Involvement of a Flavosemiquinone in the Enzymatic Oxidation of Nitroalkanes Catalyzed by 2-Nitropropane Dioxygenase

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2-Nitropropane dioxygenase (EC 1.13.11.32) catalyzes the oxidation of nitroalkanes into their corresponding carbonyl compounds and nitrite. In this study, the ned-2 gene encoding for the enzyme in Neurospora crassa was cloned, expressed in Escherichia coli, and the resulting enzyme was purified. Size exclusion chromatography, heat denaturation, and mass spectroscopic analyses showed that 2-nitropropane dioxygenase is a homodimer of 80 kDa, containing a mole of non-covalently bound FMN per mole of subunit, and is devoid of iron. With neutral nitroalkanes and anionic nitronates other than propyl-1- and propyl-2-nitronate, for which a non-enzymatic free radical reaction involving superoxide was established using superoxide dismutase, substrate oxidation occurs within the enzyme active site. The enzyme was more specific for nitronates than nitroalkanes, as suggested by the second order rate constant determined with 2-nitropropane and primary nitroalkanes with alkyl chain lengths between 2 and 6 carbons. The steady state kinetic mechanism with 2-nitropropane, nitroethane, nitrobutane, and nitrohexane, in either the neutral or anionic form, was determined to be sequential, consistent with oxygen reacting with a reduced form of enzyme before release of the carbonyl product. Enzyme-monitored turnover with ethyl nitronate as substrate indicated that the catalytically relevant reduced form of enzyme is an anionic flavin semiquinone, whose formation requires the substrate, but not molecular oxygen, as suggested by anaerobic substrate reduction with nitroethane or ethyl nitronate. Substrate deuterium kinetic isotope effects with 1,2-[2H4]nitroethane and 1,1,2-[2H3]ethyl nitronate at pH 8 yielded normal and inverse effects on the kcat/Km value, respectively, and were negligible on the kcat value. The kcat/Km and kcat pH profiles with anionic nitronates showed the requirement of an acid, whereas those for neutral nitroalkanes were consistent with the involvement of both an acid and a base in catalysis. The kinetic data reported herein are consistent with an oxidase-type catalytic mechanism for 2-nitropropane dioxygenase, in which the flavin-mediated oxidation of the anionic nitronates or neutral nitroalkanes and the subsequent oxidation of the enzyme-bound flavin occur in two independent steps.

2-Nitropropane dioxygenase (EC 1.13.11.32) from Neurospora crassa is a flavin-dependent enzyme, which catalyzes the oxidative denitriﬁcation of nitroalkanes to their corresponding carbonyl compounds and nitrite. The enzyme was ﬁrst characterized by Little (1) in 1951 as an oxidase, but was later classiﬁed as a dioxygenase based on the observation that the oxygen atom of the organic product formed upon oxidation of propyl-2-nitronate originates from molecular oxygen and not from water (2). To date, 2-nitropropane dioxygenase has been isolated from N. crassa (1) and Hansenula mrakii (3). Whereas the two enzymes have similar molecular weights of about 40,000, they differ in their prosthetic group content in that FMN and FAD are found in the N. crassa and H. mrakii enzymes, respectively (2, 4).

A study on the enzymatic oxidation of nitroalkanes by 2-nitropropane dioxygenase is of considerable interest for both applied and fundamental reasons. Because of the low pK values for deprotonation of the α-carbon of the organic product formed upon oxidation of nitroalkanes, it has been observed to occur physiologically in N. crassa (1), H. mrakii (17), and Fusarium oxysporum (18); however the implications of the reaction in biological systems are not yet fully understood. Consequently, a characterization of the biochemical and mechanistic properties of 2-nitropropane dioxygenase might shed light on the role of nitroalkane oxidation by living organisms. From a mechanistic standpoint, 2-nitropropane dioxygenase is unique in that it can effectively utilize both the neutral (nitroalkane) and anionic (nitronate) forms of the nitroalkane substrate (2). In contrast, the numerous reported studies on the enzymatic oxidation of nitroalkanes catalyzed by flavin-dependent enzymes have only characterized the reaction using a single form of substrate (19–21). Therefore, 2-nitropropane dioxygenase offers the unique opportunity for a direct comparison of the enzyme-catalyzed oxidation of nitroalkanes.

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Catalytic Mechanism of 2-Nitropropane Dioxygenase

in their neutral and anionic forms. To date, because of the
limited amount of enzyme that can be obtained in a pure form
upon extraction from natural sources, minimal biochemical,
structural, or mechanistic information is available on 2-nitro-
propane dioxygenase.

In this study, the ncd-2 gene encoding for 2-nitropropane
dioxygenase was cloned into the expression vector pET20b(+),
expressed in Escherichia coli strain BL21(DE3), and the
resultant enzyme was characterized in its kinetic and biochem-
ical properties. The data presented herein indicate that the
enzymatic oxidation of neutral nitroalkanes and anionic nitro-
trones catalyzed by 2-nitropropane dioxygenase occurs through
an oxidase-style catalytic mechanism in which the
flavin-mediated oxidation of the substrate and the subsequent
oxidation of the enzyme-bound flavin occur in two independent
steps. Evidence is presented that the enzyme-catalyzed oxida-
tion of the nitroalkane substrate involves the unusual forma-
tion of an anionic flavin semiquinone species as a catalytic
intermediate.

