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Purification and Properties of Nitroalkane-Oxidizing Enzyme from *Hansenula mrakii*

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A nitroalkane-oxidizing enzyme was purified about 1,300-fold from a cell extract of *Hansenula mrakii* grown in a medium containing nitroethane as the sole nitrogen source by ammonium sulfate fractionation, diethylaminoethyl-cellulose column chromatography, hydroxyapatite column chromatography, and Bio-Gel P-150 column chromatography. The enzyme was shown to be homogeneous upon acrylamide gel electrophoresis and ultracentrifugation. The enzyme exhibits absorption maxima at 274, 370, 415, and 440 nm and a shoulder at 470 nm. Balance studies showed that 2 mol of 2-nitropropane is converted into an equimolar amount of acetone and nitrite with the consumption of 1 mol of oxygen. Hydrogen peroxide is not formed in the enzyme reaction. In addition to 2-nitropropane, 1-nitropropane and nitroethane are oxidatively denitrified by the enzyme, but nitromethane is inert to the enzyme. The nitroalkanes are not oxidized under anaerobic conditions.

Although considerable effort has been devoted to the isolation, identification, and characterization of nitro compounds as microbial secondary metabolites (17, 18), the metabolism of nitro compounds has remained unsolved. During the course of an investigation of the microbial assimilation of alkyl nitro compounds, we found that 2-nitropropane, 1-nitropropane, and nitroethane are oxidatively decomposed by an intracellular enzyme (or enzymes) of *Hansenula mrakii* and other organisms utilizing these nitro compounds to yield nitrite and the carbonyl analogues (5). The present study of purification and some properties of the enzyme from *H. mrakii* was undertaken to shed light on the enzymological aspects of microbial metabolism of alkyl nitro compounds.

MATERIALS AND METHODS

Chemicals. Diethylaminoethyl (DEAE)-cellulose was purchased from Serva, Heidelberg, Germany; Bio-Gel P-150 was from Bio-Rad Laboratories, Richmond, Calif. Hydroxyapatite was prepared according to the method given previously (6). Catalase and peroxidase were purchased from Sigma Chemical Co., St. Louis, Mo., and 3-methyl-2-benzothiazolone hydrazone-hydrochloride (MBTH) was from Aldrich Chemical Co., Inc., Wis. The other chemicals were analytical-grade reagents and were obtained from Nakarai Chemicals, Kyoto, Japan.

Growth of organism. *H. mrakii* IFO 0895 was grown 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. Nitroethane was sterilized separately by filtration with a Seitz filter. The pH of the medium was adjusted to 5.5 with 1 N HCl. *H. mrakii* was inoculated in a 2-liter flask containing 500 ml of the medium. After incubation at 28 C for 20 h with shaking on a reciprocating shaker, the subculture was inoculated into 22 liters of the medium placed in a 30-liter jar fermentor. Growth was at 25 C for 20 h under aeration. The harvested cells were washed twice with 0.85% NaCl. The yield of cells was about 10 g, wet weight, per liter of the medium. The washed cells were stored at −20 C until used.

Enzyme assay and analytical methods. The standard reaction mixture consisted of 50 μmol of 2-nitropropane, 1-nitropropane, or nitroethane, 100 μmol of potassium phosphate (pH 8.0 for 2-nitropropane and 1-nitropropane and pH 7.0 for nitroethane), and enzyme in a final volume of 1.0 ml. Enzyme was replaced by 0.01 M potassium phosphate (pH 7.0) in a blank. The mixture was incubated aerobically at 37 C for 20 min with shaking. The reaction was terminated by the addition of 0.1 ml of glacial acetic acid. After centrifugation, 0.1-ml aliquots of the supernatant liquid were used for the determination of nitrite and acetone. Nitrite was determined by the Little method (8). Acetone was determined spectrophotometrically with MBTH by the method of Paz et al. (13). 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 catalyzes the formation of 1 nmol of nitrite or acetone per min. Specific activity was expressed as units per milligram of protein.

2-Nitropropane was determined with a Shimadzu gas chromatograph, GC-4BM. The column (3.5 mm

[ID] by 2 m [length]) was packed with 20% Carbowax terminated with terephthalic acid on Chromosorb W (60 to 80 mesh) and was operated at 120 C.

Oxygen uptake was measured manometrically. The main compartment of a Warburg flask contained 125 μmol of 2-nitropropane and 250 μmol of potassium phosphate (pH 8.0) in a final volume of 2.0 ml. In the side arm, enzyme and 5 μmol of potassium phosphate (pH 7.0) were placed to a final volume of 0.5 ml. After equilibration at 30 C for 10 min, the reaction was started by the addition of enzyme solution, and readings were made over the next 1 h.

