The Nonoxidative Conversion of Nitroethane to Ethylnitronate in *Neurospora crassa* 2-Nitropropane Dioxygenase Is Catalyzed by Histidine 196[†]

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Received May 29, 2008; Revised Manuscript Received July 10, 2008

ABSTRACT: The deprotonation of nitroethane catalyzed by *Neurospora crassa* 2-nitropropane dioxygenase was investigated by measuring the formation and release of ethylnitronate formed in turnover as a function of pH and through mutagenesis studies. Progress curves for the enzymatic reaction obtained by following the increase in absorbance at 228 nm over time were visibly nonlinear, requiring a logarithmic approximation of the initial reaction rates for the determination of the kinetic parameters of the enzyme. The pH dependence of the second-order rate constant k_{cat}/K_m with nitroethane as substrate implicates the presence of a group with a pK_a of 8.1 ± 0.1 that must be unprotonated for nitronate formation. Mutagenesis studies suggest that this group is histidine 196 as evident from the inability of a H196N variant form of the enzyme to catalyze the formation of ethylnitronate from nitroethane. Replacement of histidine 196 with asparagine resulted in an ~15-fold increase in the k_{cat}/K_m with ethylnitronate as compared to the wild-type, which results from the inability of the mutant enzyme to undergo nonoxidative turnover. The results presented herein are consistent with a branched catalytic mechanism for the enzyme in which the ethylnitronate intermediate formed from the H196-catalyzed deprotonation of nitroethane partitions between release from the active site and oxidative denitrification to yield acetaldehyde and nitrite.

A variety of enzymes have been shown to catalyze the oxidative denitrification of either (neutral) nitroalkanes or (anionic) nitronates to their corresponding aldehyde compounds and nitrite (*1*−*14*). These include 2-nitropropane dioxygenase (*1*−*6*) and nitroalkane oxidase (*8, 9*), both of which catalyze a physiological denitrification reaction, as well as D-amino acid oxidase (*10*), glucose oxidase (*11*), propionate-3-nitronate oxidase (*12*), and horseradish peroxidase (*13*), all of which can utilize nitroalkanes in nonphysiological reactions. While the catalytic mechanisms of these enzymes are diverse, they all involve the oxidation of an enzyme-bound form of nitronate during turnover. For nitroalkane oxidase and *Neurospora crassa* 2-nitropropane dioxygenase, which are the only two enzymes capable of effectively utilizing the neutral from of substrate in catalysis (*2, 3, 8, 9*), this oxidation reaction requires the generation of an enzyme-bound nitronate through the initial abstraction of a proton from the α-carbon of the nitroalkane substrate. The proton abstraction step in the nitroalkane oxidase reaction has been extensively characterized (*14*−*16*), where it has been shown through both mutagenesis and crystallographic studies to be catalyzed by an aspartate residue in the active site of the enzyme (i.e., Asp-402). The identity of the catalytic base in *N. crassa* 2-nitropropane dioxygenase has yet to be elucidated, although a conserved histidine residue has been proposed to be suitably positioned to deprotonate nitroalkanes in the X-ray crystallographic structure of the enzyme from *Pseudomonas aeruginosa* (*17*).

N. crassa 2-nitropropane dioxygenase is the only flavin-dependent enzyme reported to date with the ability to effectively utilize either neutral nitroalkanes or anionic nitronates as substrate in an oxidative denitrification reaction (*2, 3*). An anionic flavosemiquinone is transiently formed in both reactions from a single electron transfer reaction involving the enzyme-bound nitronate, either directly after the anionic substrate binds in the active site of the enzyme or upon deprotonation of the neutral substrate by a catalytic base in the active site of the enzyme (*2, 3*). Studies of the secondary kinetic isotope effects using [1-^{2}H]ethylnitronate as substrate have suggested that at pH ≤7.5 a significant fraction of the enzyme-bound anionic substrate is protonated in the active site of the enzyme to form nitroethane, which is then released to the solvent rather than undergoing oxidative denitrification (Scheme 1) (*3*). Based on the structural similarities between ethylnitronate and nitroethane, a similar partitioning has also been proposed for the ethylnitronate intermediate formed during the enzymatic turnover with nitroethane (*3*), although no direct evidence is available in support of such a hypothesis. The current study was conducted with the dual purpose of testing the proposed role of the conserved histidine (i.e., His-196) in the deprotonation reaction of nitroethane catalyzed by *N. crassa* 2-nitropropane dioxygenase and of evaluating whether a fraction of the resulting ethylnitronate is released from the

[†] This work was supported in part by Grant PRF 47636-AC4 from the American Chemical Society (G.G.) and a Molecular Basis of Disease Fellowship (K.F.).

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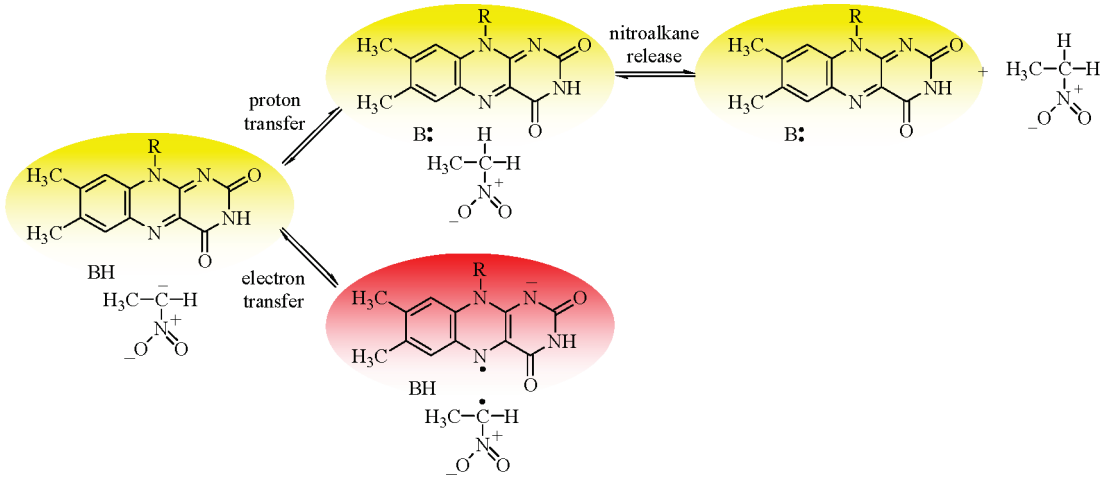
10.1021/bi801013e CCC: $40.75
Published on Web 08/09/2008

