Identification of an Essential Tyrosine Residue in Nitroalkane Oxidase by Modification with Tetranitromethane†

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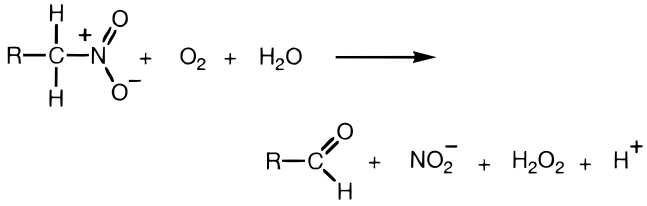
Received September 20, 1999; Revised Manuscript Received November 2, 1999

ABSTRACT: The flavoprotein nitroalkane oxidase from *Fusarium oxysporum* catalyzes the oxidation of nitroalkanes to the respective aldehydes or ketones with production of nitrite and hydrogen peroxide. The enzyme is irreversibly inactivated by incubation with tetranitromethane, a tyrosine-directed reagent, at pH 7.3. The inactivation is time-dependent and shows first-order kinetics for two half-lives of inactivation. Further inactivation can be achieved upon a second addition of tetranitromethane. A saturation kinetic pattern is observed when the rate of inactivation is determined versus the concentration of tetranitromethane, indicating that a reversible enzyme−inhibitor complex is formed before irreversible inactivation occurs. Values of 0.096 ± 0.013 min^{-1} and 12.9 ± 3.8 mM were determined for the first-order rate constant for inactivation and the dissociation constant for the reversibly formed complex, respectively. The competitive inhibitor valerate protects the enzyme from inactivation by tetranitromethane, suggesting an active-site-directed inactivation. The UV−visible absorbance spectrum of the inactivated enzyme is perturbed with respect to that of the native enzyme, suggesting that treatment with tetranitromethane resulted in nitration of the enzyme. Comparison of tryptic maps of nitroalkane oxidase treated with tetranitromethane in the presence and absence of valerate shows a single peptide differentially labeled in the inactivated enzyme. The spectral properties of the modified peptide are consistent with nitration of a tyrosine residue. The amino acid sequence of the nitrated peptide is L-L-N-E-V-M-C-(NO_2-Y)-P-L-F-D-G-G-N-I-G-L-R. The possible role of this tyrosine in substrate binding is discussed.

The flavoprotein nitroalkane oxidase from the fungus *Fusarium oxysporum* (ATCC 695) catalyzes the oxidation of nitroalkanes to the corresponding aldehydes, hydrogen peroxide, and nitrite (Scheme 1). The study of an enzyme capable of oxidizing nitroalkanes is of considerable interest. Nitroalkanes are widely used as industrial solvents, chemical intermediates, explosives, and fuels (*1*). Several nitroalkanes have been shown to be toxic and/or carcinogenic (*1*). Thus, an enzymatic activity that converts these compounds into less harmful species is of interest for bioremediation. In addition, many antibiotics contain nitro groups and many leguminous plants produce nitrated toxins (*2*−*4*). Therefore, it is possible that the physiological role for nitroalkane oxidase is the conversion of such compounds to less harmful species, thereby inactivating the natural defenses of the host organisms. Finally, from a chemical standpoint, the formation of nitronates in solution is a well-characterized chemical reaction and is considered a model system for the formation of carbanions in solution (*5*). Thus, the study of the mechanism of nitroalkane oxidase provides an opportunity to compare the enzyme-catalyzed oxidation of nitroalkanes with the reaction in solution.

Nitroalkane oxidase is isolated with the flavin in the form of an *N*(5)-nitrobutyl dihydroflavin adenine dinucleotide and is consequently not active (*6*, *7*). Conversion of the nitrobutyl−flavin adduct to flavin adenine dinucleotide (FAD) yields active enzyme (*7*, *8*). The FAD-containing enzyme is active on a broad spectrum of primary and secondary nitroalkanes (*9*). The partial sequence of the gene encoding the N-terminal half of nitroalkane oxidase[1] and the amino acid composition of the enzyme (*8*) do not match any known protein or open reading frame, suggesting that nitroalkane oxidase has not been previously described. While other flavoproteins, such as D-amino acid oxidase (*10*), glucose oxidase (*11*), and 2-nitropropane dioxygenase (*12*, *13*), can catalyze the oxidation of the anionic form of the nitroalkane substrate, nitroalkane oxidase is unique in that it requires the neutral form of the substrate (*7*, *14*). This suggests that the physiological role of nitroalkane oxidase is to oxidize nitroalkanes. Consistent with this conclusion, the enzyme is produced in large amounts when *F. oxysporum* is grown on nitroethane as the sole carbon source (*15*). Recently a