EXPERIMENTAL PROCEDURES

Materials—The plasmid pUCncd containing the ncd-2 gene encoding
for 2-nitropropane dioxygenase in N. crassa was a generous gift from
Drs. Nobuyoshi Esaki and Tatsuo Kurihara, Kyoto University, Japan.
Pfu DNA polymerase was from Stratagene. Restriction endonucleases
NdeI and EcoRI, T4 DNA ligase, and calf intestinal alkaline phospha-
tase were from Promega. DNAse I and the Rapid DNA ligation kit were
from Roche Applied Science. Luria-Bertani agar and broth, ampicillin,
FMN, phenylmethylsulfonyl fluoride, superoxide dismutase, lysozyme,
and nitroalkanes were from Sigma-Aldrich. Ammonium sulfate and
MgCl2 were from ICN Biomedicals. EDTA was from Fisher. The plas-
mid vector pET20b(+) was from Novagen. Primers and products de-
derived from primer extension amplification were purified using mini kits
from Qiagen. E. coli strain BL21(DE3) (Novagen) was used for protein
expression, and the strain XL1-Blue (Stratagene) was used during cloning
procedures. Both strains were stored at -80 °C as 7% dimethyl sulfoxide suspensions. The Hi-Prep 16/10 Octyl Fast Flow column and
the DEAE-Sepharose used in packing the DEAE column were from
Amersham Biosciences. All other reagents were of the highest purity
commercially available.

Instruments—DNA sequencing was carried out using an Applied
 Biosystems Big Dye kit on an Applied Biosystems model ABI 377 DNA
sequencer at the DNA Core Facility of the Biology Department of
Georgia State University. Oligonucleotides were custom synthesized on
an Applied Biosystems model 380B DNA Synthesizer by the Gene
Technology Laboratory of the Biology Department of Texas A&M
University, College Station. UV-visible absorbance spectra were recorded
using an Agilent Technologies diode-array spectrophotometer Model
HP 8453, equipped with a thermostatted water bath. Fluorescence
emission spectra were recorded with a Shimadzu Spectrofluorometer
Model RF-5301 PC, thermostatted at 15 °C. MALDI-TOF1 spectra were
recorded using an ABI Voyager DE-pro mass spectrophotometer.
Stopped flow experiments were carried out using a Hi-Tech SF-61
Double Mixing Stopped flow system.

Cloning of ncd-2 into pET20b(+)—Plasmid pUCncd containing the
ncd-2 gene for 2-nitropropane dioxygenase was used directly to trans-
form E. coli strain XL1-Blue competent cells, using the heat shock
method of Inoue et al. (22), and the pUCncd plasmid was isolated through a QIAquick Spin miniprep kit, according to the manufacturer's
instructions. The extracted pUCncd plasmid was used as a DNA tem-
plate for primer extension amplification of the ncd-2 gene, using oligo-
 nucleotide sense and antisense primers containing NdeI and EcoRI
restriction endonuclease sites designed to anneal to the 5'- and 3'-ends
of the gene, respectively. The engineered NdeI (CGAGTACATATGACT-
GACCCCCTTAGGC) and EcoRI (TACCGAATTCTTACACGCG-
GCACCCCTTAGGC) restriction sites (underlined) were designed to
facilitate directional cloning into the corresponding sites in pET20b(+).
The ncd-2 gene was ligated into pET20b(+) to construct plasmid pET/
2NPncd using a Rapid DNA Ligation kit (Roche Applied Science),
and the plasmid was used directly to transform E. coli strain XL1-
Blue competent cells. The correct construct was sequenced in both
directions using oligonucleotide primers designed to bind to the DNA
regions of pET flanking the inserted gene and was used to transform E.
coli strain BL21(DE3) competent cells for protein expression.

1 The abbreviation used is: MALDI-TOF, matrix-assisted laser des-
orption/ionization-time of flight.
enzymatic activity. Alkyl nitronates were prepared as described above, except that the nitroalkane solution was allowed to react for 24 h at room temperature, with a 1.2 molar excess of KOH. Enzymatic assays were initiated by the addition of substrate, in order to minimize changes in the ionization state of the nitroalkane.\(^2\) When both the organic substrate and oxygen were varied, the assay mixtures were equilibrated with the appropriate \(\text{O}_2/\text{N}_2\) 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. When the pH was varied, 50 mM sodium phosphate was used as a buffer between pH 6 and 9.5. Deuterium substutate kinetic isotope effects were determined using \(1.2\)–\(\text{[H}_2\)nitroethane or \(1.2,2\)-\(\text{[H}_3\)ethyl nitronate as substrate in air-saturated potassium phosphate, pH 8. Activity assays were carried out by alternating substrate isopomers. The presence of superoxide during turnover of the enzyme with different nitroalkane substrates was monitored by measuring the rate of oxygen consumption at a fixed concentration of substrate in either the presence or absence of 125 units of superoxide dismutase, in air-saturated 50 mM Tris-Cl, pH 8 at 30 °C. Production of hydrogen peroxide during turnover was monitored by measuring the rate of oxygen consumption at a fixed concentration of substrate, either the presence or absence of 75 units of catalase.

Stopped Flow Analysis of 2-Nitropropane Dioxygenase in Turnover—2-Nitropropane dioxygenase was mixed with ethyl nitronate at a final concentration of 5 mM in 50 mM potassium phosphate and 1% ethanol, pH 7.4, in a stopped flow spectrophotometer thermostatted at 15 °C. Traces were recorded for 60 s, while following the reaction at 370 and 445 nm. Spectra of the enzyme in turnover with substrate were obtained by following absorbance changes at a single wavelength between 300 and 600 nm at 10-nm intervals and plotting the absorbance versus wavelength for any given time.

