnitronate, or 1-[^2H]-ethylnitronate as the substrate was not attained across the entire pH range tested. Consequently, the pH dependence of the $^Dk_{cat,NE}$ and $^Dk_{cat,ENate}$ values could not be determined.

[6] Instability of the enzyme at pH values above 10 prevented an accurate determination of the kinetic parameters at high pH. While the available kinetic data clearly indicate the presence of a group that needs to be unprotonated for catalysis with nitroethane and 1,1-[$^2H_2$]-nitroethane as the substrate, with a pK_a value of 9.3 or higher, the lack of kinetic data above pH 10 did not allow for an accurate determination of the pK_a value for the group that must be protonated for catalysis.

Table 1: Steady-State Kinetic Parameters for 2-Nitropropane Dioxygenase at pH 6, 8, and 9[a]

pH	k_{cat} (s^{-1})	K_a[b] (mM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	K_{O_2}[b] (μM)	k_{cat}/K_{O_2} ($\mu M^{-1}s^{-1}$)	K_{ia} (mM)	R^2
			Ethylnitronate				
6	130 ± 3	5.1 ± 0.2	$25\,400 \pm 1400$	20 ± 2	6.2 ± 0.6	0.8 ± 0.4	0.990
8[c]	57 ± 1	3.4 ± 0.1	$16\,900 \pm 30$	≤ 5	≥ 11	19 ± 1	0.998
9	25 ± 1	9.5 ± 0.3	2500 ± 100	45 ± 2	5.3 ± 0.2	9.4 ± 0.6	0.988
			Nitroethane				
6	nd[d]	nd[d]	40 ± 3	10 ± 3	3.2 ± 0.9	50 ± 20	0.998
8[c]	11 ± 1	19 ± 1	560 ± 10	≤ 5	≥ 2	11 ± 1	0.998
9	4.9 ± 0.1	13 ± 1	375 ± 40	≤ 5	≥ 1	40 ± 5	0.998

[a] Enzyme activity was measured at varying concentrations of both the organic substrate and oxygen in 50 mM Tris-Cl at 30 °C. Data were fit to eq 1. [b] K_a is the Michaelis constant for the organic substrate, and K_{O_2} is the Michaelis constant for oxygen. [c] From ref 1. [d] Not determined because the saturation of the enzyme was not achieved.

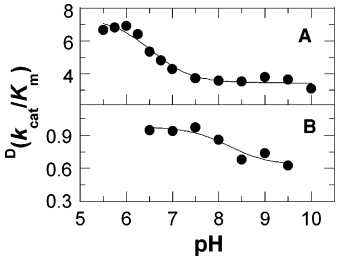


FIGURE 2: pH dependence of the kinetic isotope effects on k_{cat}/K_{NE} and k_{cat}/K_{ENate}. (A) pH dependence on the overall kinetic isotope effect with 1,1-[$^2H_2$]-nitroethane as the substrate. (B) pH dependence of the α-secondary kinetic isotope effect with 1-[^{2}H]-ethylnitronate as the substrate. Kinetic isotope effects were calculated by taking the ratio of k_{cat}/K_m obtained for the unlabeled substrate to that of the labeled substrate at 30 °C. The data were fit with eq 5.

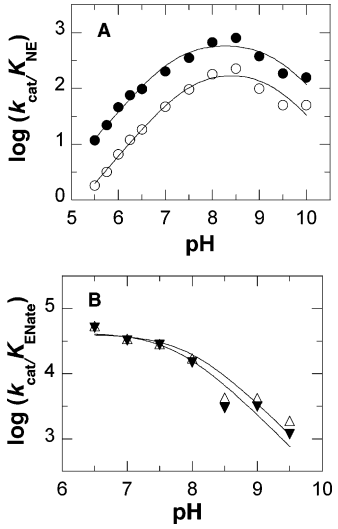


FIGURE 3: pH dependence of k_{cat}/K_m with either nitroethane or ethylnitronate as the substrate. (A) pH dependence of k_{cat}/K_{NE} with either nitroethane or 1,1-[$^2H_2$]-nitroethane as the substrate. Enzymatic activity was measured in air-saturated 50 mM sodium pyrophosphate in the pH range of 5.75−10 at varying concentrations of either nitroethane (●) or 1,1-[$^2H_2$]-nitroethane (○) at 30 °C. (B) pH dependence of k_{cat}/K_{ENate} with either ethylnitronate (▼) or 1-[^{2}H]-ethylnitronate (△) as the substrate. Enzymatic activity was measured at varying concentrations of both ethylnitronate and oxygen in 50 mM sodium pyrophosphate in the pH range of 6.5−9.5 at 30 °C. Data were fit with eqs 4 and 6 for nitroethane and ethylnitronate, respectively.

pH profiles of k_{cat}/K_{ENate} with ethylnitronate and 1-[^{2}H]-ethylnitronate were 7.8 ± 0.2 and 8.0 ± 0.2, respectively, for an amino acid group that needs to be protonated for catalysis (Figure 3B).