Scheme 1: Branching of the Michaelis Complex Formed during Turnover of *N. crassa* 2-Nitropropane Dioxygenase with Ethylnitronate as Substrate[a]



[a] Note that under initial velocity conditions in the absence of exogenous nitroethane the kinetic step of nitroalkane release is practically irreversible. For clarity, further conversion of the anionic flavosemiquinone enzyme through the oxidative denitrification pathway is not shown.

active site of the enzyme rather than proceeding through oxidative catalysis. Toward these aims, a kinetic assay to monitor the time-dependent formation of ethylnitronate from nitroethane was developed for the wild-type *N. crassa* 2-nitropropane dioxygenase and was used to determine the effects of the replacement of histidine 196 with asparagine in the active site of the enzyme. The kinetic data presented herein are consistent with histidine 196 acting as a base in the deprotonation of nitroethane catalyzed by *N. crassa* 2-nitropropane dioxygenase and with the (partial) release of the ethylnitronate that is formed enzymatically from nitroethane from the active site of the enzyme before oxidation of this intermediate occurs during turnover.

EXPERIMENTAL PROCEDURES

Materials. Escherichia coli strain Rosetta(DE3)pLysS was from Novagen (Madison, WI). The QIAprep spin miniprep kit was from Qiagen (Valencia, CA), and the QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Oligonucleotides used for site-directed mutagenesis and for sequencing of the mutant gene were from Sigma Genosys (The Woodlands, TX). Luria−Bertani agar and broth, ampicillin, phenylmethanesulfonyl fluoride (PMSF), lysozyme, and nitroethane were from Sigma-Aldrich (St. Louis, MO). Isopropyl β-D-thiogalactopyranoside (IPTG) was from Promega (Madison, WI). Ammonium sulfate and magnesium chloride were from ICN Biomedicals (Irvine, CA). EDTA was from Fisher Scientific, whereas DNase I and RNase A were from Roche Biomedicals (Indianapolis, IN). The Hi-Prep 16/10 Octyl Fast Flow column and the DEAE-Sepharose used in packing the DEAE column were from GE Healthcare (Barrington, IL). All other reagents were of the highest purity commercially available.

Expression and Purification of N. crassa 2-Nitropropane Dioxygenase. The enzyme was obtained utilizing the protocol described previously (*2*) with the following modifications. *E. coli* strain BL21(DE3) cells harboring plasmid pET/2NPDnc were used to inoculate 6 × 1.25 L of Luria−Bertani broth containing 50 μg/mL ampicillin, and the cultures were incubated at 37 °C until an OD_{600} of 0.6 to 0.8 was reached, at which point IPTG was added to a final concentration of 0.1 mM. After 6 h of induction at 37 °C, the cells were harvested and treated in the same manner described previously (*2*). Purification of *N. crassa* 2-nitropropane dioxygenase was achieved through a simplified protocol involving a 70% ammonium sulfate fractionation step and a single anion-exchange chromatographic step as described previously (*2*), but at pH 7 to facilitate the binding of the enzyme to the column. Fractions of the highest purity were pooled, dialyzed against 50 mM potassium phosphate, pH 7.4, and stored at −20 °C until use. The simplified procedure resulted in the obtainment of highly purified enzyme (>95%) as judged by SDS−PAGE.

Preparation and Purification of N. crassa 2-Nitropropane Dioxygenase H196N. A QuikChange kit was used to prepare a mutant form of *N. crassa* 2-nitropropane dioxygenase in which histidine 196 was replaced with asparagine. The method used was according to the manufacturer's instructions, using a pET/2NPDnc plasmid (*2*) as a template, and the oligonucleotides For.H196N (5′GGATCGATGCGGGAGGGAATCAGCTTGCTACAGGG3′) and Rev.H196N (5′CTCCCTGTAGCAAGCTGATTCCCTCCCGCATCGATCCC3′) as forward and reverse primers (underlined letters indicate the site of mutation). The DNA was then sequenced at the DNA Core Facility at Georgia State University using an Applied Biosystems big dye kit on an Applied Biosystems model ABI 377 DNA sequencer, which confirmed the presence of the mutant gene in the correct orientation. *E. coli* strain Rosetta(DE3)pLysS competent cells were transformed with plasmid pET/2NPDnc-H196N by electroporation and were stored at −80 °C as a 7% DMSO suspension.

E. coli strain Rosetta(DE3)pLysS cells harboring plasmid pET/2NPDnc-H196N were used to inoculate 5 × 1.25 L of Luria−Bertani broth containing 50 μg/mL ampicillin plus 34 μg/mL chloramphenicol, and the cultures were incubated at 37 °C until an OD_{600} of 0.6 to 0.8 was reached. The mutant enzyme was then expressed through induction with IPTG at a final concentration of 0.2 mM at 37 °C for 6 h. The cells