Scheme 1



† This work was supported in part by NIH Grant GM 58698.

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[1] G. Gadda, and P. F. Fitzpatrick, unpublished results.

10.1021/bi9921743 CCC: $19.00
Published on Web 01/12/2000

biochemical and biophysical characterization of the FAD-containing form of nitroalkane oxidase was carried out (*8*). In the absence of crystallographic data, an effective strategy to identify active-site residues has been the use of irreversible inhibitors. In this paper we report a kinetic and structural characterization of the inactivation of the FAD-containing form of nitroalkane oxidase by tetranitromethane, a tyrosine-directed reagent (*16*).

MATERIALS AND METHODS

Materials. Nitroethane, 5,5′-dithiobis(2-nitrobenzoic acid), and FAD were from Sigma. Tetranitromethane and valerate were from Aldrich. TPCK-treated trypsin was purchased from Worthington. Nitroalkane oxidase was purified from *Fusarium oxysporum* (ATCC 695) as previously described (*9*). The activated FAD-containing form of the enzyme was prepared according to Gadda and Fitzpatrick (*8*) 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*) with bovine serum albumin as standard. The concentration of bound FAD was determined spectrophotometrically at 446 nm using an ϵ_{446} value of 11 700 M^{-1} cm^{-1} (*8*). 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, and 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 (*9*). UV−Visible absorbance spectra were recorded on a Hewlett-Packard model HP 8453 spectrophotometer. Stock solutions of tetranitromethane were prepared just prior to use.

Nitroalkane oxidase (10−20 μM) was incubated with tetranitromethane (3−24 mM) in 0.5 mM FAD and 35 mM potassium phosphate, pH 7.3, at 30 °C. At different times, aliquots were withdrawn and assayed polarographically 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 tetranitromethane. Gel filtration to remove residual tetranitromethane after a reaction was carried out on a Sephadex G-25 column equilibrated with 25 mM potassium phosphate, pH 7, at 4 °C. The irreversibility of tetranitromethane inactivation of nitroalkane oxidase was determined by incubating the modified enzyme isolated by gel filtration for 4 h in 0.5 mM FAD and 25 mM potassium phosphate, pH 7, at 30 °C. At different times aliquots were withdrawn and assayed for enzymatic activity. The cysteine contents of the inactivated enzymes isolated by gel filtration were determined with 5,5′-dithiobis(2-nitrobenzoic acid) after the samples were incubated for 15 min at 55 °C in 4 M urea and 25 mM potassium phosphate, pH 7. An ϵ_{413} value of 13 600 M^{-1} cm^{-1} was used (*18*).

To identify the peptides whose modification by tetranitromethane resulted in enzyme inactivation, 29 μM enzyme was incubated with 5 mM tetranitromethane in the presence and absence of 25 mM valerate in a total volume of 0.64 mL. After 1 h, a second 5 mM aliquot of tetranitromethane was added, and the mixture was allowed to react for one additional hour. After gel filtration to remove the unreacted reagents, trichloroacetic acid to a final concentration of 10% (w/v) was added and the samples were incubated on ice for 30 min before centrifugation at 12000*g* for 20 min. The pellets were washed with 10% trichloroacetic acid followed by an ice-cold acetone/HCl (39:1) mixture, resuspended in 0.1 mL of 8 M urea, 4 mM $CaCl_2$, and 0.4 M ammonium bicarbonate, pH 8, and allowed to stand for 1 h at 37 °C. The solutions were then diluted with 0.3 mL of water before 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 mixture was allowed to react for a further 15 h at 37 °C. The digestion was stopped with trifluoroacetic acid (1% final concentration). 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 × 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% eluent B over 90 min. Peptides were collected manually. Automated Edman degradation of purified peptides was carried out on a Hewlett-Packard G1000A protein sequencer at the Protein Chemistry Laboratory of Texas A&M University.