Solvent Viscosity Effects. The effects of solvent viscosity on the kinetic parameters of 2-nitropropane dioxygenase with nitroethane as the substrate were determined at pH 10, to establish whether the decreased $^D(k_{cat}/K_{NE})$ value at this pH was the result of slow substrate binding. As shown in Figure 4, increasing the viscosity of the reaction mixture had no effect on the kinetic parameters with nitroethane. Similarly, no effects of solvent viscosity were observed on the normalized k_{cat}/K_m and k_{cat} values with ethylnitronate at pH 6.5 (Figure 4), at which $^D(k_{cat}/K_{ENate})$ values near unity were observed with this substrate. Table 2 summarizes the effects of solvent viscosity on the kinetic parameters of the enzyme.

pH Dependence of m-Nitrobenzoate Inhibition. A survey of a number of compounds established that *m*-nitrobenzoate was a competitive inhibitor with respect to ethylnitronate as the substrate for 2-nitropropane dioxygenase (Figure 5A), with a K_{is} value of 9 ± 1 mM at pH 7.4 and 30 °C. To establish a thermodynamic pK_a for a group within the active site of the enzyme (*20*), the pH dependence of the inhibition by *m*-nitrobenzoate was determined at atmospheric oxygen with ethylnitronate at 30 °C. As shown in Figure 5B, a plot of K_{is} versus pH revealed a pK_a of 7.6 ± 0.1 for a group that needs to be protonated for inhibition.

DISCUSSION

The mechanistic data presented in this study, along with the results of a recent kinetic investigation (*1*), are consistent with 2-nitropropane dioxygenase being a catalyst for both the oxidative denitrification of nitroalkanes and nitronates to the corresponding carbonyl compounds and for the non-oxidative tautomerization between their anionic and neutral forms. In the oxidative denitrification pathway with nitroethane as the substrate (Scheme 4), after the formation of an oxidized enzyme−nitroalkane complex (k_1), catalysis is initiated by the removal of a proton from the substrate α carbon by an active-site base (k_3), which is likely His196 based on the crystal structure of the enzyme from *P. aeruginosa* (*16*), yielding an enzyme-bound alkyl nitronate. After partial reduction of the enzyme-bound flavin through a single-electron transfer from the alkyl nitronate (k_5), the resulting anionic flavosemiquinone reacts with molecular oxygen to form a superoxide anion. The resulting superoxide anion then reacts with the nitro radical to yield a nitroperoxide anion species, which will simultaneously or perhaps subsequently be protonated in an acid-catalyzed reaction (k_9). The resulting peroxynitroethane is then released from the active site of the enzyme (k_{11}) and undergoes a nucleophilic

