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Purification and Properties of Nitroalkane Oxidase from *Fusarium oxysporum*

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A nitroalkane-oxidizing enzyme, which was inducibly formed by addition of nitroethane to the medium, was purified to homogeneity from an extract of *Fusarium oxysporum* (IFO 5942) with an overall yield of about 20%. The enzyme catalyzed the oxidative denitrification of 1-nitropropane as follows: $CH_2(NO_2)CH_2CH_3 + O_2 + H_2O \rightarrow OHCCH_2CH_3 + HNO_2 + H_2O_2$. In addition to 1-nitropropane, 3-nitro-2-pentanol, 2-nitropropane, and nitrocyclohexane are good substrates; the enzyme is designated "nitroalkane oxidase" (EC class 1.7.3). The enzyme has a molecular weight of approximately 185,000 and consists of four subunits identical in molecular weight (47,000). Flavine adenine dinucleotide was required for the enzyme activity and could be replaced in part by riboflavine 5'-phosphate. The maximum reactivity was found at about pH 8.0. The enzyme was inhibited significantly by $HgCl_2$, KCN, *p*-chloromercuribenzoate, and *N*-ethylmaleimide. The Michaelis constants are as follows: 1-nitropropane, 1.54 mM; 2-nitropropane, 7.40 mM; nitroethane, 1.00 mM; 3-nitro-2-pentanol, 3.08 mM; nitrocyclohexane, 0.90 mM; and flavine adenine dinucleotide, 1.33 μM.

Various organic nitro compounds, including physiologically active substances, e.g., chloramphenicol, occur naturally (15, 16). The metabolism of nitro compounds, in particular, the enzymological aspect, is still obscure. In studies of the microbial assimilation of nitro compounds (7), we demonstrated that 2-nitropropane and some other nitroalkanes are oxidatively denitrified by several microorganisms, e.g., *Hansenula mrakii* and *Fusarium oxysporum* grown in a medium containing nitroethane as a nitrogen source, and also by an intracellular enzyme from them. We have purified a 2-nitropropane-oxidizing enzyme from *H. mrakii* to homogeneity to characterize it (8). The enzyme, which contains 1 mol of flavine adenine dinucleotide (FAD) and 1 g-atom of nonheme iron per mol of enzyme, catalyzes the following oxygenation of 2-nitropropane and is designated 2-nitropropane dioxygenase (EC class 1.13.11) (6): $2CH_3CH(NO_2)CH_3 + O_2 \rightarrow 2CH_3COCH_3 + 2HNO_2$.

In this paper, we describe the purification of a nitroalkane-oxidizing enzyme, designated "nitroalkane oxidase," from *F. oxysporum*, another assimilator of nitroalkanes, and some properties of the fungal enzyme.

MATERIALS AND METHODS

Chemicals. Diethylaminoethyl-cellulose was purchased from Serva, Heidelberg, Germany; Sephadex G-200 was from Pharmacia, Uppsala. Hydroxyapatite was prepared according to the method described previously (9). Catalase and peroxidase were purchased from Sigma Chemical Co., St. Louis, Mo.; 3-methyl-2-benzothiazolone hydrazone-hydrochloride, 3-nitropropionate, 3-nitro-2-butanol, 2-nitro-1-butanol, 3-nitro-2-pentanol, and nitrocyclohexane were from Aldrich Chemical Co., Inc., Milwaukee, Wis.; and FAD was from Yamasa Co., Choshi, Japan. The purity of FAD, riboflavine 5'-phosphate (FMN), and riboflavin was examined by paper chromatography, using the solvent systems *tert*-amyl alcohol-formic acid-water (3:1:1) and collidine saturated with water (14). The other chemicals were analytical-grade reagents and were obtained from Nakarai Chemicals, Kyoto, Japan.