were harvested by centrifugation and were suspended at pH 8 with 4 volumes of 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, 0.2 mg/mL lysozyme, 20 μg/mL DNase I, 20 μg/mL RNase A, and 10 mM $MgCl_2$, in 50 mM Tris-HCl, and were incubated on ice for 30 min. After sonication and collection of the cell-free extract by centrifugation for 20 min at 4 °C, the sample was brought to 60% ammonium sulfate saturation and was incubated on ice for 20 min. The supernatant collected after centrifugation was then loaded directly onto an Octyl Fast Flow column (3.5 × 20 cm) equilibrated with 2.75 M ammonium sulfate in 50 mM potassium phosphate containing 10% glycerol, pH 7.4, connected to an Åkta*prime* Amersham Biotech system (Barrington, IL). Protein elution was carried out with a linear gradient from 2.75 to 0 M ammonium sulfate over 500 mL at a flow rate of 2 mL/min. Fractions of the highest purity were pooled, dialyzed against 5 mM potassium phosphate containing 10% glycerol, pH 7.4, and loaded onto a DEAE Fast Flow column (4 × 30 cm) equilibrated with the same buffer. The column was eluted with a linear gradient from 0 to 500 mM ammonium sulfate developed over 1 L at a flow rate of 2 mL/min. Fractions of the highest purity were pooled, concentrated using polyethylene glycol 10000, and dialyzed against 50 mM potassium phosphate at pH 7.4. The purified enzyme was stored at −20 °C until use.

Spectrophotometric Studies. Stock solutions of nitroethane (100 mM) were prepared in water and were diluted to concentrations ranging from 0.1 to 1 mM. Absorbance spectra in the 200−300 nm region were recorded for each concentration of nitroethane at 30 °C using a cuvette with a 1 cm path length on an Agilent Technologies diode-array spectrophotometer model HP 8453 (Santa Clara, CA). The molar extinction coefficient was determined from a plot of the absorbance at 202 nm versus concentration of nitroethane using the Lambert−Beer equation. The determination of the molar extinction coefficient of ethylnitronate at 228 nm was carried out in a similar manner using concentrations ranging from 10 to 100 μM. Ethylnitronate was prepared in water upon incubating a solution of nitroethane with a 1.2 molar excess of potassium hydroxide for at least 24 h at room temperature.

The UV−visible absorbance spectra recorded during the reductive half-reaction of the wild-type and H196N variant forms of *N. crassa* 2-nitropropane dioxygenase with ethylnitronate as substrate were obtained using a TgK Scientific SF-61 stopped-flow spectrophotometer equipped with a photodiode array detector. Spectra were recorded in 50 mM sodium pyrophosphate at pH 7 and 30 °C after anaerobically mixing the enzyme with ethylnitronate with final concentrations of 10 μM and 0.5 mM, respectively. Anaerobic mixing of the enzyme with substrate was achieved using the method described previously (*3*).

Kinetic Studies. The kinetic parameters for the nonoxidative reaction catalyzed by *N. crassa* 2-nitropropane dioxygenase with nitroethane as substrate were determined by monitoring the increase in absorbance at 228 nm resulting from mixing the enzyme at a concentration of 1.07 μM in air-saturated 50 mM sodium pyrophosphate with substrate (in the concentration range from 1 to 3 mM). The pH dependence of the kinetic parameters was determined in the range from 6 to 10. The enzyme concentration used in the assays was expressed per enzyme-bound FMN content[1] using an ε_{444nm} value of 11850 M^{-1} cm^{-1} (*2*). The results reported are the averages of three independent measurements, which typically differed by ≤3%. Steady-state turnover of *N. crassa* 2-nitropropane dioxygenase (1.07 μM) with 3 mM nitroethane was also determined by monitoring oxygen consumption using a computer interfaced Oxy-32 oxygen monitoring system (Hansatech Instrument Ltd.), equipped with a thermostated water bath. Assays were carried out at pH 6, 8, and 10 and were allowed to proceed for at least 120 s after mixing the enzyme with substrate. The enzymatic activity of the H196N variant of 2-nitropropane dioxygenase with nitroethane or ethylnitronate as substrate was measured by monitoring oxygen consumption after mixing 2.17 μM enzyme with 1 mM substrate in air-saturated 50 mM sodium pyrophosphate at pH 10. The kinetic parameters for the oxidative reactions of *N. crassa* 2-nitropropane dioxygenase and the H196N variant form the enzyme with ethylnitronate as substrate were determined in 50 mM sodium pyrophosphate containing 1% ethanol at pH 9.5 and 30 °C as previously described in ref *3*. Substrate concentrations ranged from 1 to 50 mM, whereas enzyme concentrations were ≤110 nM.

Data Analysis. Kinetic data were fit using KaleidaGraph (Synergy Software, Reading, PA) or Enzfitter (Biosoft, Cambridge, U.K.) software. Initial rates for the nonenzymatic formation of ethylnitronate from nitroethane were determined from fits of the stopped-flow traces with eq 1, where A_0 is the initial absorbance at 228 nm, k_{obs} is the pseudo-first-order rate constant for the change in absorbance at 228 nm, and t is time. The k_{obs} values were then converted into rates of product formation using the experimentally determined $\Delta\varepsilon_{228nm}$ of 8520 M^{-1} cm^{-1}, which corresponds to the difference between the extinction coefficients for ethylnitronate (ε_{228nm} value of 8625 M^{-1} cm^{-1}; this study) and nitroethane (ε_{228nm} value of 105 M^{-1} cm^{-1}; this study). Initial rates for the enzymatic formation of ethylnitronate from nitroethane were determined by fitting the nonlinear reaction progress curves to the empirical equation developed by Fei and Lu (*18*) (eq 2). A_0 is the initial absorbance at 228 nm, b is defined as a shape parameter, t is time, and x is the scale of the logarithmic curve. The initial rate is given by the slope of a tangent line to the curve that intersects through the origin and is found by taking the derivative of the absorbance at 228 nm (A_{228}) with respect to t when the latter is set equal to zero. The resulting value is converted to initial rates of product formation using the experimentally determined $\Delta\varepsilon_{228nm}$ value and after correcting for the 1 cm path length used (l) (eq 3). Initial rates determined in this fashion reflect the overall rate of ethylnitronate formation and are converted to enzymatic reaction rates by subtraction of the nonenzymatic component of the reaction determined in parallel under the same conditions. The resulting data were fit with eq 4, where k_{cat}/K_m is the second-order rate constant for the enzymatic reaction and S is the concentration of nitroethane. The pH dependence of the k_{cat}/K_m values was determined

