Biochemical and Physical Characterization of the Active FAD-Containing Form of Nitroalkane Oxidase from *Fusarium oxysporum*[†,‡]

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ABSTRACT: Nitroalkane oxidase from *Fusarium oxysporum* catalyzes the oxidation of nitroalkanes to aldehydes with production of nitrite and hydrogen peroxide. The enzyme has a molecular weight of 47 955 ± 39, as determined by MALDI-TOF mass spectrometry; under nondenaturing conditions, the aggregation state of the enzyme is best described by a tetramer−dimer self-associating model, with an association constant of $(8.5 \pm 4.4) \times 10^6$ M^{-1} (pH 7.0 and 4 °C). The amino acid composition and the N-terminal amino acid sequence do not match any known protein or open reading frame. The inactive 5-nitrobutyl-1,5-dihydroflavin found in the enzyme as purified was converted to FAD, allowing characterization of the active FAD-containing enzyme. With nitroethane as substrate, the V_{max} and K_m values are 655 ± 45 min^{-1} and 2.9 ± 0.5 mM at pH 8.0 and 30 °C, respectively. One mole of FAD per mole of monomer enzyme is required for catalysis. No activity can be detected with amino acids or α-hydroxy acids as substrates. Reversible removal of the FAD cofactor yields inactive enzyme. The properties of the FAD cofactor in nitroalkane oxidase are within the range described for other oxidases. The UV−visible absorbance spectrum of the active enzyme shows maxima at 446, 384, and 274 nm; the extinction coefficient at 446 nm is 11.7 mM^{-1} cm^{-1}. The neutral form of the flavin semiquinone, with maxima at 536 and 342 nm, is kinetically stabilized. The UV−visible absorbance spectrum of the reduced enzyme is typical of the anionic form of a flavin, with a peak centered at 335 nm. The affinity of the enzyme for sulfite is low (K_d value of 13.8 ± 0.9 mM at pH 7.0 and 25 °C); this result, along with the stabilization of the neutral flavin semiquinone, suggests the presence of a weak positive charge near the N(1)−C(2)═O of FAD. The reduction potential of the enzyme is −367 mV. Benzoate and phenylacetic acid are competitive inhibitors, with K_{is} values of 5.1 ± 0.6 and 13.1 ± 2.3 mM, respectively. Binding of benzoate to nitroalkane oxidase results in spectral changes similar to those observed with D-amino acid oxidase. The absorbance spectrum of the flavin bound to nitroalkane oxidase is pH-dependent, with a pK_a value of 8.4.

Flavoprotein oxidases catalyze the removal of a hydride equivalent from a substrate, transferring electrons initially to the flavin cofactor and then to molecular oxygen to form hydrogen peroxide and the oxidized product. A great deal of study has been directed toward understanding how the hydride equivalent is removed from the substrate. Three different mechanisms are generally accepted for the various oxidases. A carbanion mechanism has been proposed for the α-hydroxy acid oxidases, which comprise lactate oxidase (*1*), glycolate oxidase (*2*), and flavocytochrome b_2 (*3*). With these enzymes, there is strong evidence that the first step in catalysis is the removal of the substrate α-proton to yield the corresponding carbanion (for a review, see *4*). In contrast, both a hydride transfer and a radical mechanism have been proposed for the less characterized alcohol oxidase group of oxidases, which includes glucose oxidase, cholesterol oxidase, and methanol oxidase (*5*−*7*). Finally, monoamine oxidase is proposed to utilize a radical mechanism for substrate oxidation, either abstracting an electron from the amine to initiate catalysis (*8*) or removing a hydrogen atom from the α-carbon (*9*). This variety of mechanisms is remarkable in that the same cofactor, i.e., the isoalloxazine ring of either flavin adenine dinucleotide (FAD)[1] or FMN, is present in all of these enzymes. How the protein microenvironment modulates the reactivity of the flavin is not yet understood.

The flavoprotein nitroalkane oxidase from the plant pathogen *Fusarium oxysporum* (ATCC 695) was first described by Kido et al. (*10*). The enzyme catalyzes the oxidation of primary and secondary nitroalkanes to aldehydes or ketones, with concomitant production of hydrogen peroxide and nitrite (Scheme 1). It was initially reported that

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[1] Abbreviations: FAD, flavin adenine dinucleotide; 5-deaza-FAD, 5-deazaflavin adenine dinucleotide; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; HPLC, high-pressure liquid chromatography.

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Scheme 1

$$RCH_2NO_2 + O_2 + H_2O \longrightarrow RCHO + HNO_2 + H_2O_2$$

the enzyme was colorless, but required added flavin for activity (*10*). We recently showed that the enzyme as purified contains FAD in the form of a 5-nitrobutyl-1,5-dihydroflavin and is not active; the mechanism of formation of this N(5)-adduct has been reported and is consistent with a carbanion mechanism (*11*−*12*). The nitrobutylflavin can be converted to FAD, yielding active enzyme, consistent with a requirement for FAD in catalysis (*11*).

While other flavoprotein oxidases have been reported to oxidize nitroalkanes (*13*), these all require the nitroalkane anion. In contrast, nitroalkane oxidase uses the neutral form of the substrate for catalysis (*11, 14*). This suggests that the physiological role of nitroalkane oxidase is to oxidize nitroalkanes. In support of such a conclusion, this enzyme is produced in high yield when nitroethane is the sole source of carbon during the growth of the fungal cells (*10*). It is well documented that naturally occurring nitrocompounds often possess toxic activity against bacteria and fungi (for a review, see *15*). Thus, one can speculate that the physiological role of nitroalkane oxidase is conversion of these antibiotics into less harmful compounds, inactivating the natural defense of host organisms. In addition to the agricultural and clinical rationale that this activity provides for studying nitroalkane oxidase, the oxidation of neutral nitroalkanes provides the potential for understanding the formation of carbanion intermediates by enzymes. The formation of nitroalkane anions in solution is a well-characterized chemical reaction (*16*); study of the mechanism of nitroalkane oxidase provides the unique opportunity to compare enzyme-catalyzed formation of carbanions with the identical nonenzymatic reaction.

As a prerequisite for detailed mechanistic studies, a thorough investigation of the general properties of the active form of the enzyme is necessary. We report here the characterization of the fully activated FAD form of nitroalkane oxidase.

MATERIALS AND METHODS

Materials. FAD, benzoate, glycine, D,L-lactate, glycolate, xanthine, xanthine oxidase, benzyl viologen, methyl viologen, sodium dithionite, and sodium sulfite were from Sigma; D,L-alanine was from USB Corp.; nitroethane was from Aldrich; phenylacetic acid was from Fluka. All other reagents were of the highest purity commercially available. FAD from Sigma was purified by reverse phase HPLC according to Light et al. (*17*) using a Waters instrument equipped with a Lambda Max Model 481 detector set at 450 nm and a μ-Bondapack C-18 (0.39 × 150 mm) column (Waters). 5-Deaza-FAD was a generous gift from Dr. Vince Massey of the University of Michigan. Nitroalkane oxidase was purified from *Fusarium oxysporum* (ATCC 695) as previously described (*14, 18*). The enzyme as purified has the cofactor in the form of a 5-nitrobutyl-FAD and is not active (*11*). The activated FAD-containing form of nitroalkane oxidase was prepared according to Gadda et al. (*11*), except that the incubation in the presence of 0.3 mM FAD was allowed to proceed for 90−100 h to allow full conversion of the native flavin to FAD. This procedure gave a typical yield of 60−80%; the activated enzyme contained 95−100% of the flavin in the form of FAD, as judged by UV−visible spectroscopy. The enzyme was stored at −70 °C in the presence of 0.3 mM FAD to prevent formation of the less stable apoprotein. Excess FAD was removed by gel filtration using a Sephadex G-25 column (PD-10, Pharmacia) just before using the enzyme for spectral studies.