Data Analysis. The time course of inactivation of nitroalkane oxidase by tetranitromethane was analyzed by fitting the residual activity (A) at a given time (t) to eq 1, where A_0 is the initial activity and k_{obs} is the observed rate of inactivation. The rates of inactivation of the enzyme were fit to eq 2, where k_{inact} is the first-order rate constant for the conversion of the reversibly formed enzyme−inhibitor complex to irreversibly inactivated enzyme, K_I is the dissociation constant for the complex, and [I] is the concentration of tetranitromethane.

$$A = A_0 e^{-k_{obst}} \quad (1)$$

$$k_{obs} = \frac{k_{inact}[I]}{[I]+K_I} \quad (2)$$

RESULTS

Inactivation of Nitroalkane Oxidase with Tetranitromethane. Treatment of nitroalkane oxidase with tetranitromethane at pH 7.3 and 30 °C resulted in a time-dependent loss of enzymatic activity, as shown in Figure 1. Since nitroalkane oxidase has a low affinity for FAD, with a K_d value of 1.4 μM at pH 7.5 (*8*), a saturating concentration of FAD was present during the incubations to prevent the formation of the apoprotein. Valerate, a competitive inhibitor of nitroalkane oxidase with a K_i value of 0.6 mM[2] protected the enzyme from inactivation (Figure 1), suggesting that the inactivation is active-site-directed. The inactivation showed first-order kinetics for about two half-lives but reached a limiting residual activity at longer times (data not shown). The addition of a second aliquot of tetranitromethane after two half-lives of inactivation resulted in a further time-dependent decrease in the activity of nitroalkane oxidase,

[2] G. Gadda, and P. F. Fitzpatrick, *Biochemistry*, in press.

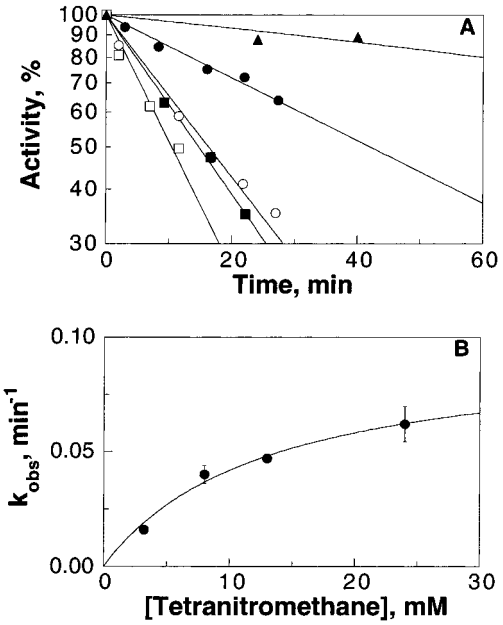


FIGURE 1: Time-dependent inactivation of nitroalkane oxidase by tetranitromethane. Nitroalkane oxidase (10 μM) was incubated with different concentrations of tetranitromethane in 0.5 mM FAD and 35 mM potassium phosphate, pH 7.3, at 30 °C. At different times, aliquots were withdrawn and assayed for enzymatic activity with 20 mM nitroethane as described under Materials and Methods. (A) Time course of inactivation; tetranitromethane concentrations were (●) 3.2 mM, (○) 8 mM, (■) 13 mM, (□) 24 mM, and (▲) 20 mM tetranitromethane 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 tetranitromethane. The curve is a fit of the data to eq 2.

suggesting that the decrease in the rate of inactivation was due to hydrolysis of tetranitromethane. The rate of inactivation was dependent on the concentration of tetranitromethane, as shown in Figure 1. A saturation curve was observed in a plot of the observed rates of inactivation versus the concentration of tetranitromethane, indicating the formation of a reversible enzyme−inhibitor complex before inactivation occurs. By fitting the data to eq 2, values of 0.096 ± 0.013 min^{-1} and 12.9 ± 3.8 mM were calculated for the limiting rate of inactivation (k_{inact}) and the apparent dissociation constant for the reversibly formed complex (K_I), respectively. Tetranitromethane was also tested as a substrate for nitroalkane oxidase. No oxygen consumption was observed when 5 mM tetranitromethane was added to an assay reaction mixture containing the enzyme at pH 7.5 and 30 °C.