[1] The choice of defining the enzymatic activity of *N. crassa* 2-nitropropane dioxygenase as a function of the FMN content of the protein stems from the observation that the nonoxidative turnover in which ethylnitronate is formed from nitroethane occurs only with the holoenzyme (K. Francis and G. Gadda, unpublished observation). Expressing the enzymatic activity in terms of FMN content also corrects for any variation that may exist in the flavin to protein stoichiometry between different preparations of the wild-type and mutant enzymes.

by fitting the initial rate data to eq 5, which describes a curve with a slope of +1 and a plateau region at high pH. *C* is the pH-independent value of the k_{cat}/K_m value. When initial rates were determined by measuring oxygen consumption, the data were fit to eq 6, where K_a and K_b represent the Michaelis constants for the nitronate substrate (A) and oxygen (B) and k_{cat} is the turnover number of the enzyme (*e*).

$$v_0 = A_0 + k_{obs}t \quad (1)$$

$$A_{228} = A_0 + b\ln\left(1 + \frac{t}{x}\right) \quad (2)$$

$$v_0 = \left(\frac{b}{x}\right)\left(\frac{1}{\Delta\varepsilon_{228nm}l}\right) \quad (3)$$

$$\frac{v_0}{e} = \left(\frac{k_{cat}}{K_m}\right)S \quad (4)$$

$$\log\left(\frac{k_{cat}}{K_m}\right) = \log\left(\frac{C}{1 + \frac{10^{-pH}}{10^{-pK_a}}}\right) \quad (5)$$

$$\frac{v_0}{e} = \frac{k_{cat}AB}{K_aB + K_bA + AB + K_{ia}K_b} \quad (6)$$

RESULTS

Assay for the Formation of Ethylnitronate from Nitroethane. As a prerequisite for studies of the deprotonation of nitroethane catalyzed by *N. crassa* 2-nitropropane dioxygenase, the UV absorbance properties of the neutral and anionic forms of the substrate were studied at 30 °C. The UV absorbance spectra of nitroethane and ethylnitronate exhibit absorbance maxima at 202 and 228 nm, respectively (Supporting Information, Figure S1). Molar extinction coefficients for nitroethane of 1240 ± 10 M^{-1} cm^{-1} and 105 ± 2 M^{-1} cm^{-1} were determined at 202 and 228 nm using the Lambert−Beer law. Similarly, a molar extinction coefficient of 8625 ± 335 M^{-1} cm^{-1} was determined for ethylnitronate at 228 nm. These results establish that the formation of ethylnitronate from nitroethane could be monitored directly by measuring the increase in absorbance at 228 nm over time, as illustrated in Figure 1 for a nonenzymatic reaction mixture consisting of 50 mM sodium pyrophosphate and 0.15 mM nitroethane at pH 8 and 30 °C. In a similar fashion, ethylnitronate accumulated over time upon incubating *N. crassa* 2-nitropropane dioxygenase with 0.15 mM nitroethane in 50 mM sodium pyrophosphate at pH 8 and 30 °C could be monitored using the assay (data not shown).

Enzymatic Formation of Ethylnitronate from Nitroethane. The reaction progress curves for the formation of ethylnitronate from nitroethane were measured upon mixing 3 mM nitroethane with 50 mM sodium pyrophosphate in either the absence or presence of 1.07 μM *N. crassa* 2-nitropropane dioxygenase at pH 8 and 30 °C to determine if the enzyme catalyzes the deprotonation reaction. As shown in Figure 2, the rate of absorbance increase at 228 nm over time was higher for the reaction containing the enzyme than that containing only sodium pyrophosphate, consistent with *N. crassa* 2-nitropropane dioxygenase catalyzing the formation and release of ethylnitronate with nitroethane as substrate. Moreover, while the nonenzymatic reaction yielded linear progress curves, those for the enzymatic reaction were visibly

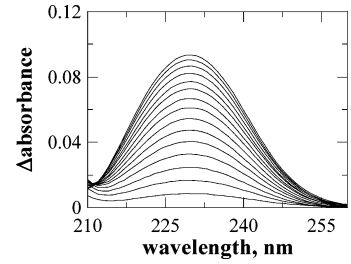


FIGURE 1: Nonenzymatic formation of ethylnitronate from nitroethane at pH 8 and 30 °C. A solution of 50 mM sodium pyrophosphate was mixed with 0.15 mM nitroethane. The UV absorbance spectrum of the reaction mixture was recorded 20 s after mixing, and additional spectra were recorded over 15 min at 1 min intervals. Difference spectra were constructed by subtracting the spectrum recorded 20 s after mixing from those recorded at later times and are shown in order of increasing incubation time from the bottom to the top spectrum.

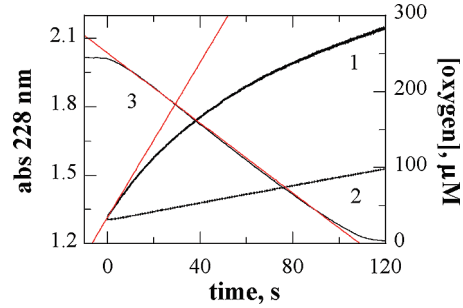


FIGURE 2: Reaction progress curves for the enzymatic and nonenzymatic formation of ethylnitronate from nitroethane. A 3 mM solution of nitroethane in water was mixed with either 1.07 μM *N. crassa* 2-nitropropane dioxygenase in 50 mM sodium pyrophosphate (trace 1) or with 50 mM sodium pyrophosphate alone (trace 2) at pH 8 and 30 °C, and the absorbance at 228 nm was monitored over 120 s. For clarity, the starting absorbance of the nonenzymatic reaction was arbitrarily increased to match that of the enzymatic reaction. Trace 3 shows a control experiment in which the progress curve for the enzymatic reaction carried out under the same conditions was followed by monitoring the concentration of oxygen over time using a Clark oxygen electrode. The red lines represent linear fits of traces 1 and 3 as tangents to the reaction progress curves at the initial stages of the reaction.