Data Analysis—Data were fit with KaleidaGraph software (Synergy Software, Reading, PA) or Enzfitter software (Biosoft, Cambridge, UK). Kinetic parameters determined in atmospheric oxygen were obtained by fitting the data to the Michaelis-Menten equation for one substrate. When initial rates of reaction were determined by varying the concentrations of both the nitroalkane substrate and oxygen, the data were fit to Equations 1 and 2, which describe a sequential and ping-pong steady state kinetic mechanism, respectively. \(k_{\text{cat}}\) and \(K_{\text{m}}\) represent the Michaelis constants for the nitroalkane substrate (A) and oxygen (B), respectively and \(k_{\text{cat}}\) is the turnover number of the enzyme (e). The pH dependence of the steady state kinetic parameters was determined by fitting initial rate data obtained at varying concentrations of organic substrate to Equations 3 and 4, which describe a bell-shaped curve with a slope of +1 at low pH and a slope of −1 at high pH, and a curve with a slope of −1 and a plateau region at low pH, respectively. \(C\) is the pH independent value of the kinetic parameter of interest. Substrate deuterium kinetic isotope effects were determined by fitting the data to Equation 5, which describes isotope effects on \(k_{\text{cat}}\) and \(k_{\text{cat}}/K_{\text{m}}\).

\[
\begin{align*}
\frac{v}{e} &= \frac{k_{\text{cat}}AB}{K_B + K_A + AB + K_{K_A}} & (\text{Eq. 1}) \\
\frac{v}{e} &= \frac{k_{\text{cat}}AB}{K_B + K_A + AB + K_{K_A}} & (\text{Eq. 2})
\end{align*}
\]

\[
\log Y = \log\left(\frac{C}{1 + \frac{10 \text{ pH}}{10 \text{ pH} + 10 \text{ pH}^2}}\right) & (\text{Eq. 3})
\]

\[
\log Y = \log\left(\frac{C}{1 + \frac{10 \text{ pH}}{10 \text{ pH} + 10 \text{ pH}^2}}\right) & (\text{Eq. 4})
\]

\[
\frac{v}{e} = \frac{k_{\text{cat}}A}{K_A + F_e \times E_{k}\text{k}} + A(1 + F_e \times E_{k}\text{kat}) & (\text{Eq. 5})
\]

\(^2\)The second-order rate constants for deprotonation of the nitroalkanes used in the present study have values comprised between 5 and 6 \(\text{M}^{-1} \text{s}^{-1}\) (48), ensuring that in assays initiated with fully protonated nitroalkanes a negligible amount of anionic substrate is present during the time required to determine initial rates (typically ~30 s). Similarly, the second-order rate constants for protonation of alkyl nitronates have values in the 15–75 \(\text{M}^{-1} \text{s}^{-1}\) range (5), ensuring a negligible amount of neutral substrate in assays initiated with fully unprotonated alkyl nitronates.
TABLE I
Purification of recombinant 2-nitropropane dioxygenase

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>μmol O₂ min⁻¹</td>
<td>μmol O₂ min⁻¹ mg⁻¹</td>
</tr>
<tr>
<td>Cell-free extract</td>
<td>1000</td>
<td>13,600</td>
<td>13</td>
</tr>
<tr>
<td>70% (NH₄)₂SO₄ saturation</td>
<td>730</td>
<td>12,000</td>
<td>16</td>
</tr>
<tr>
<td>DEAE-Sepharose FF</td>
<td>100</td>
<td>4900</td>
<td>49</td>
</tr>
<tr>
<td>Octyl-Sepharose FF</td>
<td>34</td>
<td>2500</td>
<td>80</td>
</tr>
</tbody>
</table>

* Starting from 51 g of wet cell paste.

Enzyme activity was determined with 10 mM ethyl nitronate in air-saturated 50 mM Tris-Cl, pH 8, and 25 °C.

oxidation of nitroalkanes catalyzed by 2-nitropropane dioxygenase might involve a non-enzymatic free radical mechanism that is initiated and propagated by the enzymatic formation and release of superoxide. If this were the case, one would expect the rate of oxygen consumption to decrease significantly when the enzymatic activity of 2-nitropropane dioxygenase is measured in the presence of superoxide dismutase, because the superoxide would be converted to oxygen and hydrogen peroxide. Consequently, the effect of superoxide dismutase on the reaction catalyzed by 2-nitropropane dioxygenase with several nitroalkanes as substrate was determined. As shown in Table II, with all the substrate tested except propyl-1- and propyl-2-nitronate, the rate of oxygen consumption did not change when superoxide dismutase was present in the reaction mixture, consistent with the oxidation of the nitroalkane substrates catalyzed by 2-nitropropane dioxygenase occurring at the enzyme active site and not involving a free radical chain reaction. With propyl-1- and propyl-2-nitronate as substrate for the enzyme, the enzymatic activity in the presence of superoxide dismutase decreased by 65 and 50%, respectively, suggesting that the oxidation of these nitronates catalyzed by 2-nitropropane dioxygenase has a significant non-enzymatic component. Steady State Kinetic Mechanism—The steady state kinetic mechanism of 2-nitropropane dioxygenase with a number of substrates in either the neutral or anionic form was determined by varying the concentration of both the organic substrate and oxygen at pH 8 and 30 °C. As shown in Fig. 5 for the case of butyl-1-nitronate, with all the substrates tested double reciprocal plots of the initial rate of oxygen consumption as a function of substrate concentration yielded converging lines, consistent with oxygen reacting with an enzyme species reversibly connected to the enzyme species that binds the organic substrate. Consistent with the observed kinetic pattern, the