Properties of Nitroalkane Oxidase Inactivated with Tetranitromethane. The inactivated enzyme was isolated by gel filtration after 105 min of incubation with 5 mM tetranitromethane. The resulting enzyme was 34% active and showed an increase of absorbance in the 300−400 nm region of the UV−visible absorbance spectrum, suggesting that treatment with tetranitromethane resulted in nitration of nitroalkane oxidase (Figure 2). A stoichiometry of 0.7 mol of bound FAD/mol of enzyme was calculated from the absorbance at 446 nm, consistent with an affinity of the modified enzyme for FAD that is unchanged with respect to that of the untreated enzyme.[3] No recovery of activity was detected when the inactivated enzyme isolated by gel filtration was incubated for 4 h with 0.5 mM FAD at 30 °C, indicating that the modification is irreversible. The valerate-protected enzyme isolated by gel filtration after 105 min of

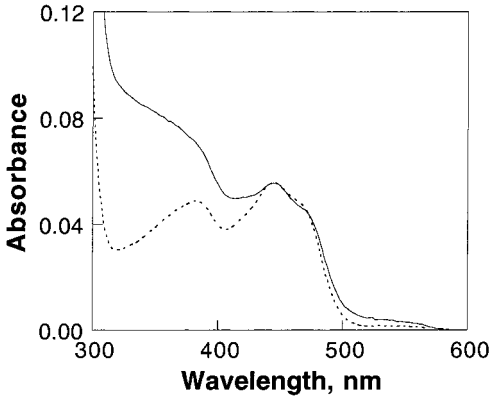


FIGURE 2: UV−Visible absorbance spectra of nitroalkane oxidase after treatment with tetranitromethane in the presence and absence of valerate. Nitroalkane oxidase was isolated by gel filtration after incubation with tetranitromethane as described in the text. The spectra were taken in 25 mM potassium phosphate, pH 7, and normalized for the absorbance at 446 nm. Solid line, spectrum of nitroalkane oxidase treated with tetranitromethane; dotted line, spectrum of nitroalkane oxidase treated with tetranitromethane in the presence of valerate.

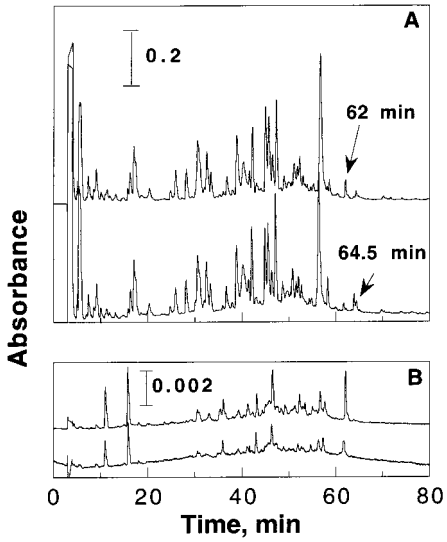


FIGURE 3: HPLC of tryptic digests of nitroalkane oxidase treated with tetranitromethane in the presence and absence of valerate. Nitroalkane oxidase was incubated with tetranitromethane in the presence and absence of valerate as described under Materials and Methods. Tryptic digests of each sample were separated by reverse-phase HPLC as described under Materials and Methods. Peptide elution was monitored at 214 and 360 nm with a photodiode array spectrophotometer detector. The peptides eluting at 62 and 64.5 min found only in the inactivated and valerate-protected samples are indicated by an arrow. (A) Elution profile at 214 nm; (B) elution profile at 360 nm. Upper line, sample treated with tetranitromethane; bottom line, sample treated with tetranitromethane in the presence of valerate.

incubation with tetranitromethane retained 78% of the initial activity and showed a UV−visible absorbance spectrum similar to that of the untreated enzyme (Figure 2). Thus, differential nitration of nitroalkane oxidase was observed in the presence and absence of valerate. To determine whether

[3] A stoichiometry of about 0.7 for the FAD bound to the inactivated enzyme is expected if the affinity of the inactivated enzyme for FAD is the same as that for the unmodified enzyme, since the K_d value for FAD of the unmodified enzyme is 1.4 μM at pH 7.5 and 4 °C (*8*) and the concentration of the enzyme treated with tetranitromethane used in this experiment was 9 μM.