nonlinear across the pH range from 6 to 10, as illustrated in Figure 2 for the case of pH 8, suggesting a role of the enzyme in the formation of ethylnitronate from nitroethane. The nonlinear reaction progress curves indicate that the concentration of enzyme turning over with the substrate progressively decreases over the time course of the assay. In principle, a progressive, time-dependent decrease in the concentration of enzyme turning over (e.g., ES complex) can be due to a decrease in the concentration of either the enzyme or the substrate. The curvature of the time courses being the result of a decrease in the enzyme concentration, either due to enzyme instability or inhibition, could be ruled out based on the observation that the rate of oxygen consumption during turnover of the enzyme with nitroethane under the same conditions maintained linearity for at least 90 s (Figure 2). Instead, the progressive decrease in the amount of enzyme turning over with the substrate could be readily explained with the time-dependent, enzymatic depletion of nitroethane when the reaction occurs at subsaturating concentrations of substrate, i.e., with $[S] \ll K_m$. In support of this rationale, only the second-order rate constants k_{cat}/K_m for the enzymatic formation and release of ethylnitronate from nitroethane could be estimated in this study (*vide infra*), but not turnover

numbers (k_{cat}) and Michaelis constants (K_m) for the reaction. Similar results demonstrating that the overall rates of ethylnitronate formation are higher in the presence of the enzyme as compared to the corresponding nonenzymatic reactions were obtained at each pH tested in the range from 6 to 10 (data not shown). As for the case of pH 8, enzyme instability or inhibition could be ruled out as a possible cause for the curved time courses at pH 6 and 10 based on the linear reaction progress curves obtained by monitoring oxygen consumption over prolonged times (Supporting Information, Figure S2).

Determination of Initial Rates for the Enzymatic Formation of Ethylnitronate from Nitroethane. The curvature observed in the time courses for the enzymatic production of ethylnitronate from nitroethane (Figure 2) indicates that the concentration of the enzyme undergoing turnover is not constant over time, thereby preventing the use of linear regression analysis to determine the initial rates of the reaction. Consequently, an alternative method based on a logarithmic approximation of the initial rates (eqs 2 and 3) that was originally developed by Fei and Lu (*18*) was used to determine initial rates of ethylnitronate formation and release from the enzyme. As shown in the example of Figure 2 (trace 1), this approach accurately determines the slope of a tangent line to the progress curve at the initial stage of the reaction. The slope of this line has the same units (abs/time) and meaning as that determined from traditional linear regression analysis (*19*) and therefore accurately represents the true initial rate of the reaction. A value of 1.6 ± 0.1 μM s^{-1} was determined with 3 mM nitroethane at pH 8 and 30 °C from the logarithmic fit of the data, irrespective of whether the progress curves were acquired for time intervals comprised between 60 and 180 s (data not shown). For comparison, the rate of the nonenzymatic reaction determined under the same conditions from linear reaction progress curves by using eq 1 (Figure 2) was found to be significantly lower, with a value of 0.27 ± 0.02 μM s^{-1}. Consequently, a value of ~1.3 μM s^{-1} was determined for the enzymatic formation of ethylnitronate under these conditions (e.g., 1.07 μM enzyme, 50 mM sodium pyrophosphate, pH 8, and 30 °C), by subtracting the rate of the nonenzymatic reaction from the overall reaction rate that includes both the enzymatic and nonenzymatic components.

pH Dependence of the k_{cat}/K_m Values with Nitroethane As Determined by Monitoring Ethylnitronate Formation. The deprotonation of nitroethane to yield ethylnitronate catalyzed by *N. crassa* 2-nitropropane dioxygenase was further examined by determining the second-order rate constants k_{cat}/K_m for the nonoxidative reaction as a function of pH. The k_{cat}/K_m values were determined by measuring reaction rates using the method described above at varying concentrations of nitroethane. As shown in Figure 3A, the rate of the reaction increased linearly with the concentration of nitroethane in the range from 1 to 3 mM, allowing for the determination of a k_{cat}/K_m value of 450 ± 10 M^{-1} s^{-1} for the enzymatic reaction at pH 8 and 30 °C. Since concentrations of nitroethane >3 mM could not be used due to the high absorbance in the UV region of the resulting reaction mixtures, neither k_{cat} nor K_m values could be determined using this approach. As shown in Figure 3B, the k_{cat}/K_m values for the enzymatic reaction increased with increasing pH and reached a plateau at high pH. A pH-independent upper limiting value for k_{cat}/K_m of 900 ± 150 M^{-1} s^{-1} and a pK_a of 8.1 ± 0.1 were determined from the fit of the curve in Figure 3B to eq 5, suggesting that an unprotonated group is required for the catalytic formation of ethylnitronate from nitroethane.

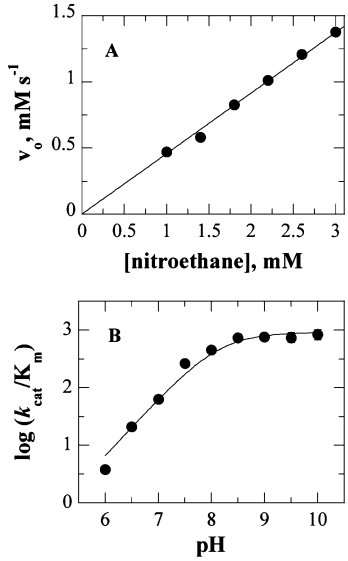


FIGURE 3: pH dependence of k_{cat}/K_m with nitroethane as substrate for *N. crassa* 2-nitropropane dioxygenase. Panel A: Nitroethane (1−3 mM) was mixed with 1.07 μM enzyme in 50 mM sodium pyrophosphate in a stopped-flow spectrophotometer at pH 8 and 30 °C, and absorbance changes at 228 nm were observed over 120 s. Reaction rates determined through fits of the traces with eq 2 were plotted as a function of nitroethane concentration. Each data point is the average of three independent measurements and is the enzymatic reaction rate subtracted by the nonenzymatic reaction rate determined under the same conditions in parallel experiments. Panel B: The pH dependence of k_{cat}/K_m was determined in the range from 6 to 10 at 30 °C by measuring rates of nitronate formation at varying concentrations of nitroethane. The data were fit with eq 5 ($R^2 = 0.981$).