Assays. Enzyme concentrations were determined by the method of Bradford (*19*) using bovine serum albumin as standard. Enzyme activity was measured in the presence of 0.3 mM FAD in air-saturated 0.2 M Tris, pH 8.0, by monitoring the rate of oxygen consumption with a Yellow Spring Instrument Model 5300 Clark electrode thermostated at 30 °C. To minimize the amount of nitroethane anion in the assay mixture, assays were started by adding the substrate. When the enzyme activity was measured at pH 7.0, 0.1 M ACES, 52 mM Tris, 52 mM ethanolamine was used as buffer. Glycine, glycolate, D,L-lactate, D,L-alanine, and sodium benzoate were tested as substrates for nitroalkane oxidase at a concentration of 40 mM in air-saturated 0.2 M Tris, pH 8.0 at 30 °C. For determination of inhibition patterns, inhibitors were incubated with nitroalkane oxidase for 5 min in the assay mixture before starting the reaction by the addition of nitroethane (0.1−6.0 mM). Phenylacetic acid was prepared in 95% ethanol; the enzymatic activity of nitroalkane oxidase was not affected by the final concentration of ethanol (4%) used in the assay. A unit of enzymatic activity corresponds to the consumption of 1 μmol of oxygen/min.

UV−visible absorbance spectra were recorded using a Hewlett-Packard Model HP 8452A spectrophotometer equipped with a thermostated water bath. Fluorescence emission spectra were recorded with an SLM Model 8000 spectrofluorometer thermostated at 25 °C. For experiments involving anaerobiosis, the sample was placed in a 1-mL cell equipped with sidearms and made anaerobic by several cycles of evacuating and flushing with purified nitrogen obtained by passage through a column of BASF catalyst heated at 120 °C. The same cell was equipped with a gastight syringe for titrations with dithionite.

Methods. The apoprotein form of nitroalkane oxidase was prepared using a variation of the method developed by Massey and Curti (*20*). Activated enzyme (2−8 mL) was dialyzed against six 24-h changes of 500 mL of 2 M KBr, 1 mM EDTA, 20% glycerol, 5 mM 2-mercaptoethanol, 0.25 M potassium phosphate, pH 7.5, at 4 °C, followed by two changes of the same buffer devoid of KBr. The apoenzyme was stored at −70 °C and was stable for several weeks. The FAD dependence of the specific activity of apo-nitroalkane oxidase was determined in 0.2 M Tris, pH 8.0, at 30 °C with 2 mM nitroethane. Different amounts of FAD (1.2−97 μM) were incubated for 5 min with 0.07 μM apoprotein in the assay mixture before starting the reaction by addition of substrate. The affinity of the enzyme for FAD was studied by ultrafiltration in 1 mM EDTA, 20% glycerol, 5 mM 2-mercaptoethanol, 0.25 M potassium phosphate, pH 7.5, at 4 °C; 4 μM apoprotein was mixed with 10−20 μM FAD and filtered through a Centricon 30 (Amicon); the concentration of free FAD in the ultrafiltrate was determined spectrophotometrically.

The extinction coefficient of FAD bound to nitroalkane oxidase was determined in 1 mM EDTA, 25 mM potassium

phosphate, pH 7.0, from the change in absorbance following the release of FAD after heating the sample at 96−100 °C for 30 min. The precipitated protein was removed by centrifugation for 10 min at 14000*g*, and the UV−visible absorbance spectrum of the supernatant was recorded; an ϵ_{450} value of 11.3 mM^{-1} cm^{-1} was used for free FAD (*21*).

The molecular mass of the enzyme was determined by MALDI-TOF mass spectrometry, using a Voyager Elite XL mass spectrometer (PerSpective Biosystems, Framingham, MA) equipped with delay extraction. Activated nitroalkane oxidase (∼0.6 mg) was dialyzed against two 500-mL changes of 5 mM ammonium acetate, pH 7.0, at 4 °C and concentrated to a final volume of 0.13 mL using a Savant DNA SpeedVac Model DNA100. Samples were then analyzed in the Laboratory for Biological Mass Spectrometry (Department of Chemistry, Texas A&M University). MALDI-TOF spectra were acquired in the positive ion mode, using α-cyano-4-hydroxysuccinamic acid as the matrix. Samples were prepared for MALDI-TOF using the overlayer method of sample preparation previously described (*22*).

Sedimentation equilibrium ultracentrifugation was performed using a Beckman XL-A ultracentrifuge equipped with an Epon charcoal-filled 1.2 cm two-sector centerpiece cell. Protein samples (3.4−6.8 μM) in 25 mM potassium phosphate, 1 mM EDTA, 0.1 M NaCl, pH 7.0, at 4 °C, were centrifuged at 6000 rpm until an equilibrium distribution of protein in the sample cell was achieved (approximately 30 h). The absorbance at 280 nm was determined at each radial position with a resolution of 0.001 cm; 20 such scans were averaged. The analysis was repeated at 8000 and 10 000 rpm.

The amino acid composition and the N-terminal sequence of nitroalkane oxidase were determined at the Protein Chemistry Laboratory of Texas A&M University. 3,3′-Dithiodipropionic acid was added prior to hydrolysis in order to quantitate cysteine residues.

Reduction in the presence of nitroethane (1−35 mM) was performed in 0.1 M MOPS, pH 7.0, at 20 °C. Determination of the midpoint redox potential was done spectrophotometrically with the xanthine/xanthine oxidase system (*23*) in 0.1 M MOPS, pH 7.0, at 20 °C; benzyl viologen ($E_{m,7} = -359$ mV) was used as indicator dye in the determination. The enzyme and dye concentrations were typically 12−18 and 20−33 μM, respectively; 50−100 μM xanthine and 0.08 milliunit of xanthine oxidase were used. Reduction with sodium dithionite was conducted in 0.1 M MOPS, pH 7.0, at 20 °C. Dithionite was prepared just before use in a deaerated solution of 20 mM sodium pyrophosphate, pH 8.5. Stock solutions were titrated anaerobically with FAD in order to determine the actual concentration of dithionite.

The pH dependence of the absorbance spectrum was determined by titrating with sodium bicarbonate and potassium hydroxide a protein solution in 25 mM potassium phosphate, 1 mM EDTA, pH 7.0, at 25 °C. The rate of decay for the *N*(5)-sulfite adduct was determined spectrophotometrically at 20 °C after removing excess sulfite by gel filtration on a Sephadex G-25 column equilibrated in 0.1 M MOPS, pH 7.0, at 4 °C.