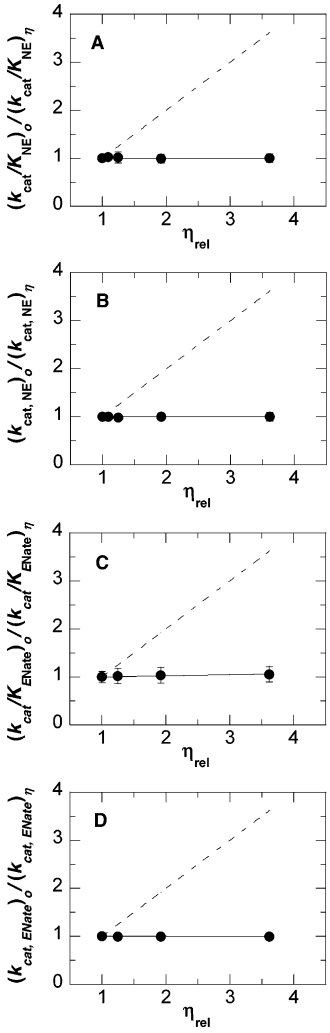


FIGURE 4: Solvent viscosity effects on the kinetic parameters of 2-nitropropane dioxygenase. (A) Effect on k_{cat}/K_{NE} with nitroethane as the substrate at pH 10. (B) Effect on $k_{cat,NE}$ with nitroethane as the substrate at pH 10. (C) Effect on k_{cat}/K_{ENate} with ethylnitronate as the substrate at pH 6.5. (D) Effect on $k_{cat,ENate}$ with ethylnitronate as the substrate at pH 6.5. The solid lines are fits of the experimental data with eq 7. The dashed lines represent a case in which the reaction is diffusion-controlled. The values of the relative viscosity at 30 °C were calculated from the values at 20 °C reported by Lide (*18*). Enzymatic activity was measured in 50 mM sodium pyrophosphate at 30 °C.

Table 2: Solvent Viscosity Effects on the Kinetic Parameters of 2-Nitropropane Dioxygenase[a]

kinetic parameter	substrate	viscosity effect (%)[b]	standard error (%)
k_{cat}	nitroethane	0.0	0.3
k_{cat}/K_m	nitroethane	0.1	0.6
k_{cat}	ethylnitronate	2.3	0.4
k_{cat}/K_m	ethylnitronate	−0.4	0.2

[a] Enzymatic activity was measured in 50 mM sodium pyrophosphate in the absence and presence of glycerol at pH 10 (nitroethane) or pH 6.5 (ethylnitronate) at 30 °C. [b] Percent increase of the kinetic parameter in the absence of viscosigen per increase in the relative viscosity. The data were fit with eq 7.

attack, presumably in a nonenzymatic fashion, yielding nitrite and acetaldehyde. The oxidative pathway with ethylnitronate as the substrate proceeds in a similar fashion as that with nitroethane, except that the initial proton abstraction step is

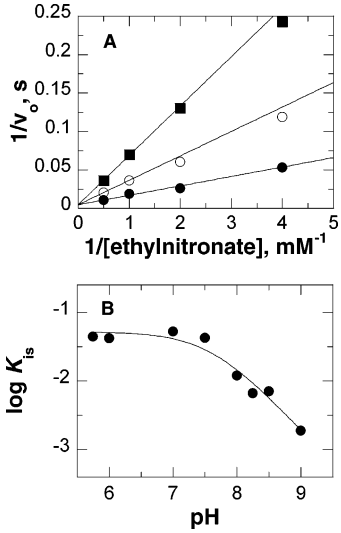


FIGURE 5: Inhibition of 2-nitropropane dioxygenase by *m*-nitrobenzoate with respect to ethylnitronate as the substrate. (A) Enzymatic activity was measured in air-saturated 50 mM potassium phosphate at pH 6 and 30 °C by varying concentrations of ethylnitronate in the presence of 0 mM (●), 38 mM (○), and 100 mM (■) *m*-nitrobenzoate. The data were fit with eq 3. (B) pH dependence of *m*-nitrobenzoate inhibition. The data were fit to eq 6.

excluded. In the non-oxidative tautomerization pathway with ethylnitronate, after the formation of the enzyme−substrate complex (k_{13}), the enzyme-bound anionic substrate is protonated in the active site of the enzyme to form nitroethane (k_4), which is then released to the solvent (k_2). A similar nitro-aci tautomerization probably occurs with nitroethane, in which, after the formation of the oxidized enzyme−nitroethane Michaelis complex (k_1) and the subsequent deprotonation of the neutral substrate (k_3), the resulting ethylnitronate dissociates from the active site of the enzyme (k_{14}) completing the catalytic cycle.[7] In the branched mechanisms of Scheme 4, the k_{cat}/K_m values for nitroethane and ethylnitronate when the enzyme is saturated with oxygen are given by eqs 10 and 11, respectively.