Identification of the Catalytic Base of N. crassa 2-Nitropropane Dioxygenase. Histidine 196 in *N. crassa* 2-nitropropane dioxygenase was replaced with asparagine through site-directed mutagenesis to evaluate whether this residue acts as the catalytic base that abstracts the proton from the α-carbon of nitroethane during turnover. The role of His-196 in the deprotonation of nitroethane was tested by monitoring the formation of ethylnitronate upon mixing the H196N variant form *N. crassa* 2-nitropropane dioxygenase with the neutral nitroalkane using the assay developed in this study. The assays were carried out at pH 10 to ensure that the catalytic base, if present in the mutant, is in the correct ionization state to react with nitroethane. In contrast to the wild-type enzyme (see above), the rate of ethylnitronate formation from nitroethane upon mixing the mutant form of *N. crassa* 2-nitropropane dioxygenase with nitroethane was not significantly different from that of the buffer-catalyzed reaction, with a value of ~0.04 min^{-1} at pH 10 and 30 °C as compared to ~0.05 min^{-1} for a control reaction ran under the same conditions in the absence of enzyme. This suggests that the H196N variant is incapable of catalyzing the deprotonation of nitroethane to ethylnitronate. The activity of the mutant enzyme with nitroethane as substrate was further investigated by testing the ability of the H196N variant to consume oxygen in the presence of the nitroalkane. As shown in Figure 4, oxygen consumption was not observed

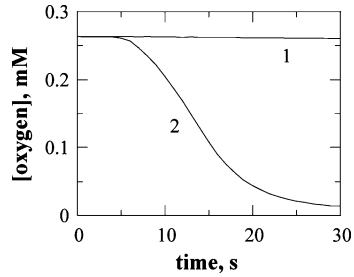


FIGURE 4: Identification of the catalytic base of *N. crassa* 2-nitropropane dioxygenase. Time courses measuring the concentration of oxygen after mixing the H196N variant form of the enzyme (2.17 μM) with either 1 mM nitroethane (trace 1) or 1 mM ethylnitronate (trace 2) at pH 10 and 30 °C were followed using a Clark oxygen electrode.

upon mixing the mutant enzyme with 1 mM nitroethane at pH 10 and 30 °C. In contrast, mixing of the H196N variant with ethylnitronate under the same conditions resulted in the complete depletion of oxygen from the assay reaction mixture within 30 s after initiating the reaction. These data suggest that the mutant enzyme is properly folded and capable of undergoing catalytic turnover with anionic substrates for which a catalytic base is not required in the wild-type (*2, 3*). The lack of rate enhancement for the formation of ethylnitronate as compared to the nonenzymatic reaction and the lack of oxygen consumption observed upon mixing the H196N enzyme with neutral substrates unequivocally establish that *N. crassa* 2-nitropropane dioxygenase loses the ability to deprotonate nitroethane when histidine 196 is replaced with asparagine.

Effect of Replacing His-196 with Asparagine on Enzyme Activity with Ethylnitronate as Substrate. The steady-state kinetic parameters with ethylnitronate as substrate for the mutant form of *N. crassa* 2-nitropropane dioxygenase were determined at pH 9.5 and 30 °C to investigate the role of histidine 196 in turnover of the enzyme with anionic substrates. As shown in Table 1, the H196N enzyme showed an ~15-fold increase in the k_{cat}/K_m value and an ~10-fold increase in the k_{cat} value when rates of oxygen consumption were measured. A $^{D}(k_{cat}/K_m)$ value with [1-^{2}H]ethylnitronate of 0.99 ± 0.09 was also determined. In contrast, the $^{D}(k_{cat}/K_m)$ value for the wild-type enzyme was 0.65 ± 0.02, in good agreement with the value of 0.63 previously reported (*3*). The data demonstrate that the H196N variant is a better catalyst than the wild-type enzyme for oxidative turnover with ethylnitronate.

Effect of Replacing His-196 with Asparagine on Flavin Reduction with Ethylnitronate as Substrate. Previous studies have demonstrated that an anionic flavosemiquinone forms during catalytic turnover of *N. crassa* 2-nitropropane dioxygenase with ethylnitronate as substrate (*2, 3*). The requirement for His-196 in the formation of the flavosemiquinone was investigated in the current study by monitoring flavin visible absorbance spectra during the reductive half-reaction of the H196N variant with ethylnitronate as substrate at pH 7 and 30 °C. As shown in Figure 5, anaerobic mixing of the H196N variant with ethylnitronate resulted in the formation of an anionic flavosemiquinone as indicated by the peaks in the visible absorbance spectra at 371 and 496 nm that developed during the course of flavin reduction. Thus, replacing histidine 196 with asparagine has no effect on the mechanism of electron transfer in the reductive half-reaction of the enzyme nor does it alter the ionization state of the catalytically relevant flavosemiquinone.

DISCUSSION

Previous studies of the *N. crassa* 2-nitropropane dioxygenase reaction with nitroethane are consistent with the formation of an enzyme−ethylnitronate complex during turnover (*2, 3*), which is produced in the enzyme-catalyzed deprotonation of the substrate by the action of an active site catalytic group acting as a base. In this study, a continuous spectrophotometric assay was developed to evaluate whether the ethylnitronate that is produced from the enzymatic deprotonation of nitroethane is released to the solvent. A logarithmic approximation of the initial rates of the enzymatic reaction was then used to assess the kinetic parameters for the nonoxidative deprotonation of nitroethane catalyzed by *N. crassa* 2-nitropropane dioxygenase and a mutant variant of the enzyme in which the conserved histidine 196 was replaced with asparagine.