Data Analysis. Data were fit to eqs 1−6, using the Kaleidagraph software from Adelbeck Software (Reading, PA). Equation 1 was used for the determination of the steady-state kinetic parameters of the enzyme when the concentration of nitroethane was varied, for the determination of the K_m value for FAD, and for the analysis of the binding of ligands to the enzyme (sulfite, benzoate). V represents the turnover number or the maximal absorbance value at the wavelength of choice in kinetic and ligand binding studies, respectively; K_a is the Michaelis−Menten (K_m) or the dissociation constant (K_d) in kinetic and ligand binding experiments, respectively; and A represents the concentration of the varied substrate or ligand. Equation 2 describes a competitive inhibition process and was used for the determination of the inhibition constant (K_{is}) for benzoate and phenyl acetic acid. Equation 3 describes a single first-order reaction and was employed for the determination of the rates of formation and of dissociation of the *N*(5)-sulfite adduct; k represents the observed first-order rate constant of formation and of dissociation of the *N*(5)-sulfite adduct, A represents the amplitude of the absorbance change at 446 nm, and B is the absorbance value at 446 nm at the end of the process. Equation 4 was used to fit the dependence of the absorbance spectrum of nitroalkane oxidase as a function of pH; A and B represent the absorbance values at 466 nm at low and high pH values. Equations 5 and 6 were used for fitting sedimentation equilibrium ultracentrifugation data. Equation 5 describes a single sedimenting species model, and eq 6 describes a self-associating species model; A_o represents the absorbance of the monomer at the reference radius r_o, r is the radius, M is the monomer molecular weight, m and n are the stoichiometry for species 1 and 2, respectively, K_a is the association constant for the self-associating species 1−species 2 equilibrium, ω is the rotor angular velocity, R is the gas constant, T is the absolute temperature, ρ is the buffer density, ν is the partial specific volume of nitroalkane oxidase, and B is the base line offset. The buffer density (ρ) was calculated from standard tables (*24*), whereas the partial specific volume (ν) was estimated using the residue values from Cohn and Edsall (*25*) and the amino acid composition of nitroalkane oxidase.

$$v = \frac{VA}{K_a + A} \tag{1}$$

$$v = \frac{VA}{K_a(1+I/K_{is}) + A} \tag{2}$$

$$k = Ae^{-kt} + B \tag{3}$$

$$y = \frac{AH + BK}{H + K} \tag{4}$$

$$A = A_o \exp\left[\frac{\omega^2}{2RT} M(1 - \nu\rho)\,(r^2 - r_o^2)\right] \tag{5}$$

$$A = A_o m \exp\left[\left(\frac{\omega^2}{2RT}\right) mM(1 - \nu\rho)\,(r^2 - r_o^2)\right] + K_a A_o n \exp\left[\left(\frac{\omega^2}{2RT}\right) nM(1 - \nu\rho)\,(r^2 - r_o^2)\right] + B \tag{6}$$

RESULTS

Physical Properties of Nitroalkane Oxidase. The molecular weight of nitroalkane oxidase was determined by MALDI-TOF mass spectrometry. Two peaks with m/z^+ values of 47 995 and 23 958 were observed, corresponding

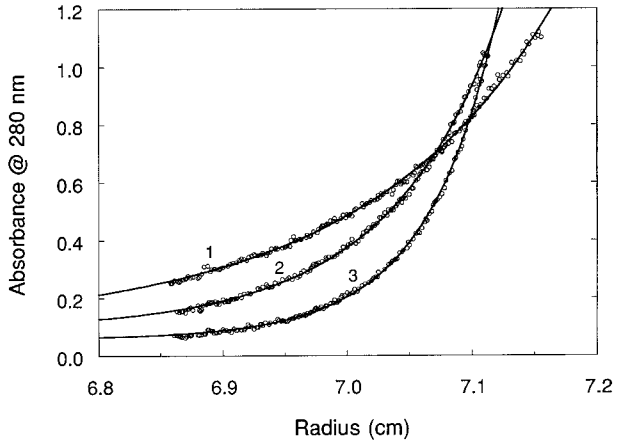

FIGURE 1: Equilibrium sedimentation analysis of native nitroalkane oxidase. Curve 1, native nitroalkane oxidase (6.8 μM) was centrifuged at 6000 rpm in 25 mM potassium phosphate, 1 mM EDTA, 0.1 M NaCl, pH 7.0, at 4 °C. After equilibrium was achieved (approximately 30 h), the absorbance at 280 nm was recorded with respect to radial position. The analysis was repeated at 8000 and 10 000 rpm. Curve 2, same sample after centrifugation at 8000 rpm. *Curve 3*, same sample after centrifugation at 10 000 rpm. The solid lines through the data represent the best fit to eq 6 for a self-associating species model describing an equilibrium between a dimer and a tetramer form of nitroalkane oxidase, where $K_a = 212$, and M is left floating.

to the singly and doubly charged species of the protein (data not shown); thus, a molecular weight of 47 955 ± 39 could be calculated for the apoprotein. Sedimentation equilibrium ultracentrifugation was used to determine the quaternary structure of the enzyme as isolated. When data collected at 6000−10 000 rpm were fit to a model describing a single species, the average molecular weight was 147 300 ± 7700. Significantly improved fits (e.g., $X^2 = 0.012$ versus 0.058 at 8000 rpm) were obtained when the data were fit to a model describing an equilibrium between a dimer and a tetramer. Models describing monomer−dimer, monomer−trimer, or monomer−tetramer equilibria did not yield equivalent improvements in the fit of the data. The average value of the association constant obtained at different rotor speeds using a dimer−tetramer model was $(8.5 \pm 4.4) \times 10^6$ M^{-1}. When the data were fit to eq 6 using this equilibrium constant, the calculated molecular weight of the monomer was 47 000 ± 1600, in excellent agreement with the results of the mass spectrometry analysis. Figure 1 shows the data and the fit to this model.

The amino acid composition of nitroalkane oxidase, determined after acid hydrolysis of the purified enzyme, is shown in Table 1. The number of tryptophan residues was calculated as 6 using the ϵ_{280} value of the apoprotein of 46.1 mM^{-1} cm^{-1} (vide infra) according to Pace et al. (*26*). Titration of the denatured enzyme after treatment with dithiothreitol gave a value of 4 cysteinyl residues per flavin (*18*). The relative molar amount of each amino acid residue was calculated setting the number of cysteinyl residues to this value; this gave a molecular weight for apoenzyme of 45 387. The calculated molar amino acid composition was used to search the databases using the search programs PROPSEARCH (*27*) and ExPASy; no significant matches were found with any known protein. The N-terminal amino acid sequence was VDFKL SPSQL EARRH AQAFA. This sequence was used to search the databases using the search programs TFASTA and BLASTP; no significant matches were found with any known protein or open reading frame.

Table 1: Amino Acid Composition of Nitroalkane Oxidase[a]

amino acid	relative molar amount (%)[b]	number of residues
Asx	7.70 ± 0.11	32.7 ± 0.5 (33)
Glx	12.04 ± 0.28	50.8 ± 1.2 (51)
Ser	7.43 ± 0.28	32.7 ± 1.2 (33)
His	2.42 ± 0.13	10.0 ± 0.5 (10)
Gly	6.35 ± 0.01	27.2 ± 0.04 (27)
Thr	6.62 ± 0.08	28.7 ± 0.3 (29)
Cys	0.98 ± 0.18	4.2 ± 0.8 (4)
Ala	10.44 ± 0.23	45.5 ± 1.0 (46)
Arg	4.79 ± 0.21	21.1 ± 0.9 (21)
Tyr	2.37 ± 0.24	10.9 ± 1.1 (11)
Val	6.47 ± 0.01	27.8 ± 0.03 (28)
Met	4.13 ± 0.11	17.3 ± 0.5 (17)
Phe	3.42 ± 0.01	14.7 ± 0.04 (15)
Ile	3.72 ± 0.03	15.9 ± 0.1 (16)
Leu	10.11 ± 0.06	43.2 ± 0.1 (43)
Lys	5.65 ± 0.25	23.4 ± 1.0 (23)
Pro	5.52 ± 0.22	23.0 ± 0.9 (23)
Trp	N. D.	5.8 (6)

[a] Each value represents the mean of two determinations after vapor-phase acid hydrolysis as described under Materials and Methods. Values were calculated assuming the number of Cys equal to 4 (*18*) and the molecular weight of nitroalkane oxidase equal to 47 955. Trp was calculated according to Pace et al. (*26*), using the value of ϵ_{280} for the apoprotein of 46.1 M^{-1} cm^{-1}. [b] Calculated from the observed picomoles. Total amounts of hydrolyzed protein analyzed in the two samples were 40 and 58 pmol, respectively.