![Fig. 2. MALDI-TOF mass spectrometric analysis of the flavin cofactor of 2-nitropropane dioxygenase. The spectrum was recorded in negative ion mode with a 50:50 methanol/acetonitrile matrix using a sample prepared by treating the enzyme with trichloroacetic acid.](Image)

![Fig. 3. UV-visible absorbance spectra of recombinant 2-nitropropane dioxygenase. 2-Nitropropane dioxygenase was irradiated with a 100-watt light bulb in the presence of 15 mM EDTA in 50 mM potassium phosphate, pH 7.4. Spectra of a 2-nitropropane dioxygenase were recorded after different intervals of irradiation. Only selected spectra are presented: curve 1, spectrum of 2-nitropropane dioxygenase as purified at a concentration of 29 μM; curve 18, same sample after 23 min of irradiation.](Image)
for substrates with different alkyl chain length was carried out by measuring initial rates of reaction with primary nitroalkanes and nitronates in air-saturated buffer. As shown in Table IV, both $k_{\text{cat}}$ and $k_{\text{cat}}/K_{m}$ values were not significantly affected by the length of the substrate alkyl chain irrespective of whether the neutral or the anionic nitroalkane was the substrate for the enzyme. In contrast, the $k_{\text{cat}}/K_{m}$ values determined with alkyl nitronates were between 2 and 35 times as large as the corresponding values determined with nitroalkanes, suggesting a preference of the enzyme for the anionic substrates. Similarly, the $k_{\text{cat}}$ values with alkyl nitronates were 3–15 times larger than those with nitroalkanes.

**pH Dependence of the $k_{\text{cat}}/K_{m}$ and $k_{\text{cat}}$ Values**—The pH dependence of the kinetic parameters of 2-nitropropane dioxygenase with nitroethane, nitrobutane, ethyl nitronate, and butyl-1-nitronate as substrate were measured in air-saturated buffer in the accessible pH range. With nitroethane and nitrobutane, both the $k_{\text{cat}}$ and $k_{\text{cat}}/K_{m}$ values yielded bell-shaped pH profiles, consistent with the involvement of two ionizable groups that must be protonated and unprotonated for the oxidation of the neutral substrates (Fig. 6). In contrast, with ethyl nitronate and butyl-1-nitronate, the $k_{\text{cat}}$ and $k_{\text{cat}}/K_{m}$ values increased to limiting values with decreasing pH, consistent with the requirement of a single ionizable group that must be protonated for catalysis with anionic substrates. The $pK_{a}$ values determined in this study are summarized in Table V.

**Substrate Deuterium Kinetic Isotope Effects**—Substrate deuterium kinetic isotope effects were measured with $[1,2-\text{H}_{2}]$-nitroethane as substrate for 2-nitropropane dioxygenase in air-saturated buffer. A $\Delta^{1}(k_{\text{cat}}/K_{m})$ value of 4.1 ± 0.5 was determined at pH 8, consistent with the CH bond of the neutral nitroalkane substrate being cleaved in a kinetically slow step during catalysis by 2-nitropropane dioxygenase. When $1,2,2-[\text{H}_{3}]$-ethyl nitronate was used as substrate for the enzyme, an inverse $\Delta^{1}(k_{\text{cat}}/K_{m})$ value of 0.76 ± 0.06 was determined, consistent with a change in the hybridization state of the $\alpha$-carbon from sp$^{3}$ to sp$^{2}$ occurring in the oxidation of alkyl nitronates catalyzed by 2-nitropropane dioxygenase. The $\Delta^{1}k_{\text{cat}}$ values were not significantly different from parent $k_{\text{cat}}$ and $k_{\text{cat}}/K_{m}$ values determined at atmospheric oxygen with primary nitroalkanes and nitronates approximating well the values that would be measured by varying the concentration of both organic substrate and oxygen. As expected based on this analysis, the $k_{\text{cat}}$ and $k_{\text{cat}}/K_{m}$ determined with the neutral and anionic forms of nitroethane, nitrobutane, and nitropropane at atmospheric oxygen (Table IV) were similar to those determined by varying the concentration of oxygen (Table III).
Catalytic Mechanism of 2-Nitropropane Dioxygenase

Table III
Steady state kinetic parameters for 2-nitropropane dioxygenase at pH 8

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$</th>
<th>$K_m$</th>
<th>$k_{cat}/K_m$</th>
<th>$K_{cat}$</th>
<th>$k_{cat}/K_{cat}$</th>
<th>$K_m$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s$^{-1}$</td>
<td>μM</td>
<td>s$^{-1}$ μM</td>
<td>μM</td>
<td>μM s$^{-1}$ μM</td>
<td>μM</td>
<td></td>
</tr>
<tr>
<td>Nitroalkanes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitroethane</td>
<td>11 ± 1</td>
<td>19 ± 1</td>
<td>560 ± 10</td>
<td>4.9 ± 0.2</td>
<td>2.2 ± 0.1</td>
<td>11 ± 1</td>
<td>0.998</td>
</tr>
<tr>
<td>Nitrobutane</td>
<td>5.9 ± 0.1</td>
<td>15 ± 1</td>
<td>400 ± 1</td>
<td>≤ 2</td>
<td>12 ± 1</td>
<td>190 ± 1</td>
<td>0.981</td>
</tr>
<tr>
<td>Nitrohexane</td>
<td>1.7 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>2,100 ± 50</td>
<td>2.4 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>30 ± 1</td>
<td>0.971</td>
</tr>
<tr>
<td>2-Nitropropane</td>
<td>4.0 ± 0.1</td>
<td>18 ± 1</td>
<td>250 ± 1</td>
<td>≤ 2</td>
<td>6.4 ± 0.1</td>
<td>420 ± 2</td>
<td>0.997</td>
</tr>
<tr>
<td>Alkyl nitronates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl nitronate</td>
<td>57 ± 1</td>
<td>3.4 ± 0.1</td>
<td>16,900 ± 30</td>
<td>2.4 ± 0.1</td>
<td>24 ± 1</td>
<td>19 ± 1</td>
<td>0.998</td>
</tr>
<tr>
<td>Butyl-1-nitronate</td>
<td>56 ± 1</td>
<td>6.5 ± 0.1</td>
<td>8,600 ± 30</td>
<td>2.4 ± 0.1</td>
<td>21 ± 1</td>
<td>86 ± 1</td>
<td>0.997</td>
</tr>
<tr>
<td>Hexyl-1-nitronate</td>
<td>21 ± 1</td>
<td>1.2 ± 0.1</td>
<td>17,200 ± 800</td>
<td>2.1 ± 0.1</td>
<td>10 ± 1</td>
<td>8 ± 1</td>
<td>0.992</td>
</tr>
<tr>
<td>Propyl-1-nitronate</td>
<td>21 ± 1</td>
<td>4.9 ± 0.1</td>
<td>5,100 ± 200</td>
<td>30 ± 1</td>
<td>0.50 ± 0.01</td>
<td>15 ± 1</td>
<td>0.983</td>
</tr>
</tbody>
</table>