Growth media, culture conditions, and mycelium production. *F. oxysporum* (IFO 5942) was cultured in a medium containing (per liter): nitroethane, 1.0 g; peptone, 5.0 g; glycerol, 5.0 g; KH_2PO_4, 2.0 g; K_2HPO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and yeast extract, 0.5 g (pH 5.5). Nitroethane was sterilized separately by filtration with a Seitz filter. *F. oxysporum* grown at 28°C for 48 h in a test tube containing 5 ml of the solution was inoculated, with shaking, in a 2-liter flask containing 700 ml of the medium and incubated at 28°C for 96 h on a reciprocating shaker. The harvested mycelium was washed twice with sterile 0.85% NaCl and transferred to a 2-liter flask containing 4 volumes of 0.2 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer (pH 8.0) and nitroethane (final concentration, 2%). After incubation at 28°C for 15 h with shaking, the mycelium was harvested and washed with the 0.85% NaCl solution.

Enzyme assay and analytical methods. The standard reaction mixture consisted of 50 μmol of 1-

nitropropane, 0.1 μmol of FAD, 200 μmol of Tris-hydrochloride buffer (pH 8.0), and enzyme in a final volume of 1.0 ml. Enzyme was replaced by water in a blank. The mixture was incubated aerobically at 37°C for 20 min with shaking. The reaction was terminated by addition of 0.1 ml of 1.0 M acetic acid. After centrifugation to remove precipitated protein when necessary, 0.1-ml samples of the supernatant solution were used for the determination of nitrite and propionaldehyde. Nitrite was determined according to the method of Ida and Morita (4). Propionaldehyde was determined spectrophotometrically with 3-methyl-2-benzothiazolone hydrazone-hydrochloride by the methods of Paz et al. (12). Absorption was measured with a Hitachi EPO-B photometer or a Carl Zeiss PMQ II spectrophotometer with a 1.0-cm light path.

One unit of enzyme was defined as the amount of enzyme that catalyzed the formation of 1 nmol of nitrite or propionaldehyde per min. Specific activity was expressed as units per milligram of protein.

Oxygen uptake was measured with a Gilson respirometer (G-14). The main compartment of a flask contained 125 μmol of 1-nitropropane, 0.3 μmol of FAD, and 500 μmol of Tris-hydrochloride buffer (pH 8.0) in a final volume of 2.0 ml. In the side arm, enzyme (80 μg) and 5 μmol of potassium phosphate buffer (pH 7.0) were placed to a final volume of 0.5 ml. After equilibration at 30°C for 10 min, the reaction was initiated by addition of enzyme solution, and readings were made over the next 1 h.

Hydrogen peroxide and protein were determined by methods described previously (8).

Molecular weight measurements. The molecular weight was estimated by gel filtration on a Sephadex G-200 column (1.5 by 125 cm) at 4°C, according to the method of Andrews (1). The column was calibrated with egg albumin (molecular weight, 43,000), bovine serum albumin dimer (136,000), lactate dehydrogenase of bovine heart (140,000), catalase of bovine liver (240,000), glutamate dehydrogenase of bovine liver (332,000), and blue dextran 2000.

The molecular weight of the enzyme subunit was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, according to Weber and Osborn (17).

RESULTS

Induction of enzyme. The mycelium of *F. oxysporum* grown in the absence of nitroethane showed only low activity of a nitroalkane-oxidizing enzyme. Figure 1 shows the growth of *F. oxysporum* in the medium containing nitroethane or peptone and nitroethane as a nitrogen source, the formation of nitrite in the culture fluid, and nitroalkane-oxidizing enzyme activity of the mycelium. The organism started growing after a short lag phase. The rate of formation of nitrite increased with an increase in the growth in the nitroethane medium, but nitrite was accumulated only in the phase of stationary multiplication in the nitroethane-peptone medium, in which mycelial yield was much higher. The mycelium grown in the nitroethane-peptone medium had a much lower specific activity than that grown in the nitroethane medium. When the mycelium grown for 96 h in the nitroethane-peptone medium was incubated subsequently with 2 or 3% nitroethane, the enzyme was produced inducibly, and the very high activity, very close to that of the mycelium grown in the nitroethane medium, was obtained after incubation for about 15 h. The inducibility of the mycelium harvested after 48 h of growth is comparatively low (Fig. 2).

Purification of enzyme. All operations were carried out at 0 to 5°C unless otherwise stated.