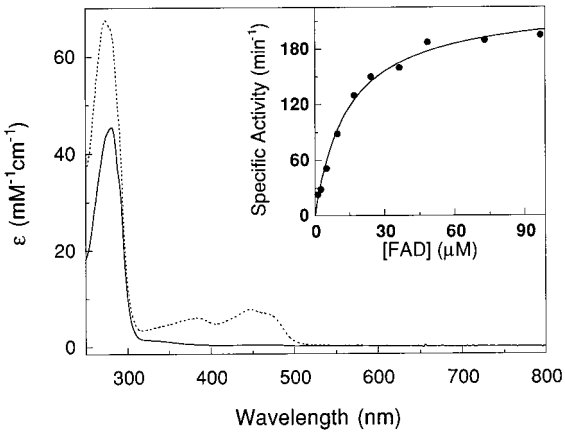

FIGURE 2: UV−visible absorbance spectrum of apo-nitroalkane oxidase and reconstituted holo-nitroalkane oxidase. Solid line, absorbance spectrum of the apoprotein form of nitroalkane oxidase in 0.25 M potassium phosphate, 1 mM EDTA, 20% glycerol, 5 mM 2-mercaptoethanol, pH 7.5, at 25 °C; dotted line, same sample after incubation with 0.5 mM FAD for 60 min on ice followed by gel filtration on Sephadex G-25 equilibrated with 10 mM potassium phosphate, 1 mM EDTA, pH 7.0. The spectrum of reconstituted holoprotein was normalized for the FAD content, based on $\epsilon_{446} = 11.67$ mM^{-1} cm^{-1}. Inset: dependence of the specific activity of apo-nitroalkane oxidase upon added FAD; experimental conditions as described under Materials and Methods.

Apoprotein Preparation and Determination of the Affinity for FAD. The apoprotein of nitroalkane oxidase was prepared by dialysis of the FAD-containing enzyme against 2 M KBr; the yield was typically 60−75%. When the native enzyme containing nitrobutylflavin was treated in the same way, no apoprotein was obtained. As shown in Figure 2, the absorbance spectrum of the apoprotein is devoid of any absorbance in the visible region, and the UV peak is centered at 280 nm. A value of 46.1 mM^{-1} cm^{-1} was determined

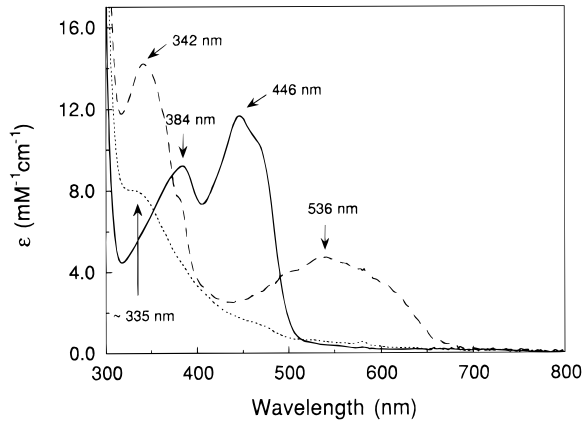

FIGURE 3: UV−visible absorbance spectrum of activated nitroalkane oxidase in the oxidized, semiquinone, and reduced state. Solid line, absorbance spectrum of oxidized nitroalkane oxidase in 25 mM potassium phosphate, 1 mM EDTA, pH 7.0, at 25 °C; dotted line, same sample after anaerobic reduction in the presence of 1 mM nitroethane; dashed line, absorbance spectrum of nitroalkane oxidase after 3.5 h incubation of the reduced enzyme with 1 mM nitroethane under anaerobiosis.

for the ϵ_{280} of the apoprotein. No fluorescence emission in the 530 nm region, typical of oxidized FAD, was detected (λ_{ex} 450 nm); the protein fluorescence emission was maximal at 340 nm (λ_{ex} 285 nm). A reconstituted holoprotein could be isolated by gel filtration after incubation of the apoprotein with 0.5 mM FAD at 0 °C; when 0.3 mM FAD was included in the assay, the specific activity of the reconstituted holoprotein was 112% that of the activated enzyme. The absorbance spectrum of the reconstituted enzyme (Figure 2) was indistinguishable from that of FAD-containing enzyme prepared directly from the native protein (vide infra).

The apoprotein was devoid of any activity when 6 mM nitroethane was used as substrate; when 0.1 mM FAD was included in the assay, the activity was restored, giving a specific activity of 354 min^{-1}, at pH 8.0 and 30 °C. The affinity of the enzyme for FAD was determined after mixing 4 μM apo-nitroalkane oxidase with 10−20 μM FAD; a K_d value of 1.4 ± 0.4 μM was found at pH 7.5 and 4 °C. When the affinity of apo-nitroalkane oxidase for the cofactor was determined kinetically at pH 8.0 and 30 °C by monitoring the rate of oxygen consumption at different concentrations of exogenous FAD, a K_m value for FAD of 14.4 ± 1.4 μM was found (Figure 2 inset). Accordingly, 0.3 mM FAD was included in the assay mixture for enzyme activity.

Activated FAD-containing enzyme was active when no added FAD was included in the assay; the activity traces in the absence of added FAD were linear only for a short time, consistent with release of FAD from the enzyme at the concentration of nitroalkane oxidase used in the assay (~0.3 μM). The specific activity of a sample containing 0.54 mol of FAD/mol of monomer of enzyme was 59% that of the same sample assayed in the presence of 0.3 mM added FAD, giving a stoichiometry of 1.09 mol of FAD/mol of monomer of enzyme for maximal activity.

Spectral Properties of FAD-Containing Nitroalkane Oxidase. The UV−visible absorbance spectrum of activated FAD-containing nitroalkane oxidase is shown in Figure 3. The absorbance maxima are centered at 446, 384, and 274 nm. After gel filtration to remove excess FAD, the stoichiometry of FAD/monomer for the activated enzyme (up to 48 μM) was in the range 0.6−0.9. The extinction coefficient at 446 nm was 11.7 mM^{-1} cm^{-1}. The ratio of the absorbances at 446 and 384 nm was 1.25. The fluorescence emission maximum was 530 nm (λ_{ex} 450 nm); the intensity was 2% that of free FAD.

When the FAD-containing enzyme was incubated anaerobically with 1 mM nitroethane, the spectrum of the fully reduced enzyme was immediately obtained (Figure 3). The single unresolved peak at 335 nm suggests that the N(1) position of the reduced flavin is unprotonated at pH 7.0 (*28*). An ϵ_{335} value of 8.0 mM^{-1} cm^{-1} was calculated for the reduced form of the enzyme. No flavin fluorescence emission was observed with the reduced enzyme, whereas the protein fluorescence emission was maximal at 342 nm (λ_{ex} 285 nm), as with the apoenzyme. Upon prolonged incubation of the reduced enzyme with nitroethane in the absence of oxygen, a species with maxima at 536 and 342 nm was formed (Figure 3). The extinction coefficient at 536 nm was 4.75 mM^{-1} cm^{-1}. An isosbestic at 395 nm was observed during formation of this long-wavelength absorbing species (results not shown). These spectral changes are consistent with formation of the neutral flavin semiquinone. The rate of the increase of absorbance at 536 nm was linearly dependent on the concentration of nitroethane over a range of 1−35 mM, with a rate constant of 0.28 mM^{-1} min^{-1} at 25 °C and pH 7.0. Upon readmission of oxygen, the absorbance spectrum of the oxidized flavin was observed; the specific activity of this sample was 94% the value of the starting material. When 28 μM FAD-containing enzyme was incubated anaerobically at pH 7.0 and 30 °C with 5 mM nitroethane in the presence of a 6-fold molar excess FAD, reduction of 25 μM FAD was immediately observed. This was followed by very slow bleaching of the flavin over several hours with a rate constant of of 0.001 min^{-1}. This stoichiometry of 0.9 FAD reduced does not support a model in which the enzyme-bound flavin can transfer electrons to FAD in solution at a catalytically relevant rate.