$^a$ $K_m$ is the Michaelis constant for the organic substrate.
$^b$ $K_{cat}$ is the Michaelis constant for oxygen.
$^c$ Measured in the presence of 21 units of superoxide dismutase.

Table IV
Substrate specificity of 2-nitropropane dioxygenase at pH 8

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$</th>
<th>$K_m$</th>
<th>$k_{cat}/K_m$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s$^{-1}$</td>
<td>μM</td>
<td>s$^{-1}$ μM</td>
<td></td>
</tr>
<tr>
<td>Nitroalkanes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitroethane</td>
<td>14 ± 1</td>
<td>29 ± 2</td>
<td>480 ± 20</td>
<td>0.995</td>
</tr>
<tr>
<td>Nitropropane</td>
<td>20 ± 1</td>
<td>24 ± 2</td>
<td>830 ± 60</td>
<td>0.998</td>
</tr>
<tr>
<td>Nitrobutane</td>
<td>6.2 ± 0.4</td>
<td>17.9 ± 2.0</td>
<td>350 ± 50</td>
<td>0.992</td>
</tr>
<tr>
<td>Nitropentane</td>
<td>4.3 ± 0.1</td>
<td>7.1 ± 0.4</td>
<td>610 ± 40</td>
<td>0.998</td>
</tr>
<tr>
<td>Nitrohexane</td>
<td>1.5 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1,100 ± 100</td>
<td>0.994</td>
</tr>
<tr>
<td>Alkyl nitronates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl nitronate</td>
<td>51 ± 3</td>
<td>3.1 ± 0.4</td>
<td>16,500 ± 2,300</td>
<td>0.986</td>
</tr>
<tr>
<td>Propyl-1-nitronate</td>
<td>60 ± 1</td>
<td>5.5 ± 0.4</td>
<td>11,400 ± 500</td>
<td>0.996</td>
</tr>
<tr>
<td>Butyl-1-nitronate</td>
<td>55 ± 2</td>
<td>10 ± 1</td>
<td>5,600 ± 500</td>
<td>0.994</td>
</tr>
<tr>
<td>Pentyl-1-nitronate</td>
<td>n.d. *</td>
<td>n.d. *</td>
<td>1,200 ± 100</td>
<td>0.995</td>
</tr>
<tr>
<td>Hexyl-1-nitronate</td>
<td>22 ± 1</td>
<td>1.4 ± 0.2</td>
<td>16,000 ± 1,000</td>
<td>0.987</td>
</tr>
</tbody>
</table>

$^a$ Measured in the presence of 500 units of superoxide dismutase.
$^b$ Not determined because saturation could not be achieved because of limited solubility of the nitroalkane substrate.

Enzyme activity was measured at varying concentrations of both organic substrate and oxygen in 50 mM Tris-Cl, pH 8 at 30 °C. Data were fit to Equations 1 and 2; with all the substrates tested the best fit was with Equation 1.

unity, with values of 1.3 ± 0.2 and 0.99 ± 0.09 with deuterated nitroethane and ethyl nitronate, suggesting that chemical steps that are isotope sensitive are masked by some later kinetic steps, such as product release, in the catalytic pathway of 2-nitropropane dioxygenase.

Anaerobic Substrate Reduction of 2-Nitropropane Dioxygenase—The steady state kinetic data determined at varying concentrations of both organic substrate and oxygen reported in this study support a kinetic mechanism in which an enzyme species in complex with the organic ligand reacts with oxygen, but do not establish whether the enzyme-bound ligand is the nitroalkane substrate or the carbonyl product of the reaction. A ternary complex involving the carbonyl product requires the enzyme to be in a reduced form, since an oxidation-reduction reaction must have necessarily occurred between the nitroalkane and the enzyme-bound flavin prior to reaction with oxygen. If this were the case, anaerobic mixing of the oxidized enzyme with the nitroalkane substrate would result in the reduction of the enzyme-bound flavin. In contrast, no changes in the oxidation state of the enzyme-bound flavin would be expected upon mixing anaerobically the oxidized enzyme with the substrate if the ternary complex involves the nitroalkane substrate, since catalysis would occur only in the presence of oxygen. As shown in Fig. 7, anaerobic mixing of the enzyme with ethyl nitronate at 15 °C resulted in the rapid formation of an anionic flavin semiquinone species with peaks centered at 371 and 476 nm, consistent with oxidation of the substrate occurring in the absence of oxygen. The enzyme-bound flavin semiquinone then slowly decayed to the hydroquinone form at a rate of 8.4 ± 0.6 s$^{-1}$, as determined from the decrease in absorbance at 371 nm over time. However, complete formation of the flavin hydroquinone species was not observed even after 94 min of incubation, consistent with the fully reduced form of the enzyme-bound flavin not being in the normal catalytic pathway of the enzyme. Similar results were obtained when nitroethane was used as the reductant (data not shown), indicating that the anaerobic one-electron reduction of the flavin did not depend on the ionization state of the substrate, but was a property of 2-nitropropane dioxygenase.