(i) Enzyme extraction. The washed mycelium (300 g, wet weight), grown and incubated with nitroethane as described in Materials and Methods, was ground thoroughly with two to three times the weight of levigated alumina in a mortar. The paste obtained was mixed with 10 mM potassium phosphate buffer (pH 7.0) to extract the enzyme, followed by centrifugation.

(ii) Ammonium sulfate fractionation. The supernatant solution (810 ml) was brought to 30% saturation (calculated at 25°C) with ammonium sulfate at pH 7.0, and the precipitate was removed by centrifugation. Ammonium sulfate was added to the supernatant solution (875 ml) to 60% saturation. The pH was kept at about 7.0 by addition of 3 N ammonia. The precipitate collected by centrifugation was dissolved in a small volume of 10 mM potassium phosphate buffer (pH 7.0) and dialyzed against the same buffer. The insoluble materials formed during dialysis were removed by centrifugation.

(iii) Diethylaminoethyl-cellulose column chromatography. The enzyme solution (86 ml) was chromatographed on a diethylaminoethyl-cellulose column (2 by 10 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0).

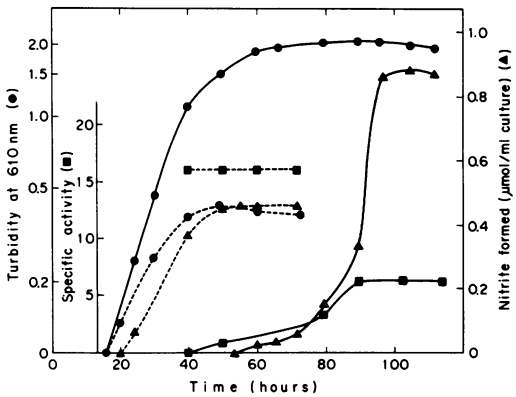

FIG. 1. *Time course of growth and nitrite formation. The organism was grown in nitroethane-peptone medium* (——) *and nitroethane medium* (-----).

After application of the enzyme and washing of the column with 1,000 ml of the buffer containing 0.05 M NaCl, the elution was carried out with the buffer containing 0.2 M NaCl. Fractions (5 ml) were collected at a flow rate of 50 ml/h. The fractions containing activity were precipitated by addition of ammonium sulfate (70% saturation) and dialyzed against 100 volumes of 1 mM potassium phosphate buffer (pH 7.0).

(iv) Hydroxyapatite column chromatography. The enzyme solution (55 ml) was placed on a hydroxyapatite column (2 by 8 cm) equilibrated with the above-mentioned buffer. The column was washed with 300 ml of 0.1 M potassium phosphate buffer (pH 7.0). The enzyme was eluted with 0.2 M potassium phosphate buffer (pH 7.0) at a flow rate of 15 ml/h. The fractions containing activity were concentrated by addition of ammonium sulfate (70% saturation) and dissolved in a small volume of 10 mM potassium phosphate buffer (pH 7.0).

(v) Sephadex G-200 column chromatography. The enzyme solution (2.5 ml) was applied to a Sephadex G-200 column (1.5 by 125 cm) buffered with 10 mM potassium phosphate buffer (pH 7.0) and eluted with the same buffer. The fractions containing activity were concentrated by addition of ammonium sulfate (70% saturation) and dissolved in a small volume of 10 mM potassium phosphate buffer (pH 7.0), followed by dialysis against the same buffer. A protocol for the purification procedure is presented in Table 1.

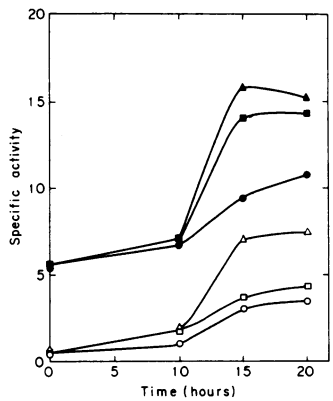

FIG. 2. *Time course of induction of enzyme. The mycelium, grown for 48 h in nitroethane-peptone medium, was incubated in the presence of 1%* (○), *2%* (△), *or 3%* (□) *nitroethane. Mycelium similarly grown for 96 h was also incubated in the presence of 1%* (●), *2%* (▲), *or 3%* (■) *nitroethane.*

Homogeneity of enzyme. Disc gel electrophoresis in 7.5% polyacrylamide gel was performed by a modification of the procedure of Davis (3) as described previously (8). The enzyme (70 μg) was applied on top of spacer gel in 1 M sucrose. The enzyme migrated toward the anode as a single band. The section of the unstained gel corresponding to the protein band was cut out and crushed in 10 mM potassium phosphate buffer (pH 7.0) to extract the protein. The protein exclusively contained the activity.