When dithionite was used as the reductant, the absorbance spectrum of the fully reduced flavin was immediately observed after a single addition of 2.06 electron equiv. No further spectral changes were observed over 3.5 h incubation of the reduced enzyme at 20 °C. However, when substoichiometric amounts of dithionite, i.e., ~0.1 electron equiv, were added successively at short intervals, an intermediate formed transiently after each addition. This species was characterized by increased absorbance at 342 and 536 nm and bleaching of the peak at 446 nm, consistent with formation of the neutral flavin semiquinone (Figure 4). Upon each addition of dithionite, formation of the semiquinone was maximal after 2−5 min; this species then slowly decayed (k_{obs} = 0.07 min^{-1}).[2] Sequential additions of dithionite were consequently made at 5 min intervals to maximize the amount of semiquinone formed. Upon readmission of oxygen, the absorbance spectrum of the oxidized flavin was observed. When 1 electron equiv of dithionite was added in a single step to the oxidized enzyme, the absorbance

[2] Maximal production of this blue flavin semiquinone was observed when successive substoichiometric additions of dithionite were made at ~5 min intervals, i.e., before the previous reaction was complete. Up to 70−80% of the theoretical amount of semiquinone was observed at pH 7.0 and 20 °C by using this procedure.

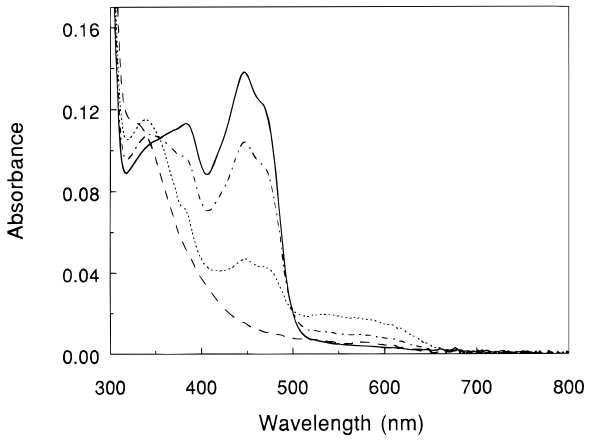

FIGURE 4: Reaction of activated nitroalkane oxidase with substoichiometric dithionite. Activated nitroalkane oxidase (11.73 μM in FAD content) in 0.1 M MOPS, pH 7.0 and 20 °C, was added anaerobically to substoichiometric amounts of dithionite (~2.4 μM for each addition). Each successive addition of dithionite was done 5 min after the previous one, i.e., before equilibrium was achieved and disproportionation fully occurred. Only selected spectra are shown. Solid line, oxidized enzyme; dashed−dotted line, same sample after total addition of 23.5 μM dithionite; dotted line, after total addition of 70.4 μM dithionite; dashed line, after total addition of 126.7 μM dithionite.

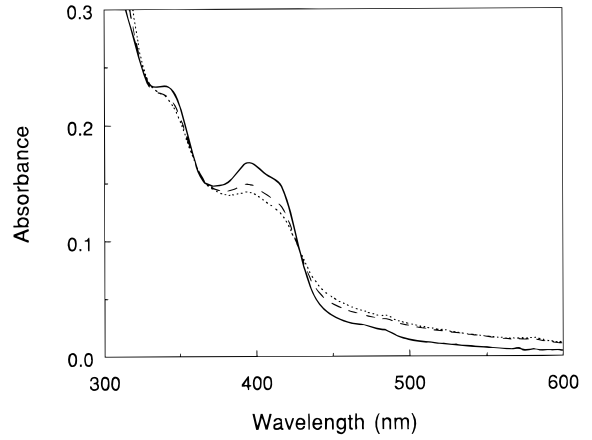

FIGURE 5: UV−visible absorbance spectrum of apo-nitroalkane oxidase in the presence of 5-deaza-FAD. Solid line, absorbance spectrum of the apoprotein form of nitroalkane oxidase (56.8 μM) in the presence of 5-deaza-FAD (19.4 μM) in 0.25 M potassium phosphate, 1 mM EDTA, 20% glycerol, 5 mM 2-mercaptoethanol, pH 7.5, at 15 °C; dashed line, same sample after addition of 2.5 mM nitroethane; dotted line, same sample after addition of 9.7 mM nitroethane.

spectrum observed was that of an equimolar mixture of reduced and oxidized enzyme. When an electron carrier, such as methyl or benzyl viologen, was present during a reduction with successive substoichiometric amounts of dithionite, no semiquinone was observed and the absorbance spectrum of the fully reduced flavin species was obtained directly (data not shown).

To examine further the role of the flavin in catalysis, the apoenzyme was reconstituted with 5-deaza-FAD (Figure 5). A shift to 396 nm of the flavin peak, as compared to 400 nm for unbound 5-deaza-FAD, and a better resolution of the absorbance spectrum in the 410−420 nm region were observed, establishing that the modified flavin bound to the protein. Nitroethane was added to the 5-deaza-FAD-containing nitroalkane oxidase in the absence of oxygen (Figure 5). In contrast to the results with the native FAD-containing

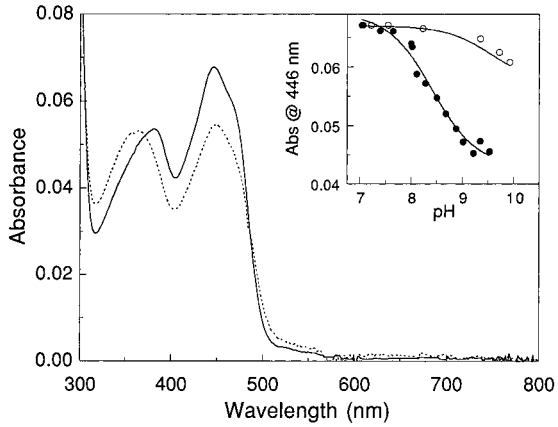

FIGURE 6: Dependence of the UV−visible absorbance spectrum of activated nitroalkane oxidase on pH. Solid line, absorbance spectrum of the enzyme (5.8 μM in FAD content) at pH 7.0; dotted line, same sample at pH 9.65. Both spectra were recorded in 25 mM potassium phosphate, 1 mM EDTA at 25 °C. Inset: pH titration of nitroalkane oxidase under the same experimental conditions both in the presence and in the absence of 40 mM sodium benzoate; solid circles, ligand-free enzyme; open circles, enzyme−benzoate complex.

enzyme, only a slight decrease in the spectrum of the flavin was observed. The peak at 396 nm decreased about 20% in the presence of 9.7 mM nitroethane; half-maximal bleaching at 396 nm was observed at 1.5 ± 0.002 mM nitroethane at pH 7.5 and 15 °C (data not shown). No oxygen consumption was detected at pH 7.0 and 30 °C when the 5-deaza-FAD-containing enzyme was tested with 6 mM nitroethane as substrate.

pH Dependence of the Absorbance Spectrum of FAD-Containing Nitroalkane Oxidase. The absorbance spectrum of FAD-containing nitroalkane oxidase showed a marked dependence on pH, as shown in Figure 6. At pH 7.0, the 446 nm peak was well resolved, whereas at pH 9.6 both the near-UV and the visible peaks were unresolved and shifted outward to 358 and 450 nm, respectively. A pH titration of the absorbance spectrum gave a pK_a value of 8.4 ± 0.1 for the spectral change (Figure 6, inset). On the basis of the extinction coefficient at pH 7.0, the extinction coefficient at 450 nm at pH 9.6 was calculated to be 9.04 mM^{-1} cm^{-1}. A likely candidate for the group responsible for this pK_a is the N(3) position of the FAD, which has a pK_a value of 10.4 in unbound FAD (*29*). Decreased absorbance is consistent with deprotonation of FAD at the N(3) position. In the presence of 40 mM benzoate, a competitive inhibitor of nitroalkane oxidase (vide infra), no substantial absorbance changes were observed up to pH 9.0 (Figure 6, inset). Denaturation of the enzyme ensued at pH values above 10.0, preventing the determination of the pK_a value of the N(3) position of FAD of the benzoate−enzyme complex. However, from visual inspection of the titration curve, a value in the range of free FAD can be estimated.