Ethylnitronate is released to the solvent during enzymatic turnover of *N. crassa* 2-nitropropane dioxygenase with nitroethane, as indicated by the progressive, nonlinear increase in absorbance at 228 nm after mixing the enzyme with nitroethane. Initial rates for the formation and release of ethylnitronate from the active site of the enzyme could be approximated despite the nonlinearity of the reaction progress curves. This was achieved using the method originally developed by Fei and Lu (*18*), which involves fitting the entire reaction progress curve to a logarithmic function. The slope of a tangent line to the curve that intersects through the origin is analogous to that found using traditional methods to determine initial rates of enzymatic reactions that rely on a steady-state accumulation or depletion of a reaction species (*19*). Both methods measure changes in the concentration of either a substrate or product with respect to time during the initial stages of the reaction. The advantage of the logarithmic approximation to determine the initial rate is that it involves fitting the entire time course as opposed to arbitrarily considering only a portion of the curve that may appear linear to the observer. The logarithmic approximation is generally applicable to any enzymatic reaction resulting in nonlinear progress curves due to the concentration of substrate being significantly smaller than the K_m value, provided that the curvature is not the result of enzyme instability or inhibition.

The kinetic studies of the formation and release of ethylnitronate from *N. crassa* 2-nitropropane dioxygenase in turnover with nitroethane along with previous results on the flavin-dependent oxidative denitrification of nitroethane catalyzed by the enzyme (*2, 3*) demonstrate a branched catalytic mechanism for the enzyme with the nitroalkane as substrate (Scheme 2). The catalytic cycle is initiated by the base-catalyzed deprotonation of nitroethane to yield ethylnitronate. This enzyme-bound nitronate is then either released to the solvent or is oxidized through the transfer of a single electron to the flavin cofactor of *N. crassa* 2-nitropropane dioxygenase. The time-dependent increase in the UV absorbance at 228 nm seen upon mixing the enzyme with nitroethane is consistent with the accumulation of ethylnitronate in solution during the course of the assay. While the nonenzymatic deprotonation of nitroethane to ethylnitronate

Table 1: Steady-State Kinetic Parameters of Wild-Type and H196N *N. crassa* 2-Nitropropane Dioxygenase with Ethylnitronate as Substrate[a]

enzyme	k_{cat} (s^{-1})	K_a[b] (mM)	k_{cat}/K_a (M^{-1} s^{-1})	K_{O_2}[b] (μM)	k_{cat}/K_{O_2} (μM^{-1} s^{-1})	K_{ia} (mM)	R^2
			Ethylnitronate				
wt	16.2 ± 0.1	15.9 ± 0.3	1020 ± 20	≤5	≥1	100 ± 10	0.993
H196N	185 ± 1	11.0 ± 0.1	16800 ± 100	34 ± 1	5.5 ± 0.1	1.2 ± 0.2	0.998
			[1-^{2}H]Ethylnitronate				
wt	16.5 ± 0.2	10.6 ± 0.4	1560 ± 50	≤5	≥1	140 ± 20	0.994
H196N	195 ± 5	11.5 ± 1.1	16900 ± 1600	35 ± 4	5.6 ± 0.6	2.3 ± 1.8	0.995

[a] Enzyme activity was measured at varying concentrations of both organic substrate and oxygen in 50 mM sodium pyrophosphate at pH 9.5 and 30 °C. Data were fit to eq 6. [b] K_a refers to the Michaelis constant for the organic substrate; K_{O_2} refers to the Michaelis constant for oxygen.

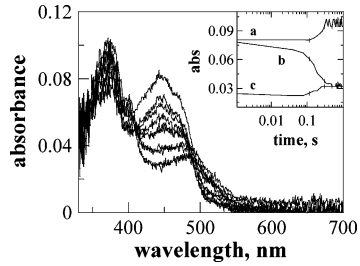
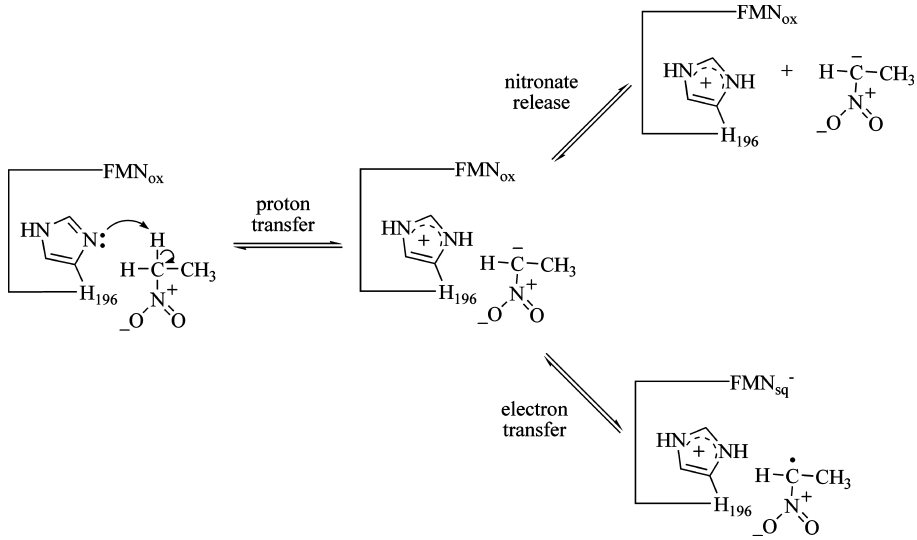