Hydrogen peroxide was determined by the method of Malmstadt and Djiioannou (11). About 13 nmol of hydrogen peroxide could be detected at the lower limit.

α-Keto acids formed from D-amino acids were determined with MBTH by the method of Soda (16).

Protein was determined by the method of Lowry et al. (10); with most column fractions, protein elution patterns were estimated by absorbance at 280 nm.

Disc-gel electrophoresis. Disc-gel electrophoresis in 7.5% polyacrylamide gel was performed by a modification of the procedure of Davis (1). The enzyme (25 μg) was applied to the top of spacer gel in 1 M sucrose. Electrophoresis was conducted at a current of 2 mA for 2.5 h in tris(hydroxymethyl)aminomethane-glycine, pH 8.3. After electrophoresis, protein was stained with 1.0% amido schwarz in 7.0% acetic acid and destained electrophoretically in 7.0% acetic acid.

RESULTS

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

Step 1. Enzyme extraction. The washed cells (4.5 kg, wet weight) were suspended in 4.5 liters of 0.01 M potassium phosphate (pH 7.0) and disrupted continuously by a Vibrogen Cell Mill with glass beads (0.13 to 0.18 mm). The intact cells and cell debris were removed by centrifugation.

Step 2. Ammonium sulfate fractionation. The supernatant solution (8.3 liters) was brought to 60% saturation with ammonium sulfate, and the precipitate was discarded by centrifugation. Ammonium sulfate was added to the supernatant liquid (11.1 liters) to 90% saturation. The pH was kept at about 7.0 by the addition of 3 N ammonia. The resulting precipitate was collected by centrifugation and was dissolved in 500 ml of 0.01 M potassium phosphate (pH 7.0). The enzyme solution was dialyzed overnight against the same buffer. The insoluble materials formed during dialysis were removed by centrifugation.

Step 3. DEAE-cellulose column chromatography. The dialyzed enzyme solution (1 liter) was applied to a DEAE-cellulose column (7 by 60 cm) equilibrated with 0.01 M potassium phosphate (pH 7.0). After the column was washed with 2 liters of the same buffer and 10 liters of the buffer containing 0.12 M NaCl, enzyme was eluted with the buffer containing 0.2 M NaCl. Fractions (15 ml) were collected at a flow rate of 120 ml/h. Active fractions were concentrated by the addition of ammonium sulfate (90% saturation) and dialyzed against 100 volumes of 1 mM potassium phosphate (pH 7.0).

Step 4. Hydroxyapatite column chromatography. The enzyme solution was placed onto a hydroxyapatite column (4 by 25 cm) equilibrated with the above-mentioned buffer. The column was washed with 0.3 liter of the same buffer and then with 2 liters of 0.05 M potassium phosphate (pH 7.0). The enzyme was eluted with 0.1 M potassium phosphate (pH 7.0) at a flow rate of 40 ml/h. The active fractions were combined and concentrated by the addition of ammonium sulfate (90% saturation). The precipitate was dissolved in a small volume of 0.01 M potassium phosphate (pH 7.0).

Step 5. Bio-Gel P-150 column chromatography. The enzyme solution was applied to a Bio-Gel P-150 column (1.5 by 150 cm) equilibrated with 0.01 M potassium phosphate (pH 7.0) and eluted with the same buffer. The active fractions were collected and concentrated by the addition of ammonium sulfate (90% saturation). The precipitate was dissolved in a small volume of 0.01 M potassium phosphate (pH 7.0) and dialyzed against the same buffer.

A summary of the purification procedure is presented in Table 1.

Purity. The purified enzyme showed a single protein band upon disc-gel electrophoresis. To determine whether the band corresponded to the enzyme activity, a gel was run in the normal way, and a thin vertical slice of the gel was stained with Coomassie brilliant blue and destained with 10% acetic acid solution in 10% methanol. The section of the unstained gel corresponding to the protein band was cut out and crushed in a small volume of 0.01 M potassium phosphate (pH 7.0). After 20 min of elution at 4 C, the gel was removed by centrifugation, and the supernatant solution was found to contain the activity of nitroalkane-oxidizing enzyme. Homogeneity of the enzyme was also demonstrated by ultracentrifugation. The enzyme sedimented as a single, symmetrical peak during a sedimentation velocity run (Fig. 1). In the sedimentation velocity experiments, the sedimentation constant ($s_{20,w}$) was determined to be 3.43, 3.40, 3.26, and 3.18S at the enzyme concentrations 0.27, 0.44, 0.48, and 0.55%, re-