Formation of an N(5)-Adduct with Sulfite. The ability to form covalent N(5)-adducts with sulfite is a characteristic of flavoprotein oxidases (*30, 31*). Figure 7 shows the absorbance spectrum of nitroalkane oxidase after a single addition of 0.36 M sodium sulfite. Formation of an N(5)-adduct with sulfite is clearly established by the bleaching of the peak at 446 nm and the appearance of a new peak in the 320 nm region. The bleaching of the flavin spectrum was

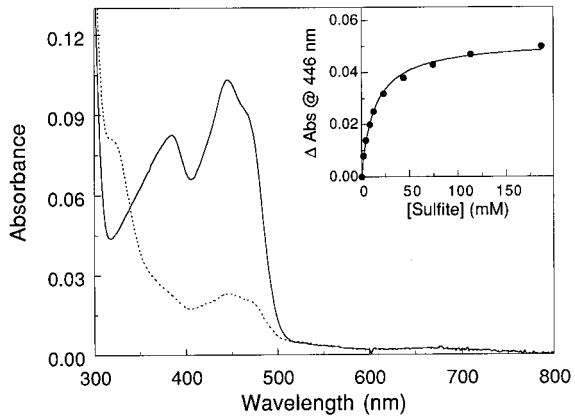

FIGURE 7: Binding of sulfite to activated nitroalkane oxidase. Activated enzyme was titrated with sulfite in 25 mM potassium phosphate, 1 mM EDTA, pH 7.0, at 25 °C. Solid line, enzyme (8.8 μM in FAD content) before the addition of sulfite; dotted line, same sample after a single addition of final 0.36 M sodium sulfite. Inset: sulfite titration of nitroalkane oxidase under the same experimental conditions.

quite slow (k_{obs} = 0.34 min^{-1} with 0.36 M sulfite at pH 7.0 and 25 °C); 20−25% oxidized flavin remained at the end of the process. A K_d value of 13.8 ± 0.9 mM was determined from the reaction with various concentrations of sulfite (Figure 7, inset). The reversibility of the sulfite adduct was studied by following the time-dependent reappearance of the absorbance spectrum of the oxidized flavin after removing excess sulfite by gel filtration. The rate of this process was 0.05 ± 0.003 min^{-1} at pH 7.0 and 20 °C; the absorbance spectrum of the oxidized FAD-containing enzyme was observed at the end of the process (data not shown).

Steady-State Kinetics with Nitroethane. Previous kinetic analyses of the unactivated enzyme gave K_m values for nitroethane and oxygen of 2.7 mM and 20 μM, and a V_{max} value of 30 min^{-1} at pH 8.0 and 30 °C (*14*). These parameters were redetermined in the presence of 0.3 mM FAD in air-saturated buffer with the fully active FAD-containing enzyme, yielding a V_{max} value of 655 ± 45 min^{-1} and a K_m value for nitroethane of 2.9 ± 0.5 mM. The activity traces were linear only for a short time, due to inactivation of the enzyme by the rapidly formed nitroethane anion (*11*). No oxygen consumption was observed when alanine, glycine, lactate, glycolate, phenylacetic acid, or benzoate was tested as a substrate under these conditions.

Binding of Benzoate and Phenylacetic Acid. The nitro and carboxyl groups are structurally similar. Accordingly, both benzoate and phenylacetic acid were tested as potential ligands of nitroalkane oxidase. As shown Figure 8A, the absorbance spectrum of nitroalkane oxidase was perturbed by benzoate. Both the peaks in the visible region of the absorbance spectrum were better resolved. The peak at 446 nm gained a shoulder at 478 nm; concomitantly, a bathochromic shift of the near-UV peak to 388 nm was observed (Figure 8A). The flavin fluorescence emission at 530 nm (λ_{ex} 450 nm) was quenched 50% upon binding of 18.7 mM benzoate to the enzyme at pH 7.0 and 20 °C. The difference spectrum obtained between the benzoate−enzyme complex and the FAD-containing nitroalkane oxidase is shown in the inset of Figure 8A. A K_d value for benzoate of 0.44 ± 0.01 mM was determined from a plot of the intensity at 478 nm vs the concentration of ligand at pH 7.0 and 10 °C (Figure 8B). Although the rate of binding was not investigated in detail, all the UV−visible spectral changes were completed within 5 min after the addition of benzoate. In steady-state kinetic assays at pH 8.0 and 30 °C, benzoate was a competitive inhibitor versus nitroethane, with a K_{is} value of 5.1 ± 0.6 mM. Phenylacetic acid was also found to be a competitive inhibitor, with a K_{is} value of 13.1 ± 2.3 mM at pH 8.0 and 30 °C (data not shown).

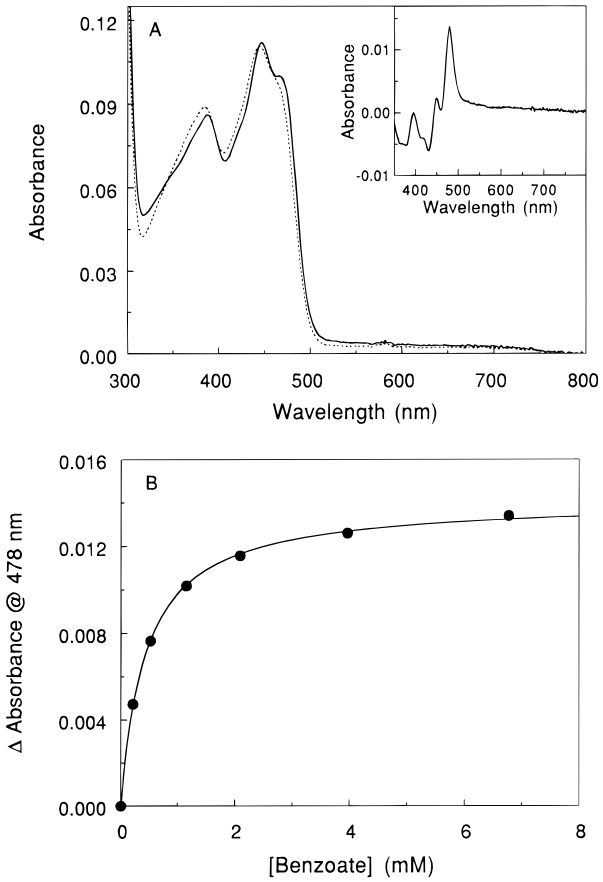

FIGURE 8: Binding of benzoate to activated nitroalkane oxidase. Activated enzyme was titrated with benzoate in 25 mM potassium phosphate, 1 mM EDTA, pH 7.0, at 10 °C. (A) Nitroalkane oxidase (9.4 μM in FAD content) before (dotted line) and after (solid line) the addition of 6.8 mM benzoate. Inset: difference spectrum after addition of 6.8 mM benzoate. (B) Benzoate titration of nitroalkane oxidase under the same experimental conditions.