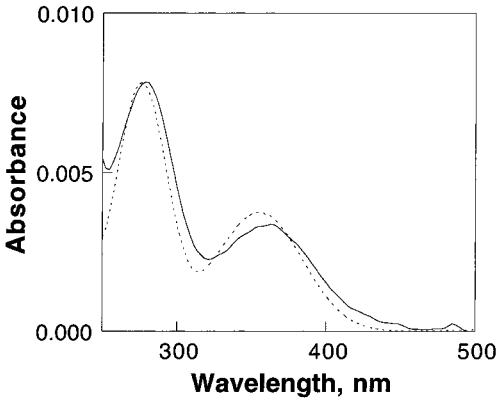


FIGURE 4: UV−Visible absorbance spectrum of the peptide eluting at 62 min in the tryptic digest of nitroalkane oxidase treated with tetranitromethane. The spectrum was taken with an in-line photodiode array spectrophotometer during the HPLC separation of the tryptic digest of nitroalkane oxidase treated with tetranitromethane. The solvent was 0.047% trifluoroacetic acid in 35% aqueous acetonitrile. The spectrum of free 3-nitrotyrosine in the same solvent is shown for comparison. Solid line, spectrum of the peptide eluting at 62 min; dotted line, spectrum of 3-nitrotyrosine.

Table 1: Amino Acid Sequences of the Peptides Eluting at 62 and 64.5 min in the Tryptic Digests of Nitroalkane Oxidase Treated with Tetranitromethane in the Absence and Presence of Valerate

	peptide eluting at 62 min of unprotected sample		peptide eluting at 64.5 min of sample protected with valerate	
cycle no.	amino acid	amount (pmol)	amino acid	amount (pmol)
1	L	91	L	152
2	L	100	L	153
3	N	117	N	174
4	E	130	E	194
5	V	90	V	137
6	M	92	M	131
7	X[a]		X[a]	
8	NO_2-Y	nq[b]	Y	141
9	P	51	P	89
10	L	49	L	76
11	F	53		
12	D	62		
13	G	51		
14	G	72		
15	N	46		
16	I	23		
17	G	52		
18	L	40		
19	R	11		

[a] Reaction of nitroalkane oxidase with *N*-ethylmaleimide results in the alkylation of the amino acid residue at position 7 when the enzyme is incubated in the absence but not in the presence of valerate (G. Gadda, and P. F. Fitzpatrick, manuscript in preparation). The mass of the alkylated peptide has been determined by mass spectroscopic analysis to be 2047.8; such a value agrees well with the value of 2048 calculated for the mass of the peptide containing a cysteine at position 7 and *N*-ethylmaleimide. Cys was not detectable in this analysis since the sample was not reduced and alkylated. [b] Not quantitated.

cysteine residues were modified by tetranitromethane, the enzymes isolated by gel filtration were titrated with 5,5′-dithiobis(2-nitrobenzoic acid) under denaturing conditions; 1.6 ± 0.1 and 1.9 ± 0.2 cysteine residues reacted with 5,5′-dithiobis(2-nitrobenzoic acid) in the enzymes treated with tetranitromethane in the absence and presence of valerate, respectively.