Enzyme Monitored Turnover with Ethyl Nitronate as Substrate—To determine whether the anionic flavin semiquinone of 2-nitropropane dioxygenase was on the direct catalytic pathway for the oxidation of the nitroalkane substrate, the enzyme was allowed to turnover in the presence of 5 mM ethyl nitronate and 0.4 mM oxygen at 15 °C, and the reaction was monitored using a stopped flow spectrophotometer. A mixture of enzyme-bound oxidized and semiquinone flavin species was rapidly established within 1.2 ms during enzymatic turnover under the conditions used, as indicated by the relative absorbance of the traces recorded at 370 and 440 nm (Fig. 8). Under these conditions, steady state enzymatic turnover persisted for ~7 s, as indicated by the lack of absorbance changes at 440 nm, after which the rapid depletion of oxygen resulted in the reduction of the enzyme-bound flavin to the flavosemiquinone form (Fig. 8). A species with $\lambda_{max}$ at ~300 nm was transiently formed in the UV-visible absorbance spectrum of the enzyme under turnover in the steady state phase, consistent with the formation and subsequent decay of a product of the reaction. It is likely that the transient species is acetaldehyde, the expected product of the oxidation of ethyl nitronate catalyzed by 2-nitropropane...
Catalytic Mechanism of 2-Nitropropane Dioxygenase

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FIG. 6. pH dependence of the \( k_{\text{cat}}/K_m \) and \( k_{\text{cat}} \) values with nitroalkanes and nitronates. Initial rates were measured in air-saturated 50 mM sodium pyrophosphate buffer in the pH range from 6 to 9.5. Kinetic parameters were determined using nitroethane (○), nitrobutane (●), ethyl nitronate (○), and nitrobutane (●) as substrate. Panel A, \( k_{\text{cat}}/K_m \) values with neutral nitroalkanes; panel B, \( k_{\text{cat}}/K_m \) values with anionic nitronates; panel C, \( k_{\text{cat}} \) values with neutral nitroalkanes; and panel D, \( k_{\text{cat}} \) value with anionic nitronates. The curves are fits of the data to Equations 3 (for nitroalkanes) and 4 (for nitronates).

TABLE V

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( k_{\text{cat}}/K_m ) ( \times 10^{-3} )</th>
<th>( k_{\text{cat}} ) ( \times 10^{-3} )</th>
<th>( \text{pK}_1 )</th>
<th>( \text{pK}_2 )</th>
<th>Eq.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitroethane</td>
<td>7.6 ± 0.3</td>
<td>7.7 ± 0.3</td>
<td>8.0 ± 0.3</td>
<td>7.7 ± 0.3</td>
<td>3</td>
</tr>
<tr>
<td>Ethyl nitronate</td>
<td>7.5 ± 0.2</td>
<td>7.7 ± 0.2</td>
<td>8.4 ± 0.2</td>
<td>7.8 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>Nitrobutane</td>
<td>7.4 ± 0.2</td>
<td>6.7 ± 0.2</td>
<td>8.4 ± 0.2</td>
<td>7.9 ± 0.2</td>
<td>3</td>
</tr>
<tr>
<td>Butyl-1-nitronate</td>
<td>8.0 ± 0.1</td>
<td>7.9 ± 0.2</td>
<td>8.0 ± 0.1</td>
<td>8.0 ± 0.1</td>
<td>4</td>
</tr>
</tbody>
</table>

* Not determined because below pH 7 substrate saturation could not be achieved due to limited substrate solubility and high \( K_m \) values.

dioxygenase, since it absorbs light at 300 nm and is known to rapidly convert in aqueous solution to its hydrated form, which absorbs at ~278 nm.

Effect of Catalase on the Enzymatic Activity of 2-Nitropropane Dioxygenase—To determine whether hydrogen peroxide is formed in the reaction catalyzed by 2-nitropropane dioxygenase, the rate of oxygen consumption during turnover of the enzyme with neutral nitroalkanes or anionic nitronates was determined in either the absence or presence of 75 units of catalase. As shown in Table II, with the exception of propyl-1-nitronate, there was no significant effect of catalase on the initial rates of reaction when the reaction was carried out in the presence of catalase, indicating that hydrogen peroxide is not formed in the catalytic pathway of 2-nitropropane dioxygenase.

DISCUSSION
In the present study, the ncd-2 gene encoding for 2-nitropropane dioxygenase from N. crassa was ligated into pET20b(+) to construct plasmid pET2/2NPDnc, which was subsequently expressed to high yields in E. coli strain BL21(DE3). The use of the heterologous bacterial expression system allowed for the obtainment of large amounts of purified enzyme with a simplified purification procedure, enabling the characterization of the biochemical and kinetic properties of 2-nitropropane dioxygenase. The original goal of the present study was to investigate the steady state kinetic mechanism of 2-nitropropane dioxygenase to define a framework for future mechanistic investigations of the enzyme aimed at a thorough understanding of the catalytic mechanism of the enzyme. In the course of the study, evidence was collected indicating the involvement of an anionic flavin semiquinone species in the oxidation reaction catalyzed by 2-nitropropane dioxygenase.

