# Iso-Mechanism of Nitroalkane Oxidase: 1. Inhibition Studies and Activation by Imidazole†

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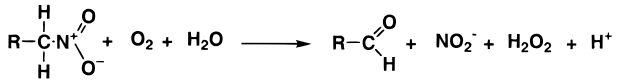
ABSTRACT: The flavoprotein nitroalkane oxidase catalyzes the oxidation of primary and secondary nitroalkanes to aldehydes and ketones, respectively, transferring electrons to oxygen to form hydrogen peroxide. The steady-state kinetic mechanism of the active flavin adenine dinucleotide-(FAD-) containing form of the enzyme has been determined with nitroethane at pH 7 to be bi-ter ping-pong, with oxygen reacting with the free reduced enzyme after release of the aldehyde product. The $V_{max}$ value is 5.5 ± 0.3 $s^{-1}$ and the $K_m$ values for nitroethane and oxygen are 3.3 ± 0.6 and 0.023 ± 0.007 mM, respectively. The free reduced enzyme forms a dead-end complex with nitroethane, with a $K_{ai}$ value of 30 ± 6 mM. Acetaldehyde and butyraldehyde are noncompetitive inhibitors versus nitroethane due to formation of a dead-end complex between the oxidized enzyme and the product. Acetaldehyde is an uncompetitive inhibitor versus oxygen, indicating that an irreversible isomerization of the free reduced enzyme occurs before the reaction with oxygen. Addition of unprotonated imidazole results in a 5-fold increase in the $V_{max}$ value, while the $V/K$ values for nitroethane and oxygen are unaffected. A 5-fold increase in the $K_{ai}$ value for nitroethane and a 6.5-fold increase in the $K_{ii}$ value for butyraldehyde are observed in the presence of imidazole. These results are consistent with the isomerization of the free reduced enzyme being about 80% rate-limiting for catalysis and with a model in which unprotonated imidazole accelerates the rate of isomerization.

Nitroalkane oxidase from the fungus *Fusarium oxysporum* (ATCC 695) is a flavoprotein that catalyzes the oxidation of nitroalkanes to the corresponding aldehydes or ketones, with production of hydrogen peroxide and nitrite (Scheme 1). The study of an enzyme able to oxidize nitroalkanes is of interest for several reasons. Many antibiotics contain nitro groups and many leguminous plants produce nitrated toxins (*1*−*3*). A possible physiological role for nitroalkane oxidase is oxidation of such compounds, thereby inactivating the natural defenses of the host organisms. Several nitroalkanes are toxic and/or carcinogenic (*4*); their wide use as industrial solvents and intermediates renders an enzymatic activity that converts these compounds to less harmful species of interest for bioremediation. Finally, from a chemical standpoint, the formation of nitronates in solution is a well-characterized chemical reaction (*5*). Thus, the study of the mechanism of nitroalkane oxidase provides the unique opportunity to compare the enzymatic and the nonenzymatic formation of nitronates.

While other flavin-containing enzymes, such as D-amino acid oxidase (*6*), glucose oxidase (*7*), and nitroalkane dioxygenase (*8*, *9*), have been shown to catalyze the oxidation of nitroalkanes to aldehydes, these all require the nitronate form of the substrate. In contrast, nitroalkane oxidase uses neutral nitroalkanes, suggesting that the physiological role

Scheme 1




of the enzyme is to oxidize nitroalkanes (*10*, *11*). This is consistent with the high yields of enzyme produced when the fungus is grown on nitroethane as the sole carbon source (*12*). The N-terminal sequence and the amino acid composition of nitroalkane oxidase do not match any known protein or open reading frame (*11*), suggesting that this enzyme has not been previously described.