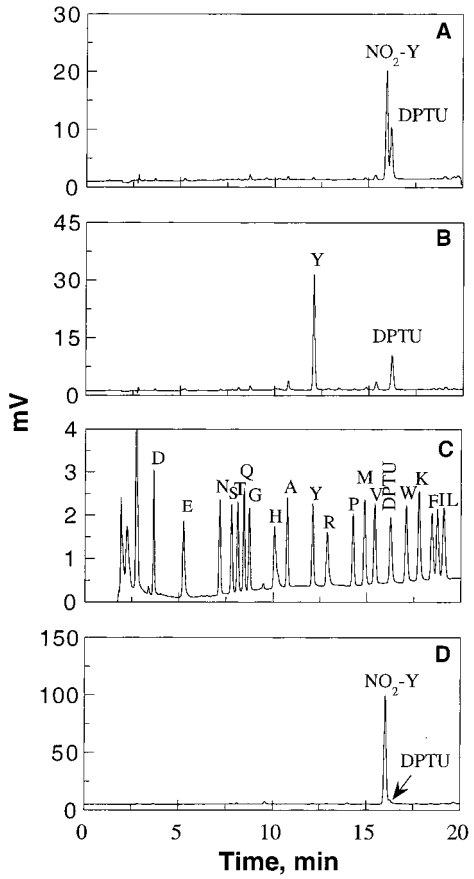


FIGURE 5: Comparison of the chromatographic profiles of the Pth-amino acid at position 8 derived from the automated Edman degradation of the peptides eluting at 62 and 64.5 min in the tryptic digests of nitroalkane oxidase treated with tetranitromethane in the absence and presence of valerate. The modified enzymes and the tryptic digests were prepared as described under Materials and Methods. The separation of the Pth-amino acids was performed on a 250 × 2.1 mm Pth-reverse-phase column (Hewlett-Packard). (A) Pth-amino acid at position 8 of the peptide eluting at 62 min in the sample prepared with tetranitromethane only; (B) Pth-amino acid at position 8 of the peptide eluting at 64.5 min in the sample prepared with tetranitromethane in the presence of valerate; (C) Pth-amino acid standards; (D) Pth-3-nitrotyrosine standard.

Identification of the Site of Modification of Nitroalkane Oxidase by Tetranitromethane. To identify the amino acid residue whose modification by tetranitromethane resulted in inactivation, nitroalkane oxidase was incubated with tetranitromethane in the absence and presence of 25 mM valerate as described under Materials and Methods. The residual activity of the enzyme incubated in the absence of valerate was 13% that of the untreated enzyme, whereas that of the valerate-protected sample was 75% that of the untreated enzyme. To identify the site of modification by tetranitromethane, both samples were digested with trypsin and the resulting tryptic digests were separated by reverse-phase HPLC (Figure 3). The two chromatographic maps were similar to each other except for the presence of an extra peptide eluting at 64.5 min in the valerate-protected sample that was missing in the tryptic digest separation of the enzyme incubated with tetranitromethane in the absence of valerate. Also, an extra peak eluting at 62 min was present in the tryptic digest chromatographic map of the enzyme inactivated with tetranitromethane alone. The UV−visible absorbance spectrum of this peptide was similar to that of