The oxidation of nitroalkanes, in both their neutral and anionic forms, by 2-nitropropane dioxygenase proceeds by an oxidase-style mechanism in which the flavin-mediated oxidation of the substrate and the subsequent oxidation of the enzyme-bound flavin occur in two independent steps. With nitronates as substrate (Scheme 2), after formation of an enzyme-substrate complex the organic substrate is oxidized through the transfer of a single electron to the enzyme-bound flavin with formation of an anionic flavin semiquinone spe-
cies (E-FMN\textsubscript{aq}\textsuperscript{−}S\textsuperscript{−}). The subsequent reaction of molecular oxygen with the E-FMN\textsubscript{aq}\textsuperscript{−}S\textsuperscript{−} complex results in the oxidation of the enzyme-bound flavin and production of the carbonyl product of the reaction, which is then released from the enzyme active site. With nitroalkanes as substrate (Scheme 3), catalysis is initiated by an enzyme-catalyzed proton abstraction from the α-carbon of the neutral substrate to yield an enzyme-bound nitronate (see below), which is then oxidized following the same catalytic pathway described for the nitronate substrates. The order of the kinetic steps involving substrate binding and product release is supported by the kinetic data observed with nitroethane, nitrobutane, nitrohexane, and 2-nitropropane as substrate for the enzyme, both in their anionic and neutral forms. Evidence for the formation of an anionic flavin semiquinone in the enzyme-substrate complex before reaction with molecular oxygen comes from the spectral investigation of the enzyme-bound flavin upon mixing the enzyme anaerobically with either ethyl nitronate or nitroethane. The stopped flow spectrophotometric analysis of the enzyme under turnover clearly establishes that the formation of the flavin semiquinone occurs in the catalytic pathway for the oxidation of the nitroalkane substrate, with the enzyme cycling between its oxidized and one-electron reduced state. The enzyme monitored turnover data also rule out the involvement of the flavin hydroquinone in the enzymatic oxidation of the nitroalkane substrate, since formation of the two-electron reduced form of the enzyme-bound flavin was observed only after complete depletion of oxygen from the reaction mixture. Consistent with formation of the enzyme-bound flavin semiquinone requiring the nitroalkane substrate in either its neutral or anionic form, but not molecular oxygen, superoxide dismutase did not have any effect on the enzymatic activity expressed as rate of oxygen consumption with nitroethane, nitropropane, nitrobutane, nitropentane, nitrohexane, 2-nitropropane, ethyl nitronate, butyl-1-nitronate, pentyl-1-nitronate, and hexyl-1-nitronate. While other flavin-dependent enzymes, such as DNA photolyase (33, 34) and luciferase (35, 36) have been previously shown to utilize neutral flavin semiquinones in their catalytic cycles, to our knowledge 2-nitropropane dioxygenase is the first example of an enzyme operating with an oxidase-like catalytic mechanism in which an anionic flavin semiquinone has been directly observed in the catalytic pathway with physiological substrates.

Oxidation of the neutral nitroalkane substrates requires a catalytic base with pH\textsubscript{K\textsuperscript{a}} of 7.5, as suggested by the pH profiles of the k\textsubscript{cat}/K\textsubscript{m} and k\textsubscript{cat} values with nitroethane and nitrobutane, showing the requirement for an unprotonated group for catalysis. Such a catalytic base does not participate in the oxidation of the anionic substrates, as indicated by the pH dependence studies of the k\textsubscript{cat}/K\textsubscript{m} and k\textsubscript{cat} values with ethyl nitronate and butyl-1-nitronate. A likely role for the catalytic base in this enzyme will have to await future structural studies.

Reaction of the enzyme-bound anionic flavin semiquinone with molecular oxygen occurs with a second order rate constant k\textsubscript{cat}/K\textsubscript{O2} in the range of 10\textsuperscript{6} to 10\textsuperscript{7} M\textsuperscript{−}1 s\textsuperscript{−}1, in agreement with studies on the oxygen reactivity in solution showing second order rate constants of 10\textsuperscript{6} M\textsuperscript{−}1 s\textsuperscript{−}1 (29). Recent ab initio theoretical calculations showed that most of the spin density in anionic flavin semiquinones is located either on the N(5) position or the benzene moiety of the isoalloxazine ring (38, 39).