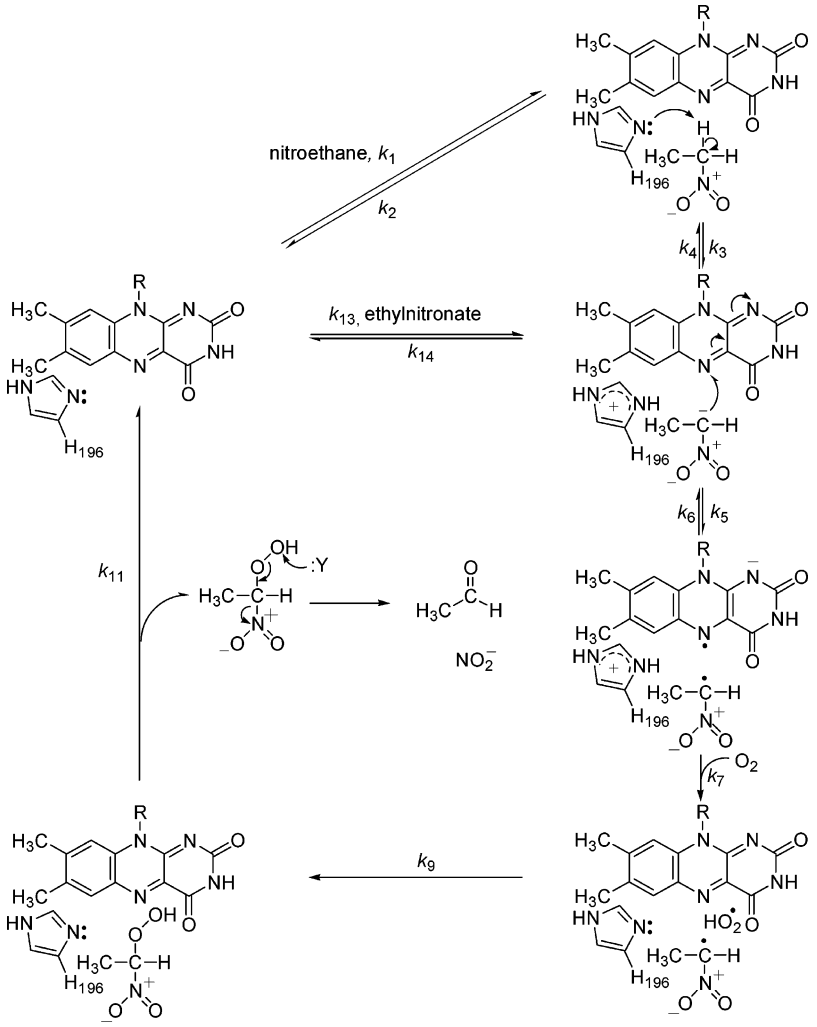
$$\frac{k_{cat}}{K_{NE}} = \frac{k_1 k_3 k_5}{k_2(k_4 + k_{14})} \quad (10)$$

$$\frac{k_{cat}}{K_{ENate}} = \frac{k_5 k_{13}}{k_4 + k_{14}} \quad (11)$$

Evidence supporting the oxidative denitrification pathways with nitroethane and ethylnitronate was reported previously

[7] While tautomerization of ethylnitronate to nitroethane is supported by the observed inverse α-secondary kinetic isotope effects on the k_{cat}/K_m value with 1-[^{2}H]-ethylnitronate at high pH and at saturating concentration of oxygen (see the main text), no direct evidence is available to support a similar tautomerization reaction when nitroethane is the substrate for the enzyme. However, given the observation that ethylnitronate binds to the enzyme in rapid equilibrium and the structural similarity between ethylnitronate and nitroethane, it is assumed here that a similar tautomerization reaction from nitroethane to ethylnitronate probably occurs with nitroethane as the substrate. In this respect, all of the conclusions concerning rate-limiting steps during turnover of the enzyme that are drawn in the present study from pH and kinetic isotope effects hold true irrespective of whether the enzyme catalyzes the tautomerization reaction with nitroethane as the substrate. Indeed, in the absence of the tautomerization reaction, the external forward commitment to catalysis with nitroethane, which equals k_3/k_2, is also negligible because $k_2 >> k_3$ (see the main text).

Scheme 4: Catalytic Mechanism of 2-Nitropropane Dioxygenase with Either Nitroethane or Ethylnitronate as the Substrate