Scheme 2

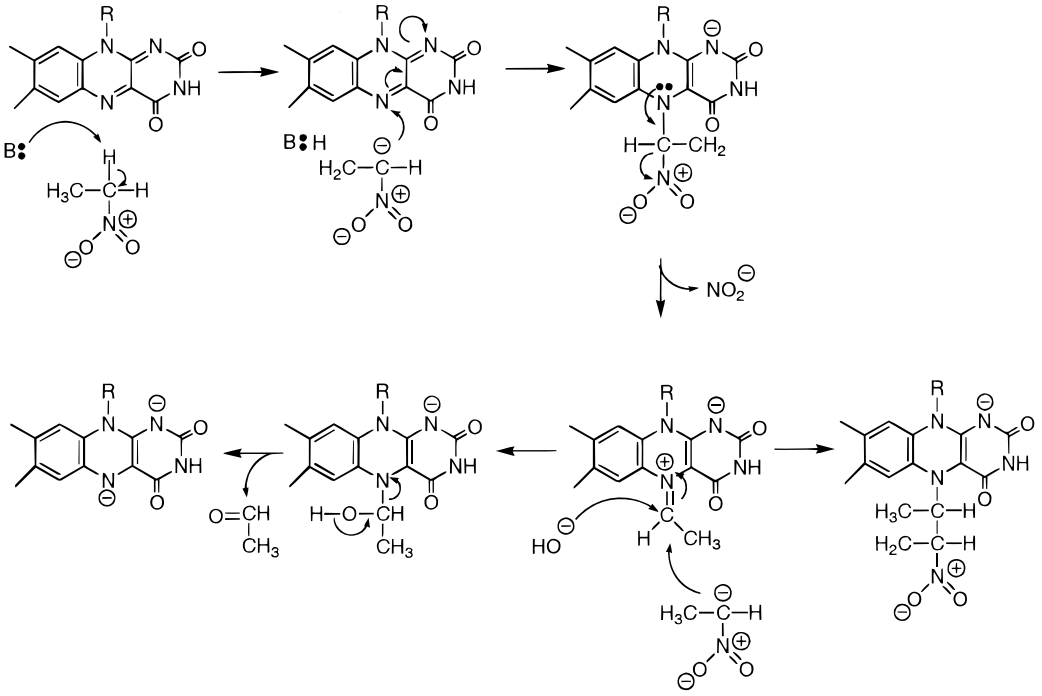
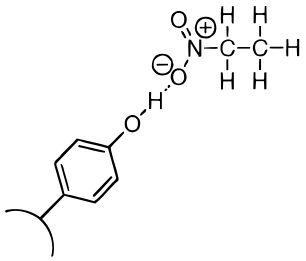


Chart 1



free 3-nitrotyrosine (Figure 4), suggesting that the target of nitration is a tyrosine residue. The amino acid sequence of the peptide eluting at 62 min was determined by automated Edman degradation analysis (Table 1). The amino acid residue eluting at cycle 8 in the automated sequence analysis of this peptide was identified as 3-nitrotyrosine by comparison of the retention time of the Pth-amino acid with that of a Pth-3-nitrotyrosine standard (Figure 5). As shown in Table 1, the amino acid sequence of the peptide eluting at 64.5 min in valerate-protected enzyme confirmed the sequence determination of the nitrated peptide and showed that a tyrosine residue is present at position 8.

DISCUSSION

The mechanisms by which carbanions are formed by enzymes have been a fundamental problem in enzymology, because of the high energetic barrier associated with this process. Pyridoxal- and thiamin-dependent enzymes lower this energetic barrier by derivatizing the substrate. However, flavoproteins must necessarily use a different strategy, since the oxidation is carried out on the underivatized substrate (*19*). Although recent models involving low-barrier hydrogen bonds provide an attractive explanation (*20*, *21*), the mechanisms by which the pK_a of the substrate is sufficiently lowered for bond cleavage are still not understood. The pK_a values for nitroalkanes are below 12 (*22*), so that little activation of the substrate is required for proton removal. For this reason, nitroalkanes have been extensively studied as model systems for the formation of carbanions in solution (*5*). These studies provide the basis for understanding the enzyme-catalyzed formation of carbanions involving much weaker carbon acids, such as α-amino acids or α-hydroxy acids. However, caution should be exerted in extrapolating results obtained in solution with nitroalkanes to enzyme-catalyzed reactions involving amino acids and hydroxy acids. An enzyme that catalyzes the cleavage of the carbon−hydrogen bond of nitroalkanes offers the unique opportunity to compare the enzyme-catalyzed formation of nitronates with the reaction in solution.

Among the flavoproteins that can catalyze the oxidation of nitroalkanes (*10*−*13*), nitroalkane oxidase is unique in that it requires the neutral form of the substrate for catalysis (*14*). The enzyme was first described by the group of Soda in 1978 as a colorless flavoprotein that catalyzes the oxidation of nitroalkanes to the corresponding aldehydes with concomitant production of hydrogen peroxide and nitrite (*15*). Recently, our group has shown that the cofactor in the enzyme as purified is in the form of a 5-nitrobutylflavin when nitroethane is used as the sole carbon source for cell growth (*6*, *7*). The mechanism of formation of the nitrobutyl−flavin adduct has been elucidated and is consistent with a mechanism for catalysis in which a base on the enzyme abstracts a proton from the α-carbon of the substrate to initiate catalysis, as shown in Scheme 2 (*7*). During the normal course of catalysis, the carbanion can attack the N(5) position of the flavin to form a covalent adduct. This adduct can lose nitrite to form an electrophilic intermediate. Attack of hydroxide is followed by loss of the aldehyde product to form the free reduced flavin. Alternatively, the highly reactive electrophilic intermediate can be intercepted by nitroethane anion preformed in solution to form the stable and inactive 5-nitrobutylflavin.

