Identification of a Cysteine Residue in the Active Site of Nitroalkane Oxidase by Modification with N-Ethylmaleimide

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Running Title: Nitroalkane Oxidase Active Site

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Summary

The flavoprotein nitroalkane oxidase catalyzes the oxidative denitrification of primary or secondary nitroalkanes to the corresponding aldehydes or ketones with production of hydrogen peroxide and nitrite. The enzyme is irreversibly inactivated by treatment with N-ethylmaleimide at pH 7. The inactivation is time-dependent and shows first-order kinetics for three half-lives. The second-order rate constant for inactivation is $3.4 \pm 0.06 \text{ M}^{-1}\text{min}^{-1}$. The competitive inhibitor valerate protects the enzyme from inactivation, indicating an active site directed modification. Comparison of tryptic maps of enzyme treated with N-[ethyl-1-$^{14}$C]-maleimide in the absence and presence of valerate shows a single radioactive peptide differentially labeled in the unprotected enzyme. The sequence of this peptide was determined to be L-L-N-E-V-M-C-Y-P-L-F-D-G-G-N-I-G-L-R using Edman degradation and MALDI-TOF mass spectrometry. The cysteine residue was identified as the site of alkylation by ion trap mass spectrometry.
The flavoprotein nitroalkane oxidase from the fungus *Fusarium oxysporum* (ATCC 695) catalyzes the oxidative denitrification of primary or secondary nitroalkanes to the corresponding aldehydes or ketones with production of hydrogen peroxide and nitrite (Scheme 1). The study of an enzyme capable of oxidizing nitroalkanes is of considerable interest from both fundamental and applied standpoints. Nitroalkanes are widely used as industrial solvents, chemical intermediates, explosives, and fuels (1). Several nitroalkanes are toxic and/or carcinogenic (1). Thus, an enzymatic activity that converts nitroalkanes into less harmful species has significant potential for bioremediation. From a chemical standpoint, the formation of nitronates in solution is a well-characterized chemical reaction (2) that serves as the basis for understanding the formation of carbanions involving much weaker carbon acids, such as amino acids and α-hydroxy acids. Therefore, the study of an enzymatic activity that carries out the oxidation of nitroalkanes provides the unique opportunity to compare the enzyme-catalyzed formation of nitronates with the reaction in solution.

Nitroalkane oxidase is isolated with the flavin cofactor in the form of an N(5)-[3-nitrobut-2-yl]-1,5-dihydroflavin adenine dinucleotide and is consequently not active (3, 4). This nitrobutyl-flavin adduct can be converted *in vitro* to flavin adenine dinucleotide (FAD) yielding active enzyme (4, 5). The FAD-containing enzyme is active on a broad range of primary and secondary nitroalkane substrates (6, 7). Although other flavoprotein oxidases, such as D-amino acid oxidase (8), glucose oxidase (9), and 2-nitropropane dioxygenase (10, 11), have been shown to be able to oxidize nitroalkanes, nitroalkane oxidase is unique in that it requires the neutral form of the substrate for catalysis (4, 12). Mechanistic studies of nitroalkane oxidase support a chemical mechanism in which an active site base with a pKₐ value of 7 removes a proton from the α-carbon of the substrate to initiate catalysis (13, 14). The resulting carbanion attacks the N(5)-position of FAD to form a covalent adduct that subsequently decays to form nitrite and the aldehyde or ketone product. This covalent adduct can be trapped by nitroethane anion during turnover of the enzyme with nitroethane, yielding 5-[3-nitrobut-2-yl]-1,5-dihydroflavin adenine dinucleotide (4). The steady state kinetic mechanism of nitroalkane oxidase has been determined...
with nitroethane (13, 14). The data are consistent with a ping-pong iso-mechanism in which the release of the aldehyde product from the reduced enzyme is followed by an irreversible isomerization to yield the enzyme species that reacts with oxygen. Despite the considerable advances in the biochemical and mechanistic characterization of the enzyme, no structural information is available beyond the sequence of part of the gene encoding for the N-terminal half of nitroalkane oxidase. In the absence of crystallographic data, an effective strategy to identify active site residues has been the use of irreversible inhibitors. This approach has been recently used to identify an essential tyrosine residue in the active site of nitroalkane oxidase (15). In the present report, we describe a kinetic and structural characterization of the inactivation of the FAD-containing form of nitroalkane oxidase by the cysteine-directed reagent N-ethylmaleimide (16).
Experimental Procedures

**Materials.** Nitroethane and FAD were from Sigma. N-Ethylmaleimide and valerate were from Aldrich. N-[Ethyl-1-\(^{14}\)C]-maleimide was from NEN\textsuperscript{TM} Life Scientific Products, Inc. TPCK-treated trypsin was purchased from Worthington. Nitroalkane oxidase was purified from *Fusarium oxysporum* (ATCC 695) as previously described (7). The activated FAD-containing form of the enzyme was prepared according to Gadda et al. (4) 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 (17) using bovine serum albumin as standard. All other reagents were of the highest purity commercially available.