FIGURE 5: Anionic flavosemiquinone formation in the reductive half-reaction of H196N *N. crassa* 2-nitropropane dioxygenase. The mutant enzyme (~10 μM final concentration) was mixed anaerobically with ethylnitronate (0.5 mM final concentration) at pH 7 and 30 °C, and UV−visible absorbance spectra were recorded using a stopped-flow spectrophotometer equipped with a photodiode array detector. The spectra shown were collected from 0.75 ms to 1 s after the end of flow. Inset: Time courses for the UV−visible absorbance of the FMN cofactor of the H196N variant at 371 (a), 442 (b), and 495 (c) nm. The time indicated is after the end of flow (2.2 ms).

is known to occur in solution (*20, 21*), the initial rates of the reaction determined in this study were higher in the presence of the enzyme than those obtained in buffer alone, clearly establishing that *N. crassa* 2-nitropropane dioxygenase catalyzes the reaction. Nitronate release during turnover of the enzyme with nitroethane as substrate thus occurs in addition to the previously described oxidative denitrification of ethylnitronate that occurs during catalysis (*2, 3*). The partitioning of the enzyme−ethylnitronate complex results in a branched catalytic mechanism of the type seen for the cytochrome P450 class of enzymes (*22*−*24*), the aromatic amino acid hydroxylases (*25*), and the hemoprotein−lipoxygenase fusion protein (*26*).

The catalytic base involved in the deprotonation of nitroethane in the reaction catalyzed by the *N. crassa* 2-nitropropane dioxygenase is histidine 196. Evidence supporting this conclusion comes from the lack of rate enhancement for ethylnitronate formation by a reaction mixture containing the H196N enzyme compared to control reactions carried out in buffer alone. The lack of enzymatic activity is not due to a deleterious effect of the mutation on the general properties of the enzyme because oxygen consumption is observed when ethylnitronate is used as a substrate for the mutant enzyme. Turnover of the mutant enzyme with ethylnitronate is expected to occur based on previous studies demonstrating that catalysis with the nitronate does not require an unprotonated group (*2, 3*). The kinetic data implicating the role of His 196 as the catalytic base in the reaction of the enzyme with nitroethane are consistent with crystallographic studies of *P. aeruginosa* 2-nitropropane dioxygenase in complex with 2-nitropropane (*17*), which shows the conserved histidine next to the α-proton of the substrate.

Scheme 2: Branching of the Enzyme−Ethylnitronate Complex during Turnover of *N. crassa* 2-Nitropropane Dioxygenase with Nitroethane as Substrate[a]



[a] Note that under initial velocity conditions in the absence of exogenous ethylnitronate the kinetic step of nitronate release is practically irreversible. For clarity, further conversion of the anionic flavosemiquinone enzyme through the oxidative denitrification pathway is not shown.

Replacement of histidine 196 with asparagine in *N. crassa* 2-nitropropane dioxygenase results in a better catalyst for the oxidative denitrification of ethylnitronate, as suggested by the ~15-fold increase in the k_{cat}/K_m value observed with the H196N variant as compared to the wild-type enzyme. This is likely due to the inability of the mutant enzyme to catalyze the nonoxidative conversion of ethylnitronate to nitroethane, as suggested by the lack of an α-secondary kinetic isotope on the k_{cat}/K_m value with [1-^{2}H]ethylnitronate. Indeed, this conversion is associated with a change in the hybridization of the α-carbon of the substrate, which would result in an inverse $^D(k_{cat}/K_m)$ value as observed in the wild-type enzyme (this study and ref *3*). In the H196N enzyme all of the productive complexes between the enzyme and the anionic substrate that form during turnover are therefore destined for oxidative denitrification, rather than branching through oxidative and nonoxidative catalysis as in the wild-type enzyme.

Histidine 196 is not required for the formation of the obligatory, anionic flavosemiquinone that is transiently formed during the reductive half-reaction in which ethylnitronate is oxidized by the wild-type enzyme (*2, 3*). Evidence supporting this conclusion comes from anaerobic stopped-flow measurements in which the H196N enzyme was mixed with ethylnitronate that showed the formation of the anionic flavosemiquinone. This, in turn, suggests that the reaction pathway for oxidative denitrification of ethylnitronate in *N. crassa* 2-nitropropane dioxygenase is unaltered upon replacing His-196 with asparagine.

In conclusion, a kinetic assay was developed to demonstrate that *N. crassa* 2-nitropropane dioxygenase utilizes a branched catalytic mechanism with nitroethane as substrate. The branch point occurs at the enzyme−ethylnitronate complex and involves either the release of the nitronate or an oxidative denitrification reaction. The partitioning of the enzyme−nitronate complex results in the formation of multiple products from independent catalytic pathways with nitroethane as substrate for the enzyme. In the nonoxidative pathway, nitroethane is deprotonated by histidine 196 to generate ethylnitronate which is subsequently released from the enzyme as a reaction product. The oxidative denitrification pathway was established in previous studies of the enzyme (*2, 3*) and involves the oxidation of ethylnitronate by the enzyme bound flavin to generate acetaldehyde and nitrite as product. The mechanistic factors that determine the partitioning of the enzyme−ethylnitronate complex between oxidation and release from the enzyme active site are the focus of a current investigation of the enzyme.

ACKNOWLEDGMENT

Parts of this work were presented at the Gordon Research Conference on Isotopes in Biological and Chemical Sciences in Ventura, CA, on Feb 17−22, 2008. The authors extend their appreciation to their colleagues for the insightful discussions. We also thank Ms. Hongling Yuan for the preparation of the H196N variant of *N. crassa* 2-nitropropane dioxygenase through site-directed mutagenesis, Ms. Stacy Collins for the development of the purification protocol for the mutant form of the enzyme, Mr. Merid Belaineh for the optimization of the expression and purification of the wild-type enzyme, and Mr. Paul Channell for helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Data summarizing the fits of the time courses for the enzymatic and nonenzymatic deprotonation of nitroethane at pH 8 and 30 °C, a table of the kinetic parameters for both reactions measured as a function of pH, and plots for the determination of the extinction coefficients of nitroethane and ethylnitronate and progress curves for oxygen consumption of *N. crassa* 2-nitropropane dioxygenase at pH 6 and 10. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI801013E