Determination of the Reduction Potential of FAD-Containing Nitroalkane Oxidase. The reduction potential of nitroalkane oxidase was determined spectrophotometrically at pH 7.0 and 20 °C by using the xanthine/xanthine oxidase system (*23*). Benzyl viologen ($E_{m,7}$ = −359 mV) was used as the indicator dye. Both the bound flavin and the dye were reduced concomitantly (Figure 9). From the slope of a plot of log $([ox]/[red])_{dye}$ vs log $([ox]/[red])_{enzyme}$, a reduction potential of −367 ± 7 mV could be calculated (Figure 9, inset).[3]

[3] According to Minnaert (*32*), a linear slope of 2 should be observed in a plot of a single redox center undergoing two-electron reduction vs an indicator dye undergoing one-electron reduction; instead, a slope of 1 was observed with nitroalkane oxidase. A similar behavior has been reported for mercuric ion reductase, and a two-site model has been proposed on the basis of the results (*33*). The unusual redox behavior of nitroalkane oxidase has not been investigated further at this time.

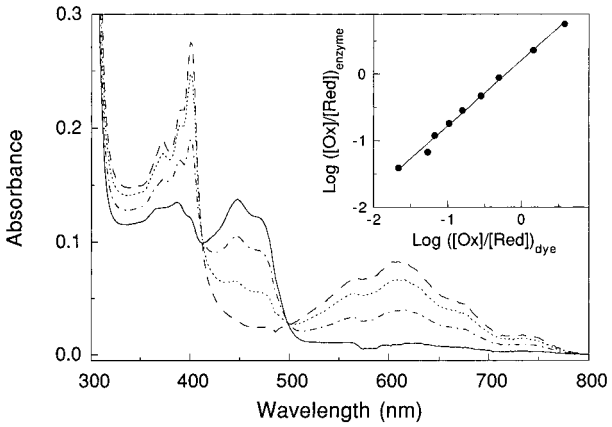

FIGURE 9: Midpoint redox potential determination of activated nitroalkane oxidase. Activated nitroalkane oxidase (11.8 μM in FAD content) was incubated anaerobically with 19 μM benzyl viologen in the presence 0.1 mM xanthine, 0.1 μM xanthine oxidase in 25 mM potassium phosphate, 1 mM EDTA, pH 7.0 at 20 °C. Solid line, spectra at time 0; dashed−dotted line, spectrum after 20 min; dotted line, spectrum after 40 min; dashed line, spectrum after 200 min. Inset: plot of log $([ox]/[red])_{\text{nitroalkane oxidase}}$ vs log $([ox]/[red])_{\text{benzyl viologen}}$.

DISCUSSION

The mechanisms by which enzymes cleave carbon−hydrogen bonds have been a fundamental problem in enzymology. Systems in which the bond is cleaved by removal of a proton are especially intriguing. In many instances, the pK_a of the proton to be removed is close to 30, while the amino acid residue which acts as the base has a pK_a less than 10. One strategy to deal with the energetic barrier which this difference in pK_a values presents is to derivatize the substrate, decreasing the pK_a. Thiamin- and pyridoxal-dependent enzymes utilize such a strategy. The flavoprotein oxidases represent a separate group of enzymes in which a proton is proposed to be removed from an underivatized substrate (*34*). The mechanism by which the substrate pK_a is decreased sufficiently for bond cleavage is unknown, although recent models involving strong hydrogen bonds provide an attractive explanation (*35*−*36*).

Model studies of carbanions formed by cleavage of carbon−hydrogen bonds have necessarily been limited to compounds with pK_a values significantly less than 30. The most-studied model system has been the formation of nitroalkane anions (*16*). Extensive studies of this reaction produced a great deal of experimental data regarding the effects of substituents on both rates and equilibria. As a consequence, the understanding of proton abstraction from a carbon provided by the nitroalkane anion system has served as a basis for understanding enzyme-catalyzed reactions involving much weaker carbon acids. Still, there must be some concern in extrapolating results obtained in solution with nitroalkanes to enzyme-catalyzed reactions involving amino and hydroxy acids. An enzyme which catalyzes cleavage of carbon−hydrogen bonds in nitroalkanes would resolve this quandary, allowing direct study of formation of nitroalkane anions by an enzyme and comparison with solution studies.

The enzyme nitroalkane oxidase from *Fusarium oxysporum* was first described as a colorless flavoprotein requiring added FAD for activity (*10*). High levels of the enzyme are present upon growth of the fungus on nitroethane as a carbon source, suggesting that the physiological role of this enzyme is nitroalkane oxidation. While both D-amino acid oxidase and glucose oxidase had previously been reported to oxidize nitroalkanes to aldehydes, in both cases this was a side reaction requiring the anion. In contrast, nitroalkane oxidase utilizes the neutral nitroalkane as substrate (*11*, *14*). The initial report that nitroalkane oxidase is a colorless flavoprotein was extended by the observation that the enzyme contains FAD, but that the cofactor is in a formally reduced state (*37*). These results suggested that this was a very unusual enzyme, and that its study might not be relevant to understanding the many hydroxy and amino acid oxidases. However, we have recently shown that the formally reduced cofactor in freshly purified nitroalkane oxidase is a 5-nitrobutyl-1,5-dihydroflavin (*11*). This form is not active, but will slowly decay to the active FAD. Moreover, the most likely mechanism for formation of the nitrobutylflavin is consistent with a catalytic mechanism involving a carbanion intermediate (*11*).

Consequently, nitroalkane oxidase would appear to present an attractive system for understanding the mechanism of carbanion formation by flavoprotein oxidases and by enzymes in general. The previous report that the flavin in the enzyme is unique has now been resolved. To further establish the validity of this as a model system, it is necessary to show that nitroalkane oxidase resembles other flavoprotein oxidases in the general properties of the flavin cofactor. In addition, given that other enzymes have been shown to oxidize nitroalkanes adventitiously, further confirmation that this is not a previously described enzyme is desired. We report herein a characterization of the physical and biochemical properties of fully activated FAD-containing nitroalkane oxidase.

Reversible removal of the FAD cofactor allows a direct test of the requirement of the flavin for catalysis by nitroalkane oxidase. While efforts to remove the nitrobutylflavin directly were unsuccessful, it was possible to resolve the apoenzyme by dialysis of the active FAD-containing enzyme against 2 M KBr, a method developed for D-amino acid oxidase (*20*). The apoenzyme was devoid of activity; the ability of FAD to restore completely the activity establishes the need for the cofactor for activity. One mole of FAD per mole of monomer of enzyme is required to confer catalytic ability to nitroalkane oxidase, since the specific activity of the FAD-containing enzyme when no added FAD was included in the assay agrees well with the FAD content. The stoichiometry of 1 mole of FAD reduced/mol of monomer of enzyme observed in the presence of excess FAD is further and independent evidence for the requirement of one flavin for catalysis. Thus, by these measures, nitroalkane is a typical flavoprotein oxidase.

In the initial kinetic characterization of nitroalkane oxidase, different nitroalkanes were tested as substrates and a steady-state ping-pong kinetic mechanism was determined (*14*). However, at the time of that report the nature of the flavin in the enzyme as purified had not been determined (*11*). Thus, we redetermined the steady-state kinetic parameters using nitroethane as substrate for the FAD-containing enzyme. The K_m value of 2.9 mM for nitroethane is in agreement with the previously reported values of 2.7 mM (*14*). In contrast, the turnover number was 20 times higher than that previously reported. The low activity previously

reported for the unactivated enzyme can be attributed to a small amount of the FAD-containing enzyme in those preparations. The substrate specificity was examined because of the similarities of this enzyme to other oxidases. Nitroalkane oxidase is not active on amino acids or α-hydroxy acids, based on the lack of oxygen consumption when alanine, glycine, lactate, glycolate, and phenyl acetic acid were tested as substrates.