Nitroalkane oxidase is active on a broad range of nitroalkane substrates (*13*). The best substrates are unbranched primary nitroalkanes. On the basis of the substrate specificity, the substrate binding site has been proposed to be hydrophobic and suitable to accommodate a four-carbon substrate (*13*). The enzyme as isolated contains the cofactor in the form of 5-(3-nitrobut-2-yl)-1,5-dihydroflavin adenine dinucleotide and is consequently not active (*14*, *15*). Conversion of this *N*(5)-flavin adduct to flavin adenine dinucleotide (FAD)[1] yields active enzyme (*11*, *14*). The mechanism of formation of the 5-nitrobutyl-FAD has been elucidated and is consistent with a carbanion mechanism for catalysis (*14*). Previous studies on a partially activated enzyme are consistent with a ping-pong mechanism, in which the oxidation of the reduced flavin by molecular oxygen occurs after product

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[1] Abbreviations: FAD, flavin adenine dinucleotide; Tris, tris-(hydroxymethyl)aminomethane; ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; MOPS, 3-morpholinopropanesulfonic acid.

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release (*10*). In this paper, we describe the use of substrate and product inhibition studies and the effects of imidazole to determine the steady-state kinetic mechanism of activated FAD-containing nitroalkane oxidase.

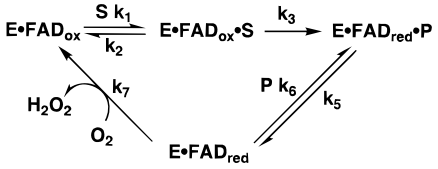
## MATERIALS AND METHODS

*Materials.* FAD was from Sigma. Nitroethane, 1-nitrobutane, acetaldehyde, and butyraldehyde were from Aldrich. Nitroalkane oxidase was purified from *Fusarium oxysporum* (ATCC 695) as described by Gadda and Fitzpatrick (*13*). The activated FAD-containing form of enzyme was prepared according to Gadda et al. (*11, 14*) and was stored at −70 °C in the presence of 0.5 mM FAD to prevent formation of the less stable apoprotein. The concentration of nitroalkane oxidase was determined by the method of Bradford (*16*) with bovine serum albumin as standard. All other reagents were of the highest purity commercially available.

*Enzyme Assays.* Unless otherwise stated, enzyme activity was measured in the presence of 0.5 mM FAD in air-saturated 50 mM potassium phosphate, pH 7, by monitoring the rate of oxygen consumption with a Yellow Springs Instruments model 5300 Clark electrode thermostated at 30 °C. Stock solutions of substrates were prepared in 95% ethanol; the final concentration of ethanol in each enzyme assay mixture was kept constant at 0.5%, so that the enzymatic activity was not affected. Unless otherwise stated, the concentration of nitroethane was kept below 25 mM to avoid substrate inhibition. To minimize the amount of nitroalkane anion in the assay mixture, assays were started by adding the substrate as the neutral nitroalkane. For determination of the steady-state kinetic parameters when both nitroethane and oxygen were varied, the assay mixtures were equilibrated with the appropriate $O_2/N_2$ mixture by bubbling with the gas for 15 min before the reaction was started by addition of the enzyme and the nitroalkane substrate. The combined volume of enzyme and substrate was kept at 1% that of the assay mixture, such that no significant perturbations of the oxygen concentration occurred. For studies of the effects of imidazole on the steady-state kinetics and of the inhibition patterns with acetaldehyde, butyraldehyde, and sodium nitrite, the enzyme was incubated for 5 min with these compounds before the reaction was started by addition of the nitroalkane substrate.

*Data Analysis.* Data were fit with the KaleidaGraph software (Adelbeck Software, Reading, PA) or the KinetAsyst software (IntelliKinetics, State College, PA). The steady-state kinetic parameters of nitroalkane oxidase were determined by fitting the data to the Michaelis−Menten equation for one substrate. When the initial reaction rates were determined by varying the concentration of both nitroethane and oxygen, the data were fit to eqs 1 and 2, which describe a ping pong and a sequential mechanism, respectively. $K_a$ and $K_b$ represent the Michaelis constants for the nitroethane substrate (A) and oxygen (B), respectively; $K_{ia}$ is the dissociation constant for the enzyme−nitroethane complex; and $V$ is the maximal velocity. Product inhibition studies were performed by varying the concentrations of either aldehyde or nitrite and one substrate at a fixed saturating concentration of the other substrate. The data were fit to eqs 3−5, which describe competitive, uncompetitive, and noncompetitive inhibition patterns, respectively. $P$ is the concentration of the product, $K_{is}$ is the inhibition constant from the slope term, and $K_{ii}$ is the inhibition constant from the intercept term. The apparent substrate inhibition constant for nitroethane ($K_{ai}$) was determined by fitting the data to eq 6.