Despite the 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 nitroalkane oxidase.[1] Thus, we have used chemical modification to study the active site of the FAD-containing form of nitroalkane oxidase. The tyrosine-directed reagent tetranitromethane (*16*) was chosen for this study because nitroalkane oxidase has affinity for substrates containing a nitro moiety. The results presented here show that nitroalkane oxidase is irreversibly inactivated by tetranitromethane. The inactivation is active-site-directed, as indicated by the protection from inactivation afforded by the competitive inhibitor valerate. Evidence for an active-site-directed inactivation also comes from the saturation kinetics observed at increasing concentrations of tetranitromethane, consistent with the formation of a reversible enzyme−tetranitromethane complex before irreversible inactivation occurs. The increased intensity of the absorbance in the 300−400 nm region of the UV−visible absorbance spectrum of the inactivated enzyme after gel filtration indicates that the inactivation involves covalent attachment of the inhibitor. Although two cysteine residues are nitrated by tetranitromethane, these residues are not directly involved in the inactivation of the enzyme. Two free thiols react with 5,5′-dithiobis(2-nitrobenzoic acid) under denaturing conditions after treatment with tetranitromethane, irrespective of the presence of valerate. Nitroalkane oxidase contains four cysteine residues (*23*); previous titrations of the enzyme with 5,5′-dithiobis(2-nitrobenzoic acid) under nondenaturing conditions established that two cysteines are located on the surface of the enzyme (*23*). A likely explanation for these results is that the two cysteine residues on the surface of the enzyme have been nitrated and that these two residues are not in the active site.

The tryptic digests of nitroalkane oxidase treated with tetranitromethane in both the presence and absence of the competitive inhibitor valerate showed that one peptide was differentially labeled by tetranitromethane in the inactivated enzyme. Both the absorbance spectrum of the nitrated peptide and the direct identification of the nitrated Pth-amino acid indicate that the site of nitration is the tyrosine residue at position 8. No direct match of the sequence of the nitrated peptide can be found in the available partial gene sequence of nitroalkane oxidase,[1] suggesting that the nitrated tyrosine is located in the C-terminal half of the protein.

The active sites of a number of flavoproteins with carboxylate-containing substrates, such as D-amino acid oxidase (*24*), flavocytochrome b_2 (*25*), *p*-hydroxybenzoate hydroxylase (*26*), and glycolate oxidase (*27*), contain a tyrosine residue that forms a hydrogen bond with the carboxylate moiety of the substrate. Mechanistic studies on mutant enzymes in which these active-site tyrosines have been replaced with different amino acids are consistent with the structural studies (*28*−*31*). Given the structural similarity of the carboxylate and nitro groups, it is possible that the tyrosine residue of nitroalkane oxidase identified in this study is involved in binding the nitro group of the substrate (Chart 1). This conclusion is supported by the observation that a reversible enzyme−tetranitromethane complex is formed before covalent inactivation by tetranitromethane occurs. Steady-state kinetic studies of nitroalkane oxidase with nitroethane show that a protein group with a pK_a value of 9.5 must be protonated for substrate binding;[2] the tyrosine identified in this study is a reasonable candidate for this residue.

In conclusion, the chemical modification studies with tetranitromethane presented herein show that a tyrosine residue, likely to be essential for substrate binding, is present in the active site of nitroalkane oxidase. This study represents the first instance in which the location of an active site residue essential for catalysis has been shown for nitroalkane oxidase. These results are a prerequisite to future mutagenesis studies aimed at a better understanding of the catalytic mechanism of this enzyme.

ACKNOWLEDGMENT

We thank Dr. Lawrence Dangott at the Protein Chemistry Laboratory of Texas A&M University for the amino acid sequence determinations.

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BI9921743