The ability of D-amino acid oxidase (*38*) and glucose oxidase (*13*) to oxidize nitroalkane anions raises the possibility that nitroalkane oxidation of similar compounds is an adventitious activity of an unidentified protein. The ability to utilize neutral nitroalkanes as substrates (*11*, *14)* and the induction of the enzyme by nitroethane (*10*) suggest that nitroalkane oxidation is the physiological role of this enzyme. The amino acid composition and N-terminal sequence reported here provide further evidence that this enzyme has not been described previously with another activity.

The properties of the FAD in nitroalkane oxidase are well within the range described for other oxidases. The visible spectrum of the FAD-containing enzyme is well resolved, with maxima at 446, 384, and 274 nm. This increased resolution has previously been attributed to a hydrophobic microenvironment at the N(3) position of the flavin in other flavoproteins (*29*). The extinction coefficient of 11.7 mM^{-1} cm^{-1} at 446 nm for the bound FAD is similar to values reported for other flavoproteins. Nitroalkane oxidase stabilizes the neutral form of the flavin semiquinone. The extinction coefficient at 536 nm falls within the range observed with other flavoprotein neutral semiquinones (*39*). The only other reported cases of neutral semiquinones observed in the flavoprotein oxidase family are glucose oxidase at acidic pH values (*40*) and pig kidney D-amino acid oxidase in the presence of the active-site ligand benzoate (*41*). In these proteins, stabilization of the neutral semiquinone has been attributed to a hydrogen bond between an amino acid residue in the active site and the N(5) of oxidized FAD. Alternatively, an apolar microenvironment around the flavin would also stabilize the neutral semiquinone rather than the anionic species. This latter explanation is consistent with the increased resolution seen in the spectrum of the oxidized enzyme.

The UV−visible absorbance spectrum of the reduced enzyme is typical of the anionic form of a flavin, with a peak centered at 335 nm. In other flavoprotein oxidases, formation of the anionic reduced cofactor has been attributed to the presence of a (partial) positive charge close to the N(1)−C(2)═O of FAD which exerts a stabilizing effect by counterbalancing the negative charge developed on the isoalloxazine ring of the reduced flavin (*31*)

Formation of adducts with sulfite is a reaction which distinguishes oxidases from other flavoproteins (*31*). Formation of a flavin−sulfite adduct by flavoprotein oxidases has also been attributed to the presence of a positive charge near the N(1)−C(2)═O of the flavin (*31*). However, the affinity of nitroalkane oxidase is relatively weak. The low affinity for sulfite and the neutral semiquinone in nitroalkane oxidase suggests that any positive charge near this region of the FAD is weak, possibly due to an α-helix dipole, as in the cases of pig kidney D-amino acid oxidase (*30*, *42*) and cholesterol oxidase (*43*−*44*).

The neutral semiquinone of nitroalkane oxidase is stabilized kinetically rather than thermodynamically. Formation of the radical requires careful addition of substoichiometric amounts of dithionite. Once formed, the semiquinone disproportionates completely with a rate constant of about 0.07 min^{-1}. In the presence of single electron carriers such as benzyl viologen or methyl viologen or with increased amounts of dithionite, disproportionation is rapid, presumably due to the ability of the one-electron carriers to catalyze the reaction. The neutral semiquinone was also observed when the reduced enzyme was incubated in the absence of oxygen with an excess of nitroethane (Figure 3). A similar phenomenon has been reported for chorismate oxidase (*45*), although in that case the same process was observed after reduction of the enzyme with dithionite. Since the slow rate of this process indicates that it is not catalytically relevant, we did not investigate it further. However, the instability of the semiquinone is consistent with it not being involved in catalysis. Secondary isotope effects with D-amino acid oxidase and nitroethane anion have shown that nitroalkane anions react with the flavin in that enzyme by a nucleophilic mechanism rather than via a radical intermediate (*46*). The lack of a stabilized semiquinone suggests that there is similarly no radical intermediate in the reaction catalyzed by nitroalkane oxidase.

No catalytic activity was detected when the FAD was replaced with 5-deaza-FAD. This is similar to results seen with other flavoprotein oxidases. The limited spectral changes seen are consistent with binding of nitroethane to the 5-deaza-FAD-containing enzyme without subsequent reduction of the flavin. Alternatively, there may be only limited reduction of the flavin due to the negative value of the redox potential of the 5-deaza-FAD-containing enzyme.

The reduction potential of FAD is lowered by about 160 mV upon binding to nitroalkane oxidase; this value is unusually low compared to the values reported for other flavoprotein oxidases. It is comparable to that of flavodoxin from *Desulfovibrio vulgaris* (*47* and references therein) and to that of iodotyrosine deiodinase (*48*). In flavodoxin, both a negative electrostatic environment and aromatic stacking interactions lower the reduction potential of the bound flavin (*47*, *49*). A similar environment may be present in nitroalkane oxidase. The highly quenched fluorescence emission of the bound FAD is consistent with stacking of an aromatic residue over the flavin ring. Definitive elucidation of the factors responsible for the low reduction potential of the bound flavin will have to await the three-dimensional structure of the enzyme.

Due to the structural similarity of the nitro and carboxyl groups, we tested benzoate and phenylacetic acid as inhibitors of nitroalkane oxidase. Both are competitive inhibitors with K_{is} values in the millimolar range. This clearly establishes that both ligands bind to the active site of the enzyme. In addition, the UV−visible spectrum of nitroalkane oxidase was perturbed by the ligand, and a better resolution of the shoulder at 478 nm was observed. The difference spectrum between the benzoate complex and free enzyme is superimposable with that of the D-amino acid oxidase−benzoate complex (Figure 8 inset and *50*). However, binding is 300-fold weaker in the case of nitroalkane oxidase. Benzoate binding to the enzyme results in quenching of the flavin fluorescence. In the D-amino acid oxidase−benzoate com-

plex, benzoate is stacked over the flavin ring (*42*). A similar arrangement in nitroalkane oxidase would be consistent with quenching of the flavin fluorescence. Given the structural similarity of the carboxyl and nitro groups, it is reasonable that similar amino acid residues are involved in binding the substrate and the inhibitor. The known structures of flavoproteins whose substrates contain a carboxyl moiety, i.e., D-amino acid oxidase (*42*), glycolate oxidase (*2*), flavocytochrome b_2 (*51*), and *p*-hydroxybenzoate hydroxylase (*52*), suggest that an arginine residue and a tyrosine residue participate in the binding of the substrate carboxylate.

The absorbance spectrum of the flavin in nitroalkane oxidase is pH dependent. A likely candidate for the group responsible for this pK_a value is the N(3) position of FAD. The pK_a of 8.4 is significantly lower than the value of 10.4 found in the free flavin (*29*). In glycolate oxidase (*53*−*54*), a hydrogen bond between an amino acid residue in the active site and the C(4)═O position of the flavin causes a similar decrease in the flavin pK_a. The increase in this pK_a value when benzoate binds to nitroalkane oxidase suggests that the flavin must be uncharged for inhibitors and presumably substrates to bind. Studies of the effects of pH on the reaction of nitroalkane anions with D-amino acid oxidase show that the flavin must be uncharged for an adduct to form between the flavin and the carbanion (*46*). This adduct is considered a model for an intermediate in the oxidation of substrates by flavoprotein oxidases (*38*). The mechanism previously proposed for formation of the nitrobutyl flavin in nitroalkane oxidase also involves a nitroalkane−flavin adduct in catalysis (*11*). The requirement for a neutral flavin for catalysis by nitroalkane oxidase is fully consistent with these earlier results.

In conclusion, the FAD-containing active form of nitroalkane oxidase from *Fusarium oxysporum* shows similarities to other flavoprotein oxidases. This suggests it will prove a useful tool for the study of carbanion formation by this class of enzymes.

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