Scheme 2




$$v = \frac{VAB}{K_aB + K_bA + AB} \quad (1)$$

$$v = \frac{VAB}{K_aB + K_bA + AB + K_{ia}K_b} \quad (2)$$

$$v = \frac{VA}{K_a[1 + (P/K_{is})] + A} \quad (3)$$

$$v = \frac{VA}{K_a + A[1 + (P/K_{ii})]} \quad (4)$$

$$v = \frac{VA}{K_a[1 + (P/K_{is})] + A[1 + (P/K_{ii})]} \quad (5)$$

$$v = \frac{VA}{K_a + A + (A^2/K_{ai})} \quad (6)$$

## RESULTS

*Steady-State Kinetics with Nitroethane.* An initial steady-state kinetic analysis was previously described for nitroalkane oxidase (*10*). In that case, the $V/K$ value for oxygen was independent of whether nitroethane, 1-nitropropane, or 1-nitropentane was the substrate. On the basis of that result, Heasley and Fitzpatrick (*10*) concluded that the steady-state kinetic mechanism for nitroalkane oxidase is ping-pong, as shown in Scheme 2. The nitroalkane substrate would react with the oxidized enzyme to form the reduced enzyme. After product dissociation, the reduced FAD would be reoxidized by oxygen, forming hydrogen peroxide. The $V/K$ value for oxygen is independent of the specific nitroalkane substrate because oxygen reacts with the enzyme after product dissociation. Consistent with this chemistry, nitroethane readily reduces the enzyme in the absence of oxygen (*11*).

The previous analysis used enzyme that was predominantly inactive due to the majority of the flavin being in the form of a 5-nitrobutyl adduct, as indicated by the very low specific activities (*10*). Methods have recently been developed to convert the 5-nitrobutyl-FAD in nitroalkane oxidase to FAD and thereby convert the inactive enzyme to the fully active form (*11*). As a first step in the analysis of the kinetic mechanism of the enzyme, the steady-state kinetic parameters have been determined for the fully active enzyme using nitroethane as substrate, by varying the concentrations of both nitroethane and oxygen at pH 7 and 30 °C. The results were fit well by eq 1, consistent with the proposed ping-pong mechanism (Table 1). If the data were fit to the equation for a sequential mechanism, eq 2, no improvement was seen in the quality of the fit as reflected in the $\sigma$ value, and the $K_{ia}$

Table 1: Steady-State Kinetic Parameters for Nitroalkane Oxidase with Nitroethane as Substrate[a]

| eq | $K_{NE}$ (mM) | $V/K_{NE}$ ($M^{-1}$ $s^{-1}$) | $K_{O_2}$ ($\mu$M) | $V/K_{O_2}$ ($mM^{-1}$ $s^{-1}$) | $V_{max}$ ($s^{-1}$) | $K_{ia}$ ($\mu$M) | $\sigma$ |
|---|---|---|---|---|---|---|---|
| 1 | $3.3 \pm 0.6$ | $1700 \pm 220$ | $23 \pm 7$ | $0.24 \pm 0.06$ | $5.5 \pm 0.3$ | | 0.299 |
| 2 | $2.7 \pm 0.6$ | $1960 \pm 360$ | $15 \pm 9$ | $0.34 \pm 0.18$ | $5.3 \pm 0.3$ | $5 \pm 6$ | 0.291 |

[a] The enzyme activity was measured in 0.5 mM FAD and 50 mM potassium phosphate buffer, pH 7, at 30 °C.