under conditions of an *atmospheric concentration of oxygen* (*1*) and is substantiated by the results presented in this study at a saturating concentration of oxygen. Briefly, an enzymic catalytic base with a pK_a of ~7.5 was observed in the pH profiles of the k_{cat}/K_m and k_{cat} values with nitroethane and nitrobutane but not in the pH profiles with their corresponding alkyl nitronates (*1*). The formation of an anionic flavosemiquinone that subsequently reacts with oxygen was suggested by enzyme-monitored turnover experiments with ethylnitronate and static anaerobic reductions of the enzyme with nitroethane or ethylnitronate (*1*). This conclusion is further supported by the stopped-flow data on the reductive half-reaction with ethylnitronate presented in this study, showing that the anionic flavosemiquinone forms during catalysis upon a single-electron transfer from the enzyme-bound anionic nitronate. The participation of a protonated group in the formation of a superoxide anion was suggested by pH profiles of the k_{cat}/K_m and k_{cat} values *at subsaturating concentrations of oxygen* with nitroethane, nitrobutane, ethylnitronate, and butyl-1-nitronate, showing the requirement for a protonated group for catalysis (*1*). The formation of a superoxide was suggested by the effect of superoxide dismutase on the rate of oxygen consumption when propyl nitronates were used as the substrate (*1*).[8] Finally, the radical recombination between the superoxide and nitroalkane radicals to form peroxynitroethane was suggested by an inverse α-secondary kinetic isotope effect of ~0.76 on the k_{cat}/K_m value, with 1-[^{2}H]-ethylnitronate as the substrate determined at pH 8 and *atmospheric oxygen*, which was interpreted as resulting from the change in the hybridization of the α carbon of the nitroalkane radical from sp^2 to sp^3 (*1*).

Evidence supporting the tautomerization pathway with ethylnitronate comes from the results presented in this study, showing an inverse α-secondary kinetic isotope effect on the k_{cat}/K_m value with 1-[^{2}H]-ethylnitronate at high pH and, most importantly, at a *saturating concentration of oxygen*. The latter condition results in the kinetic step k_5 being irreversible, because the radical pair formed by the flavosemiquinone and the nitroalkane radical does not accumulate at saturating oxygen. Consequently, the subsequent formation of peroxynitroethane cannot contribute to the k_{cat}/K_m value (*21*) when the enzyme is saturated with oxygen, ruling out the assignment of the inverse α-secondary kinetic

[8] The addition of superoxide dismutase to enzymatic assays of 2-nitropropane dioxygenase with nitroethane or ethylnitronate as the substrate had no effect on the observed rate of oxygen consumption (*1*), consistent with the superoxide anion not being released from the enzyme with these substrates.

isotope effect on k_{cat}/K_m to this step. In the oxidative pathway with ethylnitronate, there are no other kinetic steps involving a change in the hybridization of the substrate α carbon from sp^2 to sp^3, suggesting that the observed inverse α-secondary kinetic isotope effect must arise from the conversion of ethylnitronate to nitroethane within the active site of the enzyme. In this respect, 2-nitropropane dioxygenase is similar to another FMN-dependent enzyme, old yellow enzyme, which was previously shown to catalyze a non-oxidative tautomerization of nitroalkanes in the NADPH-linked reduction of nitro-olefins (*22*).

In the reactions catalyzed by 2-nitropropane dioxygenase, substrate binding and product release occur in rapid equilibrium, as suggested by the lack of solvent viscosity effects on the kinetic parameters with nitroethane or ethylnitronate as the substrate. Indeed, the lack of viscosity effects on the k_{cat}/K_{NE} and k_{cat}/K_{ENate} values suggests that dissociation of the substrate from the oxidized enzyme−substrate complex is significantly faster than the rate of chemical steps occurring within enzyme−substrate complexes belonging to k_{cat}/K_m, i.e., that k_2 and k_{14} are significantly larger than k_3 and k_5 with nitroethane and ethylnitronate, respectively. This establishes these substrates as nonsticky (*20*), with the important mechanistic implication that any observed decrease in the primary and α-secondary kinetic isotope effects on the k_{cat}/K_m values with nitroethane and ethylnitronate must necessarily be ascribed to some chemical steps occurring within enzyme−substrate complexes that contribute to k_{cat}/K_m rather than to substrate binding (*vide infra*). Similarly, the lack of solvent viscosity effects on the k_{cat} values with nitroethane and ethylnitronate establishes the products of the reactions of oxidation and tautomerization with these substrates as nonsticky (*20*).