**Methods.** Enzyme activities were measured with 20 mM nitroethane as substrate in air-saturated 0.5 mM FAD, 50 mM potassium phosphate, 16 mM imidazole, pH 7.5, by monitoring the rate of oxygen consumption with a computer interfaced Hansatech Clark oxygen electrode at 30 °C, as previously described (7). Stock solutions of N-ethylmaleimide in 25 mM potassium phosphate, pH 7, were prepared just prior to use.

Nitroalkane oxidase (10-20 µM) was incubated with N-ethylmaleimide (3-15 mM) in 0.5 mM FAD, 25 mM potassium phosphate, pH 7, at 30 °C. All incubations were carried out in the presence FAD to avoid the formation of apoprotein (5). At different times, aliquots were withdrawn and assayed for enzymatic activity. For experiments in which the effect of valerate on the rate of inactivation was measured, the enzyme was incubated for 5 min with this compound before the addition of N-ethylmaleimide. To stop the inactivation, unreacted N-ethylmaleimide was removed by gel filtration on a Sephadex G-25 column equilibrated with 25 mM potassium phosphate, pH 7, at room temperature. The irreversibility of N-ethylmaleimide inactivation of nitroalkane oxidase was determined by incubating the modified enzyme isolated by gel filtration for 3 h in 0.5 mM FAD, 25 mM potassium phosphate, pH 7, at 30 °C. At different times aliquots were withdrawn and assayed for enzymatic activity.

To identify the peptide whose modification by N-ethylmaleimide resulted in enzyme inactivation, 32 µM enzyme was incubated with 10 mM N-[ethyl-1-\(^{14}\)C]-maleimide (1.8 x 10\(^{3}\)
cpm/nmol) in the presence and absence of 25 mM valerate in a total volume of 0.5 ml. After 25
min a second 10 mM aliquot of 10 mM N-[ethyl-1-\(^{14}\)C]-maleimide was added, and the incubation
continued for an additional 25 min. The reaction was stopped by gel filtration using a Sephadex
G-25 column equilibrated with 4 mM calcium chloride, 0.4 M ammonium bicarbonate, pH 8.
After addition of solid urea to a final concentration of 8 M, the samples were incubated for 1 h at
37 °C. The solutions were then diluted with 3 volumes of water followed by the addition of
trypsin to a final concentration of 3% (w/w, trypsin/nitroalkane oxidase). After a 4 h incubation
at 37 °C, a second aliquot of trypsin (1% w/w final concentration) was added and the digestion
continued for a further 16 hours at 37 °C. The reaction was stopped by adding freshly prepared
phenylmethanesulphonyl fluoride at a final concentration of 1 mg/ml. Purification of peptides
was carried out by HPLC using a Waters instrument equipped with a model 996 photodiode
array detector and a Vydac 218TP54 (4.6 x 250 mm) reverse-phase column at a flow rate of 1 ml
min\(^{-1}\). Eluent A was 0.05% aqueous trifluoroacetic acid and eluent B was 0.04% trifluoroacetic
acid in acetonitrile. The chromatography was carried out with a linear gradient from 5% to 50%
B over 90 min. Peptides were collected manually and the amount of radioactivity incorporated in
each was determined by scintillation counting. Automated Edman degradation of purified
peptide was carried out on a Hewlett-Packard G1000A protein sequencer at the Protein
Chemistry Laboratory of Texas A&M University. MALDI-TOF mass spectroscopy of the
purified peptide was carried out using a Voyager Elite XL mass spectrometer (PerSeptive
Biosystems, Framingham, MA) at the Laboratory for Biological Mass Spectrometry at Texas A
&M University. MALDI-TOF spectra were acquired in the positive ion mode, using \(\alpha\)-cyano-4-
hydroxysuccinamic acid as matrix. Samples were prepared for MALDI-TOF using the overlayer
method of sample preparation previously described (18). Mass spectrometry of the purified
peptide was performed using an Esquire LC Ion Trap Mass Spectrometer equipped with a
nanospray source (Bruker Daltonics, Inc., Billerica, MA).