Table 2: Inhibition of Nitroalkane Oxidase by Aldehydes[a]

| varied substrate | varied product | type of inhibition | eq | $K_{ii}$ (mM) | $K_{is}$ (mM) | $\sigma$ |
|---|---|---|---|---|---|---|
| nitroethane | acetaldehyde | competitive | 3 | | $0.04 \pm 0.01$ | 0.014 |
| | | uncompetitive | 4 | $1.26 \pm 0.06$ | | 0.011 |
| | | noncompetitive | 5 | $0.22 \pm 0.05$ | $0.16 \pm 0.07$ | 0.009 |
| oxygen | acetaldehyde | competitive | 3 | | $-0.06 \pm 0.8$ | 0.304 |
| | | uncompetitive | 4 | $0.18 \pm 0.02$ | | 0.011 |
| | | noncompetitive | 5 | $0.18 \pm 0.03$ | $(7 \pm 3) \times 10^{18}$ | 0.011 |
| nitroethane[b] | butyraldehyde | competitive | 3 | | $13.5 \pm 1.8$ | 0.184 |
| | | uncompetitive | 4 | $417.6 \pm 3.0$ | | 0.259 |
| | | noncompetitive | 5 | $43.5 \pm 5.1$ | $28.3 \pm 3.1$ | 0.061 |

[a] The enzyme activity was measured in 0.5 mM FAD, 100 mM ACES, 52 mM Tris, and 52 mM ethanolamine buffer, pH 7, at 30 °C. When kept fixed, the concentration of nitroethane was 17 mM; that of oxygen was 230 mM. [b] Activity was measured in 0.5 mM FAD and 50 mM potassium phosphate buffer, pH 7, at 30 °C.

value was not significantly different from zero. The $K_m$ values in Table 1 are in agreement with the values previously reported for the unactivated enzyme (*10*). In contrast, the $V_{max}$ value is 11 times the previous number determined at pH 8, consistent with the earlier enzyme being at least 90% inactive.

*Product Inhibition Studies.* As a separate approach to examining the validity of Scheme 2, the inhibition patterns were determined for products. Critically, the mechanism of Scheme 2 predicts that the products will be uncompetitive versus nitroethane and competitive versus oxygen. As shown in Table 2, the data were fit best with acetaldehyde being noncompetitive versus nitroethane. However, the quality of the fit was not substantially better when the results were fit to eq 4, which describes an uncompetitive inhibition pattern. Consequently, the inhibition pattern versus nitroethane was also determined with butyraldehyde. In this case, the results were clearly fit best by eq 5, consistent with the aldehyde product being noncompetitive versus nitroethane. Acetaldehyde is an uncompetitive inhibitor versus oxygen (Table 2). If the data were fit to the equation for noncompetitive inhibition, no improvement was seen in the quality of the fit as judged by the value of about $10^{18}$ for the $K_{is}$ value. The inhibition pattern for nitrite was fit best with nitrite being competitive versus nitroethane, with a $K_{is}$ value of $124 \pm 23$ mM (data not shown). These results are consistent with nitrite binding to E·$FAD_{ox}$ but provide no insight into when nitrite release occurs.

The isomechanism of Scheme 3 accounts for these results. The nitroalkane substrate would react with E•$FAD_{ox}$ to irreversibly form the reduced enzyme with the aldehyde product bound (E·$FAD_{red}$·P). The irreversibility of the oxidation of nitroethane is supported by primary kinetic isotope effects studies with [1,1-$^2H_2$]nitroethane (*17*). After product dissociation, the reduced enzyme irreversibly isomerizes to yield the species E′·$FAD_{red}$. E′·$FAD_{red}$ can be reoxidized by oxygen with production of hydrogen peroxide. The noncompetitive inhibition pattern observed for aldehydes versus nitroethane is consistent with the product binding to both E·$FAD_{ox}$ and E·$FAD_{red}$. The uncompetitive inhibition pattern seen for acetaldehyde versus oxygen is due to the irreversible isomerization of E·$FAD_{red}$ after product release from E·$FAD_{red}$·P.