Removal of the proton from the α carbon of nitroethane (k_3) is fully rate-limiting for catalysis at low pH, but it is kinetically masked at high pH by the reverse commitment to catalysis ($C_{r(3)}$), which reflects the net flux of the enzyme-bound intermediates through the reverse of the kinetic step k_3. Evidence supporting this conclusion comes from the pH dependence of the $^D(k_{cat}/K_{NE})$ value with 1,1-[$^2H_2$]-nitroethane as the substrate, which decreases between limiting values with increasing pH. The magnitude of the limiting $^D(k_{cat}/K_{NE})$ value at low pH, with a value of ∼7.4 that agrees fairly well with the reported value of ∼8.5 for the kinetic isotope effect for deprotonation of nitroethane in solution (*23*), is consistent with cleavage of the CH bond of nitroethane being fully rate-limiting for catalysis at low pH. The limiting $^D(k_{cat}/K_{NE})$ value of ∼3.5 seen at high pH implies that some kinetic steps belonging to k_{cat}/K_{NE} other than CH bond cleavage become partially rate limiting with increasing pH (*20*). The lack of solvent viscosity effects on the k_{cat}/K_{NE} value with nitroethane at pH 10 suggests that nitroethane binding occurs in rapid equilibrium, i.e., with $k_2 >> k_3$. Similarly, the lack of solvent viscosity effects on the $k_{cat,NE}$ value is consistent with release of the ethylnitronate, possibly formed in the tautomerization reaction of nitroethane occurring in rapid equilibrium, i.e., with $k_{14} >> k_3$. These data immediately rule out an external forward commitment to catalysis because of substrate binding, which, with nitroethane, is given by $k_3/(k_2 + k_{14})$, as being responsible for the decreased $^D(k_{cat}/K_{NE})$ value (*20*). This, in turn, will abate any internal forward commitment to catalysis possibly arising from $k_5 >> k_4$. Thus, as illustrated in eq 12

$$^D\left(\frac{k_{cat}}{K_{NE}}\right) = \frac{^Dk_3 + {}^DEqC_{r(3)}}{1 + C_{r(3)}} \quad (12)$$

the decrease in the $^D(k_{cat}/K_{NE})$ value at high pH must necessarily arise from an increase in the $C_{r(3)}$ value. Consistent with this conclusion, a ∼0.3 pH units increase of the apparent pK_a value was seen in the k_{cat}/K_{NE} pH profiles for the group that acts as a base upon substituting nitroethane with 1,1-[$^2H_2$]-nitroethane. This is the expected result because substitution of nitroethane with a slower substrate such as 1,1-[$^2H_2$]-nitroethane, for which CD bond cleavage is ∼7.5 times slower than CH bond cleavage, will (at least partially) abate the perturbation of the kinetic pK_a value for the base seen in the k_{cat}/K_{NE} pH profiles, as illustrated in eq 13 (*20*).

$$\Delta pK_a = \log(1 + C_{r(3)}) \quad (13)$$

The formation of the anionic flavosemiquinone (k_5) is facilitated by a protonated group in the active site of the enzyme with a pK_a value of ∼7.6 that acts as an electrostatic catalyst, as suggested by the pH profiles of the k_{cat}/K_{ENate} values and *m*-nitrobenzoate inhibition. Because pK_a values determined with competitive inhibitors reflect true equilibrium dissociation constants (*20*), the observation that the pK_a value of ∼7.8 seen in the k_{cat}/K_{ENate} pH profile is not significantly different from the pK_a value of ∼7.6 observed with the competitive inhibitor *m*-nitrobenzoate establishes the latter value as the thermodynamic pK_a value for the active-site group that needs to be protonated for catalysis. The recent elucidation of the three-dimensional structure of 2-nitropropane dioxygenase from *P. aeruginosa* shows that the only ionizable group in the active site of the enzyme is a histidine residue (*16*), corresponding to His196 of the enzyme from *N. crassa*. Thus, it is likely that the pK_a value seen in the pH profiles is that of His196.

With ethylnitronate, the non-oxidative tautomerization of the anionic substrate to nitroethane (via the steps k_{13}, k_4, and k_2) is fully rate-limiting at high pH, but it is kinetically masked by the oxidative pathway at low pH. This conclusion is supported by the pH dependence of the α-secondary kinetic isotope effects on the k_{cat}/K_m value with 1-[^{2}H]-ethylnitronate as the substrate, which is assigned to the non-oxidative conversion of ethylnitronate to nitroethane (k_4) when the enzyme is saturated with oxygen (see above). The inverse kinetic isotope effect with a limiting value of ∼0.6 at high pH is consistent with the kinetic step in which the α carbon of ethylnitronate is converted from sp^2 to sp^3 being slower than other kinetic steps belonging to the oxidative pathway with ethylnitronate. This is the expected result because at high pH the effective concentration of the active-site group acting as an acid for protonation of ethylnitronate is low, yielding a significant decrease in the net flux of intermediates through the kinetic step k_4. As the pH progressively decreases, the $^D(k_{cat}/K_{ENate})$ becomes fully masked by some other kinetic step(s) belonging to the oxidative pathway that are included in the k_{cat}/K_m value. According to eq 11, k_{cat}/K_{ENate} includes substrate binding (k_{13}), flavin reduction (k_5), and both the tautomerization (k_4) and release (k_{14}) of ethylnitronate. The lack of solvent viscosity effects with