Data analysis. The time course of inactivation of nitroalkane oxidase by N-
ethylnmaleimide was analyzed by fitting the residual activity (A) at a given time (t) to equation 1,
where $A_0$ is the initial activity and $k_{obs}$ is the observed rate of inactivation.

$$A = A_0 e^{-k_{obs} t}$$  \hspace{1cm} (1)
Results

Inactivation of nitroalkane oxidase by N-ethylmaleimide. Treatment of nitroalkane oxidase with N-ethylmaleimide at pH 7 and 30 °C results in a time-dependent loss of enzymatic activity, as shown in Figure 1. The inactivation is first-order for about three half-lives, but the rates decrease at longer times (data not shown). This could be due to hydrolysis of N-ethylmaleimide to form N-ethylmaleamate, a process previously reported to occur at pH 7 and above (16). Consistent with this hypothesis, addition of a second aliquot of N-ethylmaleimide after three half-lives results in a further time-dependent loss of enzymatic activity. The initial rate of inactivation is dependent on the concentration of N-ethylmaleimide (Figure 1). A plot of the rate of inactivation versus the concentration of the reagent is linear up to 15 mM N-ethylmaleimide (Figure 1B), indicating that there is no significant formation of a reversible complex between the reagent and the enzyme prior to inactivation. The second-order rate constant for inactivation determined from this plot is 3.4 ± 0.06 M⁻¹ min⁻¹.

Valerate is a competitive inhibitor of nitroalkane oxidase with a Kᵢ value of 0.6 mM at pH 7 and 30 °C (14). In the presence of 25 mM valerate the rate of inactivation of nitroalkane oxidase by 10 mM N-ethylmaleimide decreases from 96 min⁻¹ to 0.1 min⁻¹ (Figure 1), consistent with the inactivation being active site directed.

To determine if the reaction between N-ethylmaleimide and nitroalkane oxidase is irreversible, the enzyme was separated from the remaining reagent by gel filtration when the residual activity had decreased to 6% of the initial value. The inactivated enzyme was then incubated in the absence of N-ethylmaleimide at pH 7 and 30 °C. No recovery of activity was observed after 3 hours, consistent with the modification being irreversible.

Identification of the residue modified by N-ethylmaleimide. To identify the amino acid residue whose modification by N-ethylmaleimide resulted in the inactivation of the enzyme, nitroalkane oxidase was incubated with N-[ethyl-1-¹⁴C]-maleimide in the presence and absence of valerate. The reactions were stopped when the residual activity of the enzyme incubated in the absence of valerate was 10%. At that time the valerate-protected enzyme retains about 80% of
the initial activity. Both samples were digested with trypsin, and the resulting tryptic digests were separated by reverse-phase HPLC. In our initial attempts the reaction was stopped by the addition of 10% trichloroacetic acid. When this was done, the tryptic maps of the two samples were identical (data not shown), suggesting that the modification is acid-labile. In contrast, when the reaction was quenched by using gel filtration to remove the unreacted reagent, the tryptic map of the sample lacking valerate showed an extra peak eluting at 61.4 min (Figure 2). This was the only HPLC fraction showing significant incorporation of radioactivity in either sample. The N-terminal amino acid sequence of the alkylated peptide was determined by automated Edman degradation to be L-L-N-E-V-M-X-Y-P-L (Table 1), where X indicates the absence of any phenylthiohydantoin derivative in the chromatogram. This sequence corresponds to that of a peptide in nitroalkane oxidase previously identified using tetranitromethane as an active site directed reagent, L-L-N-E-V-M-X-Y-P-L-F-D-G-G-N-I-G-L-R (15).

Mass spectrometry was used to definitively identify the residue at position 7 and the site of modification. Positive ion MALDI-TOF mass spectrometry of the modified peptide yielded a predominant peak with $m/z^+$ value of 2248.8 (Figure 3). A cysteine residue at position 7 and incorporation of a single N-ethylmaleimide moiety into the peptide would yield a $m/z^+$ value of 2249, establishing cysteine as amino acid residue X. A second peak with $m/z^+$ value of 2264.7 was observed in the mass spectrum (Figure 3), consistent with an oxidized form of the same peptide.