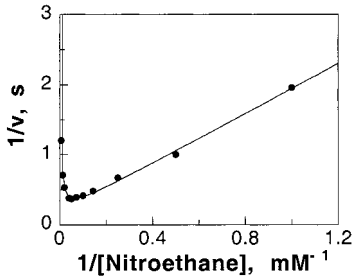
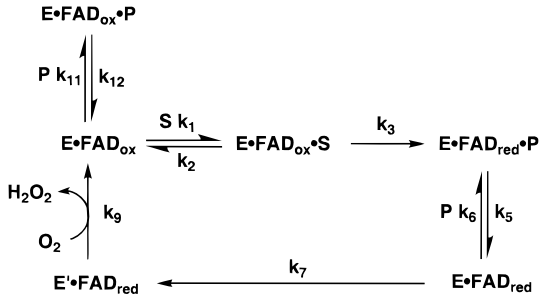



FIGURE 1: Substrate inhibition of nitroalkane oxidase with nitroethane as substrate. Initial rates were measured in air-saturated buffer in 0.5 mM FAD and 50 mM potassium phosphate, pH 7, at 30 °C. The line is a fit of the data to eq 6.

Scheme 3




*Substrate Inhibition Studies.* At concentrations of nitroethane above 25 mM, significant inhibition of the enzyme is observed (Figure 1). In general, this type of inhibition is observed when the substrate binds an enzyme form other than that which catalyzes the conversion of the substrate to the product. The most likely candidate for such an enzyme form is E·$FAD_{red}$ (Scheme 4). If this is the case, the apparent inhibition constant ($K_{ai}$) for nitroethane should be independent of the concentration of oxygen. By varying the concentration of nitroethane over the range 1−200 mM, $K_{ai}$ values of $32 \pm 14$ mM and $30 \pm 6$ mM were determined at 54 and 230 $\mu$M oxygen, respectively. These values are not significantly different from one another, consistent with

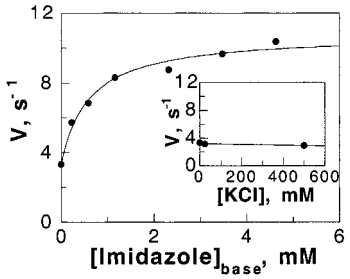
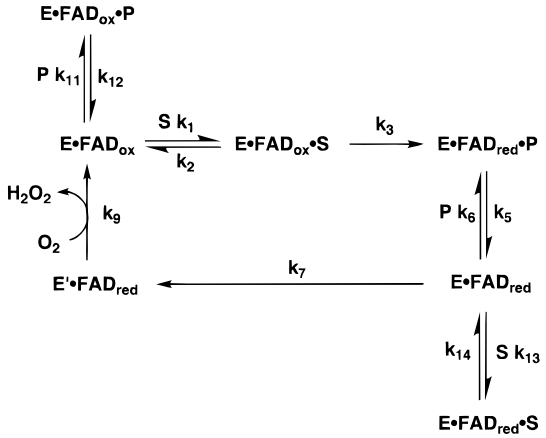



FIGURE 2: Effect of added imidazole on the $V_{max}$ value for nitroethane. Initial rates were measured with 25 mM nitroethane as a function of different concentrations of added imidazole in 0.5 mM FAD and 50 mM MOPS, pH 7, at 30 °C. Inset: Effect of KCl on the enzyme activity with 25 mM nitroethane under the same conditions as in the main panel.