Homogeneity of the enzyme was also shown by ultracentrifugation. The enzyme (4.5 mg/ml) in 10 mM potassium phosphate buffer (pH 7.0) sedimented as a single, symmetrical peak during a sedimentation velocity run at top speed (56,100 rpm) and at 20°C. The homogeneous preparation of enzyme was used throughout in the following experiments.

Cofactor requirements. The cofactor requirements of the enzyme were investigated. The enzyme showed an absolute dependence on FAD, which was only partially satisfied (about 20%) by FMN at the same concentration (100 μM). Riboflavin had no effect on the activity.

Molecular weight of enzyme and subunit structure. The molecular weight of the enzyme was determined to be approximately 185,000 by Sephadex G-200 gel filtration. The subunit structure of the enzyme was examined by disc gel electrophoresis. The enzyme was incubated with 1.0% sodium dodecyl sulfate and 2-mercaptoethanol in 10 mM sodium phosphate buffer (pH 7.0) at 37°C for approximately 2 h. The treated enzyme preparation was subjected to electrophoresis in the presence of 1.0% sodium dodecyl sulfate. There was one band of stained protein. To determine the molecular weight of the polypeptide in this band, we ran a series of marker

TABLE 1. *Purification of nitroalkane oxidase*

	Step	Total vol (ml)	Total activity (units)	Protein (g)	Sp act	Yield (%)
(i)	Mycelium extract	810	26.2×10^3	1.62	16.2	100
(ii)	Ammonium sulfate fractionation (30-60% saturation)	86	15.9×10^3	0.30	52.4	65.7
(iii)	Diethylaminoethyl-cellulose chromatography	55	11.7×10^3	0.078	149	44.7
(iv)	Hydroxyapatite chromatography	2.5	7.59×10^3	0.025	310	29.0
(v)	Sephadex G-200 chromatography	10	5.37×10^3	0.013	460	20.4

proteins treated in the same manner; serum albumin (molecular weight, 68,000), catalase (58,000), pyruvate kinase (57,000), glutamate dehydrogenase (53,000) ovalbumin (43,000), alcohol dehydrogenase (41,000), lactate dehydrogenase (36,000), carboxypeptidase A (34,600), and chymotrypsinogen A (25,700). The molecular weight of the subunit was estimated to be approximately 47,000 from a semilogarithmic plot of molecular weight against mobility, suggesting that the enzyme consists of four subunits identical in molecular weight.

Stoichiometry of the reaction. The reaction mixture, containing 1-nitropropane as a substrate with or without catalase, was incubated in a Gilson respirometer flask with two side arms at 30°C for 60 min with shaking, and oxygen consumption was measured. After termination of the reaction by addition of 0.3 ml of 1 M acetic acid, the products were determined. The results indicated that propionaldehyde, nitrite, and hydrogen peroxide were formed stoichiometrically with a consumption of oxygen and 1-nitropropane (Table 2). Addition of catalase stimulated nitroalkane oxidase activity significantly.

Stability of the enzyme. The enzyme can be stored at −20°C in 1.0 or 10 mM potassium phosphate buffer (pH 7.0) for at least 3 months without loss of activity. When the enzyme was incubated in 10 mM potassium phosphate buffer (pH 7.0) at various temperatures for 5 min, the remaining activity was 100% of the original activity level at 40°C, 86.1% at 45°C, 72.5% at 50°C, 39.4% at 55°C, 9.3% at 60°C, and none at 65°C. The enzyme was found to be relatively stable between pH 6.0 and 10.0.

Effect of pH and buffers. The pH dependence for the enzyme activity was determined in various buffers. The enzyme was most active at pH 8.0 (Fig. 3). The activity varied with the kind of buffers used, and the relative activities in 0.2 M buffers (pH 8.0) were as follows: Tris-hydrochloride, 100; Tris-maleate, 81.4; and potassium phosphate, 65.7.