The specific residue modified by N-ethylmaleimide was identified by nanospray mass spectrometry on an ion trap mass spectrometer in the positive ion mode. Species with mass/charge values of 1126 and 1132 were seen in the mass spectrum of the same peptide. The mass/charge value of the smaller species is consistent with that expected for the doubly-charged ion of the alkylated peptide, while the higher mass/charge ratio species can be attributed to an oxidized form of the same peptide. These two parental ions were further fragmented while in the trap in order to obtain the sequence of the peptide, as illustrated in Figure 4 for the $m/z^{2+}$ species of 1132. The sequence of the alkylated peptide could be determined by comparing the $m/z^+$
values of the daughter ions to the calculated values expected for the peptides produced in the fragmentation process (Table 2). The sequences of both peptides determined from the ion trap spectrometric analysis were in agreement with that from the Edman analysis. The data in Table 2 are consistent with the cysteine residue at position 7 being the site of alkylation by N-ethylmaleimide and the methionine residue at position 6 being oxidized in the species with m/z^2^+ value of 1132. Since no methionine sulfoxide was seen in the automated sequence analysis of the same peptide, the oxidized methionine probably formed during the mass spectrometric analysis.
Discussion

The mechanism by which C-H bonds are cleaved by enzymes represents a fundamental problem in enzymology, because of the high energetic barrier associated with this process. Thiamin- and pyridoxal-dependent enzymes are able to lower this energetic barrier by derivatizing the substrate. With this respect flavoprotein oxidases are particularly intriguing because cleavage of the carbon-hydrogen bond is carried out on the underivatized substrate (19). The mechanisms by which the substrate pK$_a$ is lowered to allow C-H bond cleavage is not yet fully understood, although recently attracting models invoking short hydrogen bonds have been put forward (20, 21). In the case of nitroalkanes, the pK$_a$ value for deprotonation of the α-carbon is below 12, so that little activation is required for proton removal (22). For this reason, nitroalkanes have been extensively studied as model systems for understanding the formation of carbanions in solution (2). These studies serve as the basis for the understanding of the enzymatic formation of carbanions involving much weaker carbon acids. However, caution should be exerted in extrapolating results obtained in solution with enzyme-catalyzed reactions involving much weaker carbon acids. In this context, an enzyme able to catalyze the cleavage of the C-H bond of nitroalkanes is of considerable interest because it offers the unique opportunity to compare the enzyme-catalyzed reaction with the reaction in solution. Nitroalkane oxidase from the fungus *Fusarium oxysporum* appears to be perfectly suited for this goal, since it requires the neutral form of the substrate for catalysis (4, 12).

Mechanistic studies of nitroalkane oxidase are consistent with a mechanism for catalysis in which a base on the enzyme removes a proton from the α-carbon of the substrate to form a carbanion (Scheme 2) (4,13,14). During the normal course of catalysis, the carbanion can attack the N-(5) position of the cofactor to form a covalent adduct. This adduct can lose nitrite to form a highly reactive cationic imine that can then react with hydroxide to form the aldehyde or ketone product and reduced flavin. Alternatively, the cationic imine can react with nitroethane anion in solution to form the stable and inactive 5-nitrobutyl-flavin. Despite the advances in the mechanistic and biochemical characterization of the enzyme, no structural information is
available beyond the sequence of part of the gene encoding for nitroalkane oxidase. The use of irreversible chemical modification reagents has long been employed to study the active site of enzymes whose structures are not available. By using this approach, we have recently identified a tyrosine residue in the active site of nitroalkane oxidase (15). The results presented here using N-ethylmaleimide identify a cysteine residue as also being in the active site of the enzyme.

Nitroalkane oxidase contains four cysteine residues (23). Titration of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) under non-denaturing conditions showed that two cysteine residues are located on the surface of the enzyme (23). These two cysteines can be nitrated by tetranitromethane under non-denaturing conditions with no loss of enzymatic activity (15), indicating that they are not in the active site of the enzyme.

The results presented here show that nitroalkane oxidase is irreversibly inactivated in a time- and concentration-dependent fashion by treatment with N-ethylmaleimide. The inactivation is active site directed, based on the protection from inactivation afforded by the competitive inhibitor valerate. The tryptic digests of nitroalkane oxidase treated with N-[ethyl-1-14C]-maleimide both in the presence and absence of valerate show that a single peptide is differentially labeled by N-ethylmaleimide in the inactivated enzyme. This peptide is the same as the one previously identified using tetranitromethane as an active site probe (15). The cysteine residue identified here is adjacent to the active site tyrosine nitrated by tetranitromethane (15), strengthening the conclusion that both these residues are in the active site of the enzyme. As previously reported, no direct match of the sequence of the alkylated peptide could be found with the available partial gene sequence of nitroalkane oxidase, indicating that the cysteine and tyrosine residues are located in the C-terminal half of the protein.