Scheme 4

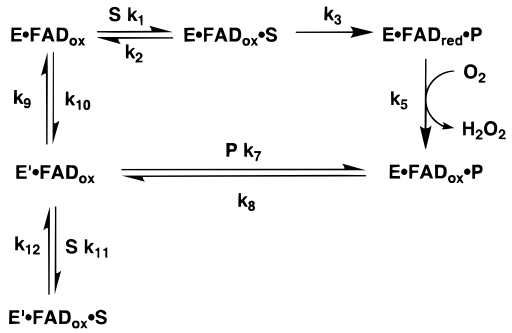



nitroethane binding the free reduced enzyme.[2] The formation of the E·$FAD_{red}$·S and E·$FAD_{ox}$·P complexes suggests that the substrate/product binding pocket is not significantly affected by the oxidation state of the bound flavin.

*Effect of Imidazole on the Activity of Nitroalkane Oxidase.* In the course of this study we observed that the activity of nitroalkane oxidase was severalfold higher when imidazole was present in the reaction assay mixture. This increase in activity showed saturation kinetics (Figure 2), suggesting that imidazole binds to the enzyme. Consistent with this conclusion, an increase of absorbance in the 500−600 nm region

[2] The observation that the apparent $K_{ai}$ value for nitroethane is independent of the concentration of oxygen rules out an alternative kinetic mechanism in which oxygen reacts with E·$FAD_{red}$·P to form E·$FAD_{ox}$·P (Scheme 5). If the oxidation of nitroethane were irreversible, such a mechanism would also fit eq 1. However, such a model predicts that the $K_{ai}$ value for nitroethane will be dependent on the concentration of oxygen.

Scheme 5



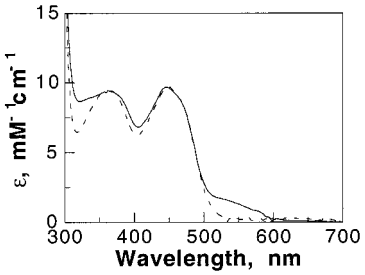




FIGURE 3: UV−Visible absorbance spectrum of nitroalkane oxidase in the presence of imidazole. Solid line: Absorbance spectrum of the enzyme in 25 mM potassium phosphate, 1 mM EDTA, and 50 mM imidazole at pH 9.7 and 10 °C. Dotted line: Absorbance spectrum of the enzyme in the same buffer devoid of imidazole.

Table 3: Effect of Imidazole on Nitroalkane Oxidase Activity with 1-Nitrobutane as Substrate[a]

| pH | $K_{act(total)}$ (mM) | $K_{act(unprotonated)}$ (mM) | $K_{act(protonated)}$ (mM) |
|---|---|---|---|
| 6 | 22 ± 8.6 | 2.2 ± 0.9 | 19.8 ± 7.7 |
| 7 | 4.9 ± 0.6 | 2.4 ± 0.3 | 2.4 ± 0.3 |
| 8 | 2.9 ± 1.2 | 2.5 ± 1.0 | 0.3 ± 0.1 |

[a] The enzyme activity was measured with 1 mM 1-nitrobutane in 0.5 mM FAD and 50 mM potassium phosphate buffer at 30 °C at the indicated pH.

of the UV−visible absorbance spectrum of nitroalkane oxidase was observed in the presence of imidazole at pH 9.7 (Figure 3). A 5% increase of the flavin fluorescence emission at 525 nm ($\lambda_{ex}$ = 450 nm) of bound FAD was also observed in the presence of imidazole at pH 7. The possibility that activation was due to the ionic strength of the buffer was ruled out by the observation that no changes were observed in the activity with nitroethane when 0.5 M KCl was added (Figure 2, inset). No activation was observed with Tris, phosphate, histidine, or ethanolamine. To determine which species of imidazole activates nitroalkane oxidase, the effect of imidazole on the activity of nitroalkane oxidase was determined as a function of pH in the range 6−8. The alternate substrate 1-nitrobutane was used in this study to avoid substrate inhibition.[3] As shown in Table 3, the $K_{act}$ values were independent of the pH when the concentration of the unprotonated imidazole was used in the calculations, consistent with this species being the active form.