TABLE 2. *Stoichiometry of oxidation of 1-nitropropane*[a]

Expt	1-Nitropropane consumed (μmol)	Oxygen consumed (μmol)	Nitrite formed (μmol)	Propionaldehyde formed (μmol)	Hydrogen peroxide formed (μmol)
1	11.7	10.8	11.8	11.0	10.3
2	26.4	13.0	23.7	25.8	0

[a] The reaction mixture contained 50 μmol of 1-nitropropane, 0.3 μmol of FAD, 500 μmol of Tris-hydrochloride buffer (pH 8.0), and enzyme (550 μg) in a final volume of 2.5 ml (in a Warburg vessel) and was incubated at 37°C for 30 min. In experiment 2, 10,000 units of catalase was added to the reaction mixture. 1-Nitropropane was determined with a Shimadzu gas chromatograph (GC-4CM). The column (3.5 mm in ID by 1 m in length) was packed with Tenax GC (60 to 80 mesh) and operated at 130°C.

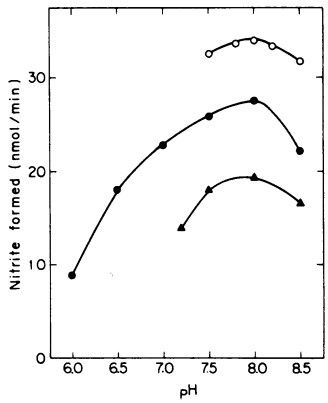

FIG. 3. *Effect of pH on nitroalkane oxidase activity. The enzyme was measured in the following buffers: Tris-hydrochloride* (○); *Tris-maleate* (●); *potassium phosphate* (▲).

Effect of temperature. The enzyme activity was assayed at various temperatures. The maximum activity was found at 40°C. The reaction velocity increased linearly when the temperature was raised in the range of 20 to 40°C and declined rapidly at over 50°C. The activation energy was calculated to be 14,000 cal (ca. 58.6 × 10^3 J)/mol from Arrhenius plot.

Substrate specificity. The ability of the enzyme to catalyze oxidation of various nitroalkanes was examined (Table 3). In addition to 3-nitro-2-pentanol, which is the preferred substrate, 1-nitropropane, 2-nitropropane, and nitrocyclohexane served as effective substrates, whereas nitromethane, 3-nitro-2-butanol, and 3-nitropropionate were oxidized slightly. Aromatic nitro compounds such as *p*-nitrobenzoic acid and *o*-nitrobenzoic acid were not substrates. Since nitrite was formed non-enzymatically from 2-nitropropane in the presence of a high concentration of FAD, e.g., 100 μM, 10 μM FAD was used when 2-nitropropane was the substrate.

Kinetics. The Michaelis constant was determined from a double-reciprocal plot for the relationship between reaction velocity and substrate or cofactor concentration. The apparent K_m values were determined as follows: 1-nitropropane, 1.54 mM; 2-nitropropane, 7.40 mM; nitroethane, 1.00 mM; 3-nitro-2-pentanol, 3.08 mM; nitrocyclohexane, 0.90 mM; and FAD, 1.33 μM.

Inhibitors. The various compounds were investigated for their inhibitory effects on enzyme activity (Table 4). The enzyme was inhibited

TABLE 3. *Substrate specificity of nitroalkane oxidase*[a]

Nitroalkanes	Relative activity
1-Nitropropane	100
2-Nitropropane	96.9
Nitroethane	51.8
Nitromethane	7.3
2-Nitroethanol	8.4
3-Nitro-1-butanol	15.7
3-Nitro-2-butanol	6.5
3-Nitro-2-pentanol	116
3-Nitropropionate	0.5
Nitrocyclohexane	99.8

[a] The reaction mixture consisted of 50 μmol of the substrates, 0.1 μmol of FAD (0.01 μmol for 2-nitropropane), 200 μmol of Tris-hydrochloride buffer (pH 8.0), and enzyme in a final volume of 1.0 ml. The activity was assayed by determining nitrite formed.