In terms of the catalytic roles of these two residues, we have previously proposed that the tyrosine residue participates in substrate binding by forming a hydrogen bond to the nitro group of the nitroalkane substrate (15). A possible role for a cysteine residue is as the active site base that abstracts a proton from the α-carbon of the substrate, as is the case with the flavoprotein dihydroorotate dehydrogenase (24, 25). The involvement of a cysteine residue in catalysis is
consistent with solvent kinetic isotope effect studies of nitroalkane oxidase, specifically an inverse effect on the V/K value of about 0.5\(^1\). Previous pH-dependence studies on the enzyme showed the presence of a base with a pK\(_a\) value of 7 in the free enzyme (14). However, the effects of D\(_2\)O on the pH dependence do not support the assignment of the cysteine as the residue responsible for this pK\(_a\). The pK\(_a\) value of a cysteine residue should shift less than 0.4 units when changing from water to D\(_2\)O (26). Instead, the pK\(_a\) value increases to 7.7 in D\(_2\)O\(^1\), strongly suggesting that the catalytic base in nitroalkane oxidase is not a cysteine residue. In the catalytic mechanism of Scheme 2, another active site base is required to deprotonate a water molecule to form the hydroxide that reacts with the cationic imine formed during catalysis. Although a serine residue would seem more appropriate for this reaction due to the pK\(_a\) value of about 15 of its functional group (27), a cysteine residue with an unusually high pK\(_a\) value could act as this base. An alternative explanation is that the active site cysteine is not directly involved in catalysis, but that enzyme inactivation stems from steric hindrance due to the presence of the N-ethylmaleimide moiety in the active site preventing substrate binding.

In summary, the chemical modification studies with N-ethylmaleimide presented here show that a cysteine residue is present in the active site of the flavoprotein nitroalkane oxidase. This amino acid residue is located next to a tyrosine residue previously identified in the active site of nitroalkane oxidase. These results are a prerequisite to future mutagenesis studies aimed at a better understanding of the catalytic mechanism of this enzyme.

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Footnotes

Figure Legends

Figure 1. Time-dependent inactivation of nitroalkane oxidase by N-ethylmaleimide. Nitroalkane oxidase (10 µM) was incubated with different concentrations of N-ethylmaleimide in 0.5 mM FAD, 25 mM potassium phosphate, pH 7 and 30 °C. At different times, aliquots were withdrawn and assayed for enzymatic activity as described in Experimental Procedures. (A) Time course of inactivation; N-ethylmaleimide concentrations were (●) 3 mM, (○) 5 mM, (■) 10 mM, (□) 15 mM, and (▲) 10 mM in the presence of 25 mM valerate. The lines are fits of the data to eq. 1. (B) Secondary plot of the observed rate of inactivation as a function of the concentration of N-ethylmaleimide.

Figure 2. HPLC of tryptic digests of nitroalkane oxidase treated with N-ethylmaleimide in the presence and absence of valerate. Nitroalkane oxidase was incubated with N-[ethyl-1-14C]-maleimide in the presence and absence of valerate as described in Experimental Procedures. Tryptic digests of each sample were separated by reverse-phase HPLC as described in Materials and Methods. Peptide elution was monitored at 214 nm (Panel A). The resulting peaks were analyzed for radioactive incorporation using a scintillation counter (Panel B). The radioactive peptide eluting at 61.4 min found only in the inactivated sample is indicated by an arrow. The upper line in each panel is the sample treated with N-ethylmaleimide alone; the bottom line is the sample treated with N-ethylmaleimide in the presence of valerate.

Figure 3. MALDI-TOF mass spectrometric analysis of the peptide modified by N-ethylmaleimide. The N-ethylmaleimide-modified peptide isolated by reverse-phase HPLC of a tryptic digest of nitroalkane oxidase inactivated by N-ethylmaleimide was analyzed by MALDI-TOF mass spectrometry as described under Experimental Procedures. Inset, expansion of the m/z region 2245-2256 of the mass spectrum.

Figure 4. Nanospray mass spectrometric analysis of the peptide modified by N-ethylmaleimide.
The parental ion with m/z²⁺ value of 1132 was fragmented in the ion trap mass spectrometer as described in Experimental Procedures.
Table 1. N-Terminal Amino Acid Sequence of the Peptide Eluting at 61.4 min in the Tryptic Digest of Nitroalkane Oxidase Treated with N-Ethylmaleimide.

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\(^a\)NEM, N-ethylmaleimide; \(^b\)M-O, oxidized methionine residue.
Scheme 1
R-C-N^+ + O_2 + H_2O → R-C=O + NO_2^- + H_2O_2 + H^+

Scheme 1
Identification of a Cysteine Residue in the Active Site of Nitroalkane Oxidase by Modification with N-Ethylmaleimide
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