*Effect of Imidazole on the Kinetic Parameters.* As a further probe of the mechanism of activation by imidazole, the kinetic parameters with a number of nitroalkane substrates were determined in the presence and absence of 12 mM unprotonated imidazole at pH 7. As shown in Table 4, the $V/K$ values for nitroalkanes did not vary significantly when imidazole was added. On the other hand, significant increases in the apparent $V_{max}$ values were observed with all the substrates tested. These results are consistent with a step after the first irreversible step being affected by imidazole. Initial rates were also measured by varying the concentrations of both nitroethane and oxygen in the presence of 12 mM unprotonated imidazole. Values of 27.7 ± 7.7 $s^{-1}$ and 0.24 ± 0.04 $\mu M^{-1}$ $s^{-1}$ were determined for $V_{max}$ and $V/K_{O_2}$,

[3] The $K_m$ value for 1-nitrobutane is less than 20 $\mu$M between pH 6 and 8, whereas the $K_{ai}$ value for 1-nitrobutane is about 50 mM at pH 7 (G. Gadda, and P. F. Fitzpatrick, unpublished results). Thus, 1 mM 1-nitrobutane is saturating and below the concentration range at which substrate inhibition is observed.

Table 4: Effect of Imidazole Base on the Kinetic Parameters of Primary and Secondary Nitroalkanes as Substrates for Nitroalkane Oxidase[a]

| substrate | $\left(\frac{V/K_{base}}{V/K_0}\right)^b$ | $\left(\frac{V_{base}}{V_0}\right)^b$ |
|---|---|---|
| nitroethane | 1.3 ± 0.2 | 3.5 ± 0.7 |
| 1-nitropropane | 1.1 ± 0.2 | 2.8 ± 0.2 |
| 1-nitrobutane | 0.9 ± 0.2 | 2.0 ± 0.2 |
| 1-nitropentane | 1.5 ± 0.3 | 2.2 ± 0.2 |
| 1-nitrohexane | 0.7 ± 0.1 | 2.7 ± 0.1 |
| 2-nitropropane[c] | 0.9 ± 0.2 | 2.1 ± 0.6 |

[a] The enzyme activity was measured in 0.5 mM FAD, 100 mM ACES, 52 mM Tris, and 52 mM ethanolamine buffer, pH 7, at 30 °C. [b] Ratio of the value determined in the presence of 12 mM added unprotonated imidazole to that with no base added. [c] The enzyme activity was measured in 0.5 mM FAD and 50 mM potassium phosphate, pH 7, at 30 °C.

respectively (data not shown). Thus, while a 5-fold increase is observed in the $V_{max}$ value in the presence of imidazole, the $V/K_{O_2}$ value is identical to that observed when no imidazole is present (Table 1), ruling out an effect on the rate of reaction with oxygen. The lack of an effect of added imidazole on the $V/K$ values for either substrate restricts the effect to steps between E·FAD$_{red}$·P and E′·FAD$_{red}$ in Scheme 4. To determine which step is affected, the effects of imidazole on the apparent $K_{ai}$ value for nitroethane were determined at pH 7.8.[4] Initial rates were determined at different concentrations of nitroethane from 1 to 400 mM. By fitting the data to eq 6, an apparent $K_{ai}$ value of 159 ± 19 mM was determined in the presence of 12 mM imidazole base (data not shown). This 5-fold increase of the apparent $K_{ai}$ value for nitroethane seen in the presence of imidazole is consistent with a decrease of the steady-state concentration of the free reduced enzyme, the species responsible for substrate inhibition. As a separate approach to determine which step is affected, the effect of imidazole on the inhibition pattern for butyraldehyde was determined with 1-nitrobutane as substrate (data not shown). The $K_{ii}$ values were 31 ± 7 mM and 202 ± 39 mM in the absence and presence of imidazole, respectively. Thus, a 6.5-fold increase in the $K_{ii}$ value was observed in the presence of imidazole, further suggesting that the steady-state concentration of free reduced enzyme decreased in the presence of imidazole.