TABLE 4. *Effect of various compounds on nitroalkane oxidase activity*[a]

Additions	Relative activity
None	100
$HgCl_2$	0
KCN	6.6
5,5'-Dithiobis-(2-nitrobenzoic acid)	28.6
p-Chloromercuribenzoate	0
N-ethylmaleimide	3.8
Cysteine	100
2-Mercaptoethanol	100
Ethylenediaminetetraacetate	100
Tiron	95.7
Epinephrine	100
NADH	100
NADPH	100

[a] The standard reaction mixture was used, except that inhibitors were added to a final concentration of 1 mM. After preincubation at 37°C for 10 min, the reaction was started by addition of enzyme. NADH, Reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

strongly by KCN and thiol reagents such as *p*-chloromercuribenzoate and *N*-ethylmaleimide. Enzyme activity was not inhibited by metal-chelating agents, e.g., ethylenediaminetetraacetate, tiron (pyrocatechol-3,5-disulfonate disodium salt), thiol compounds, and riboflavin. The activity was not influenced by reduced nicotinamide adenine dinucleotide, cytochrome *c*, and other scavengers for superoxide.

DISCUSSION

An inducible nitroalkane-oxidizing enzyme was isolated from *F. oxysporum* grown in a medium containing nitroethane and subsequently incubated with nitroethane and purified to homogeneity. The stoichiometric study of the enzymatic oxidation of 1-nitropropane showed that the enzyme catalyzes the following reaction:

$$CH_2(NO_2)CH_2CH_3 + O_2 + H_2O \rightarrow OHCCH_2CH_3 + HNO_2 + H_2O_2 \quad (1)$$

or in the presence of catalase

$$CH_2(NO_2)CH_2CH_3 + 1/2\ O_2 \rightarrow OHCCH_2CH_3 + HNO_2 \quad (1')$$

The reaction rate was enhanced by the addition of catalase. Thus, the enzyme is unambiguously different from 2-nitropropane dioxygenase of a yeast, *H. mrakii* (6). Little (10, 11) reported that nitroethane and 2-nitropropane are oxidatively denitrified by extracts of *Neurospora crassa* and pea seedlings, respectively, and tentatively proposed the following reactions:

$$CH_3CH_2NO_2 + O_2 + H_2O \rightarrow CH_3CHO + HNO_2 + H_2O_2 \quad (2)$$

$$CH_3CH(NO_2)CH_3 + O_2 + H_2O \rightarrow CH_3COCH_3 + HNO_2 + H_2O_2 \quad (3)$$

The inconsistency of the proposed reactions with the observed balance of oxygen uptake and nitrite formation in the reactions prevents a conclusion as to whether the enzymes catalyzing these reactions are classified as oxygenase or oxidase, although the *N. crassa* enzyme is described as nitroethane oxidase (EC 1.7.3.1) in *Enzyme Nomenclature* (2). Thus, the similarity of nitroalkane oxidase to the enzyme of *N. crassa* and pea seedlings is not known at present. An alternative denitrification pathway of nitroalkanes is the reaction catalyzed by glutathione *S*-transferase (5).

The nitroalkane oxidase dialyzed thoroughly shows almost no activity in the absence of FAD, which is required for maximum activity as a coenzyme. The absorption spectrum of the dialyzed enzyme still has maxima at 273, 330, and 415 nm and a shoulder at 470 nm (T. Kido, K. Soda, and K. Asada, manuscript in preparation). The fluorescence spectrum of the enzyme was shown to be identical to that of authentic free FAD. These data suggest that the inactive resolved form of enzyme also contains FAD or a closely related compound. The enzyme was not inhibited by various metal-chelating agents, suggesting that the enzyme does not contain a metal. More detailed investigations on the prosthetic groups and metal contents are currently in progress.

Recently we have obtained evidence for the participation of superoxide as an intermediate in the 2-nitropropane dioxygenase reaction (13). The nitroalkane oxidase reaction was not affected by addition of scavengers for superoxide such as reduced nicotinamide adenine dinucleotide, cytochrome *c*, and tiron. Generation of superoxide probably does not occur in the oxidase reaction. Thus, the enzyme is different from the oxygenase in this respect also.

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