ethylnitronate as the substrate at low pH immediately rules out substrate binding and release as being slow, suggesting that the non-oxidative conversion of ethylnitronate to nitroethane is masked by the kinetic step in which an electron is transferred from the enzyme-bound ethylnitronate to the flavin (k_5).

Multiple catalytic strategies exist for the oxidation of nitroalkanes by flavin-dependent enzymes as can be seen from a comparison of 2-nitropropane dioxygenase with the well-characterized nitroalkane oxidase (for a review, see ref *24*). The crystal structures of both enzymes have been recently solved (*16*, *25*), and a wealth of mechanistic data is now available (*1*, *19*). Both enzymes initiate the oxidation of neutral nitroalkanes through a base-catalyzed proton abstraction from the α carbon of the substrate. Crystallographic data of 2-nitropropane dioxygenase from *P. aeruginosa* suggest that a hisidine, corresponding to His196 in the enzyme from *N. crassa*, is the catalytic base (*16*). Consistent with this proposed role, upon replacing His196 with alanine, the enzyme completely loses the ability of oxidizing nitroethane but not ethylnitronate (Belaineh M., and Gadda, G., unpublished observations). The corresponding residue in nitroalkane oxidase is an aspartate residue, which has been shown to serve as the catalytic base in crystallographic and mutagenesis studies (*25*−*27*). Upon generation of the anionic form of the substrate, the catalytic strategies employed by the two enzymes diverge. As shown in this and previous studies (*1*), the flavin of 2-nitropropane dioxygenase reacts with the anionic substrate to generate an anionic flavosemiquinone. On the other hand, nitroalkane oxidase does not generate an observable flavosemiquinone but instead forms a flavin N(5) adduct, as evident from trapping experiments with cyanide (*28*). Prior to flavin reduction in 2-nitropropane dioxygenase, a ternary complex forms between the enzyme, the substrate radical, and oxygen. Oxygen then reacts with the flavin semiquinone, generating a superoxide anion that combines with the substrate radical, generating a peroxynitroalkane species that decays to give the product. Nitroalkane oxidase does not form a ternary complex with oxygen as suggested by its ping-pong steady-state mechanism (*29*); rather, flavin reduction is followed by expulsion of nitrite to generate a cationic imine that undergoes a nucleophilic attack to ultimately generate the product (*19*). Thus, the major difference between 2-nitropropane dioxygenase and nitroalkane oxidase is the formation of an observable flavosemiquinone versus a flavin N(5) adduct.

In conclusion, the results of the steady-state investigation with solvent, pH, and substrate kinetic isotope effects presented herein have provided further mechanistic insights on the chemical mechanism for the oxidation of nitroethane and ethylnitronate catalyzed by 2-nitropropane dioxygenase. The enzyme catalyzes both the oxidative denitrification of nitroalkanes and nitronates to the corresponding carbonyl compounds and the non-oxidative tautomerization between their anionic and neutral forms. During enzymatic turnover with neutral substrates, the rate of oxidative denitrification of nitroalkanes is limited by the cleavage of the substrate CH bond at low pH and the formation of flavosemiquinone at high pH. With anionic substrates, the non-oxidative protonation of nitronates to yield the corresponding nitroalkanes limits enzymatic turnover at high pH. These results provide a firm groundwork for future mechanistic studies aimed at the elucidation of the contribution of quantum mechanical tunneling in the cleavage of the CH bond of nitroethane and the mechanism of oxygen activation to form superoxide.

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SUPPORTING INFORMATION AVAILABLE

Derivations of eqs 10 and 11, illustrating the k_{cat}/K_m values for nitroethane and ethylnitronate as the substrate for the enzyme. This material is available free of charge via the Internet at http://pubs.acs.org.

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