## DISCUSSION

The steady-state kinetic mechanism of the activated FAD-containing form of nitroalkane oxidase has been studied with nitroethane. The results of these studies are consistent with the ping-pong isomechanism shown in Scheme 4. After formation of the E·FAD$_{ox}$·S complex, the nitroalkane substrate is oxidized to form acetaldehyde bound to the reduced enzyme.[5] Kinetic isotope effects with [1,1-$^2H_2$]-nitroethane indicate that the oxidation of nitroethane is irreversible (*17*). Following release of the products from the E·FAD$_{red}$·P complex, an irreversible isomerization of the free reduced enzyme forms the species that reacts with oxygen to produce of hydrogen peroxide. Both the oxidized and the reduced enzymes can form dead-end complexes by binding the aldehyde product or the nitroalkane substrate, respectively.

Imidazole activates nitroalkane oxidase, resulting in a 5-fold increase in the $V_{max}$ value for nitroethane. The formation of a reversible imidazole−enzyme complex is required for activation, as suggested by the saturation kinetics of activation. More definitive evidence for the formation of an imidazole−enzyme complex comes from the effects on the absorbance and fluorescence spectra of the enzyme. The increase in absorbance in the 500−600 nm region is consistent with the formation of a charge-transfer complex between the oxidized enzyme and unprotonated imidazole, suggesting that imidazole binds close to the isoalloxazine ring of the flavin. The lack of a change in the $V/K$ value for either substrate and the alleviation of both the inhibition by nitroethane and butyraldehyde in the presence of added imidazole suggest that the imidazole-sensitive step is the irreversible isomerization of E·FAD$_{red}$. The 5-fold increase in the $V_{max}$ and the $K_{ai}$ values for nitroethane and the 6.5-fold increase in the $K_{ii}$ value for butyraldehyde observed in the presence of imidazole are consistent with $k_7$ being about 80% rate-limiting for catalysis in the absence of imidazole. In agreement with this conclusion, the kinetic isotope effect on $V_{max}$ with [1,1-$^2H_2$]nitroethane increases from 2 to 7 in the presence of imidazole at pH 7 (*17*).

A slow irreversible change in the conformation of the reduced enzyme occurs after aldehyde product release; this is indicative of an isomechanism for nitroalkane oxidase. In the absence of structural information, the details of this conformational change must remain speculative. Since the pioneering work of Albery and Knowles on proline racemase (*18*−*24*), a number of enzymes have been proposed to have isomechanisms in which conformational changes are associated with proton transfers, including fumarase (*25*), triosophosphate isomerase (*26*), pepsin (*27*), diaminopimelate epimerase (*28*), and glutamate racemase (*29*). On the basis of solvent isotope effect studies, slow proton transfers to solvent have similarly been proposed for two other flavoproteins, D-amino acid oxidase (*30*) and tryptophan 2-monooxygenase (*31*). The effects of viscosity on the kinetic parameters of D-amino acid oxidase have also been interpreted in favor of a conformational change between an open conformation that binds substrates and products and a closed complex within which catalysis occurs (*32*). With nitroalkane oxidase, the unprotonated form of imidazole is required for activation, suggesting that imidazole may act as an external base by catalyzing the loss of a proton to solvent. The methods used in the study of D-amino acid oxidase and tryptophan 2-monooxygenase were insensitive to the detection of isomechanisms and not able to determine whether proton transfer to solvent is concomitant with or follows product release. However, those results are fully consistent with an enzyme isomerization following product release, raising the question as whether isomechanisms such that described here for nitroalkane oxidase are common among flavin-containing enzymes.

To our knowledge, this is the first instance in which an isomechanism has been described for a flavin-containing enzyme. In the accompanying paper (*17*), we describe the use of kinetic isotope and pH effects to study further the steady-state kinetic mechanism of nitroalkane oxidase.

[4] The apparent $K_{ai}$ value for nitroethane is independent of pH in the range 7−8.5 (*17*).

[5] Since the inhibition pattern for nitrite does not provide insight into when nitrite release occurs, it is assumed that nitrite is released at the same time as the aldehyde.

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