The N(5) locus is expected to be freely accessible to oxygen, making it a good candidate for reaction with oxygen in 2-nitropropane dioxygenase via a rapid one-electron transfer that results in the oxidation of the flavin and formation of superoxide (Scheme 4). The participation of an acid with pK\textsubscript{a} of 8.0 in the formation of a neutral superoxide species is suggested by the pH-dependence of the k\textsubscript{cat}/K\textsubscript{m} and k\textsubscript{cat} values with both nitroalkane and nitronate substrates, showing the involvement of a protonated group in catalysis. Such a chemistry is supported by recent mechanistic studies on glucose oxidase using viscosity, solvent and 18O isotope effects, suggesting that with that enzyme at high pH the association of molecular oxygen with the reduced enzyme is accompanied by the transfer of a single electron resulting in the formation of superoxide anion at the binding step (40, 41). Formation of a superoxide species in 2-nitropropane dioxygenase is supported by the effect of superoxide dismutase on the rate of oxygen consumption when propyl nitronates are used as substrates (see below). As shown in Scheme 4, the superoxide species thus generated would rapidly react with the nitroalkane radical within the enzyme active site, resulting in the formation of an α-peroxynitroalkane intermediate. Consistent with the formation of such a peroxynitroalkane species, an inverse secondary substrate deuterium kinetic isotope effect was observed on the k\textsubscript{cat}/K\textsubscript{m} value with 1,2,2-[2\textsuperscript{18}O]ethyl nitronate as substrate, which is expected for a change in hybridization of the nitronate α-carbon from sp\textsuperscript{3} to sp\textsuperscript{2} (42). The final elimination of nitrite from the peroxynitroalkane to yield the carbonyl product would then likely occur through a non-enzymatic attack by a nucleophile, such as for example the nitronate. The lack of a catalase effect on the enzymatic activity of 2-nitropropane dioxygenase rules out the alternative possibility of superoxide reacting with the enzyme-bound flavin, because if that were the case a 50% decrease in the rate of oxygen consumption would have been observed with catalase because of the formation of hydrogen peroxide following the decay of the C(4a)-peroxylavflavin intermediate (18, 43). A significant component of the oxidation of both propyl-1- and propyl-2-nitronate catalyzed by 2-nitropropane dioxygenase involves a radical chain reaction that occurs mostly off of the enzyme active site, as indicated by the effect of superoxide dismutase on the enzymatic activity with these substrates. The decreased rates of oxygen consumption observed in the presence of superoxide dismutase are readily explained with super-
oxide being normally produced in the catalytic pathway of 2-nitropropane dioxygenase, but being released from the active site only when the enzyme species that reacts with oxygen is in complex with propyl-1- and propyl-2-nitronate. The superoxide released in solution would initiate and propagate a non-enzymatic radical chain reaction with the propyl nitronates, a reaction fully established and characterized in solution (28, 44), leading to a significant amplification of the rate of oxygen consumption with these substrates. In the presence of the superoxide-scavenging enzyme superoxide dismutase, such a non-enzymatic oxidation of the propyl-nitronates would be abated, as experimentally observed. Enzymatic initiation of nitronate oxidation via facile free radical reactions triggered by the formation and release of superoxide anion was previously reported for glucose oxidase (21), horseradish peroxidase (45), and propionate-3-nitronate oxidase (46). With primary and secondary nitroalkanes and with alkyl nitronates other than the propyl ones, the 2-nitropropane dioxygenase oxidation reaction occurs enzymatically at the enzyme active site, as indicated by the lack of a superoxide dismutase effect on the rate of oxygen consumption. Therefore, it is likely that the non-enzymatic component of the oxidation of propyl nitronates is due to an adventitious release of superoxide from the enzyme active site.

Earlier studies on 2-nitropropane dioxygenase showed that the enzyme is capable of oxidizing both anionic nitronates and neutral nitroalkanes (2). The more extensive kinetic analyses reported in the present study are consistent with a broad range of substrate specificity, in that the enzyme can oxidize both 2-nitropropane and a number of primary nitroalkanes in their neutral and anionic forms. The enzyme has a higher specificity for alkyl nitronates as compared with neutral substrates, as indicated by the second order rate constant $k_{cat}/K_m$ that reflects the relative selectivity of the enzyme with different substrates. Interestingly, the specificity of the enzyme for primary nitroalkanes and nitronates is independent of the size of the substrate, since similar $k_{cat}/K_m$ values were observed with substrates with increasing length in the alkyl chain. Furthermore, substrate size does not significantly affect the overall enzymatic rate of turnover, as shown by the similar $k_{cat}$ values seen within the nitroalkane and nitronate series. These results are in stark contrast with those previously reported for another nitroalkane-oxidizing enzyme, namely nitroalkane oxidase, for which both the substrate specificity and the overall turnover number were shown to be significantly affected by the size of the nitroalkane substrate (47, 48). With that enzyme, it was proposed that substrate binding occurs at a hydrophobic site sufficiently large to accommodate nitroalkanes with a four-carbon linear chain (47). With 2-nitropropane dioxygenase the substrate binding site is likely to be less restrictive than that of nitroalkane oxidase.

In summary, in the present study the gene coding for 2-nitropropane dioxygenase from N. crassa was cloned and heterologously expressed in E. coli. The resulting enzyme was purified to high yields and was found to be a homodimer containing a mole of tightly bound FMN per mole of subunit. With neutral nitroalkanes and anionic nitronates other than propyl-1-nitronate and propyl-2-nitronate, substrate oxidation occurs at the enzyme active site. A steady state kinetic analysis showed that the preferred substrates for the enzyme are anionic nitronates as compared with neutral nitroalkanes, and that the enzyme has broad substrate specificity that is independent of substrate size. From a mechanistic standpoint, 2-nitropropane dioxygenase operates through an oxidoase-like catalytic mechanism, in which substrate oxidation occurs prior to and independently from reaction with oxygen, via the formation of an enzyme-bound anionic flavin semiquinone. To our knowledge, this represents the first account in which an anionic flavin semiquinone has been experimentally observed in the catalytic pathway for the oxidation of organic molecules catalyzed by a flavin-dependent enzyme. The availability of large amounts of recombinant 2-nitropropane dioxygenase will be instrumental for detailed mechanistic and structural studies aimed at a better understanding of the chemical mechanism of oxidation of both neutral nitroalkanes and anionic nitronates catalyzed by 2-nitropropane dioxygenase, and of the role the anionic flavin semiquinone plays in catalysis.

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REFERENCES
Involvement of a Flavosemiquinone in the Enzymatic Oxidation of Nitroalkanes
Catalyzed by 2-Nitropropane Dioxygenase
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