TABLE 1. *Purification of nitroalkane-oxidizing enzyme*[a]

Steps	Total vol (ml)	Total activity (units)	Protein (g)	Sp act	Yield (%)
1. Cell extract	8,300	34.7×10^5	310	12.0	100
2. Ammonium sulfate fractionation (60–90% saturation)	1,000	32.2×10^5	64	56.0	92.8
3. DEAE-cellulose chromatography	115	27.0×10^5	2.4	900	77.8
4. Hydroxyapatite chromatography	7	21.8×10^5	0.65	2,300	63.4
5. Bio-Gel P-150 chromatography	5	7.6×10^5	0.07	15,500	21.9

[a] 2-Nitropropane was used as a substrate.

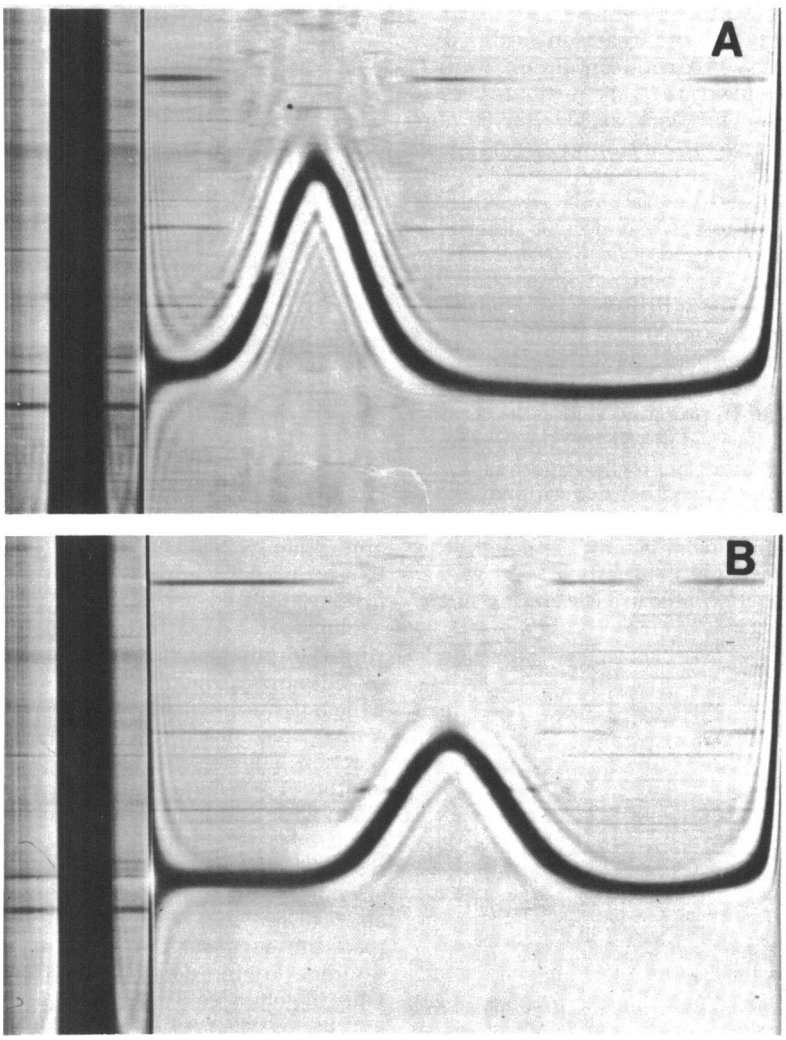

FIG. 1. *Sedimentation patterns of nitroalkane-oxidizing enzyme. Photographs were taken at 80 (A) and 152 (B) min after top speed (56,100 rpm) was achieved. The enzyme (8 mg/ml) in 0.01 M potassium phosphate buffer (pH 7.0) was centrifuged at 20 C.*

spectively. By extrapolation to zero concentration, the sedimentation coefficient ($s^0_{20,w}$) was calculated to be 3.7S.

Absorption spectrum. The absorption spectrum of nitroalkane-oxidizing enzyme has maxima at 274, 370, 415, and 440 nm and a shoulder at 470 nm (Fig. 2). No appreciable spectral shift occurred when the pH was varied between 6.0 and 8.5. The molecular absorption coefficients are 62,000 at 274 nm, 9,400 at 370 nm, 8,900 at 415 nm, 9,300 at 440 nm, and 6,900 at 470 nm. These give an absorbance ratio of 100:15.1:14.4:15.0:11.1.

When 2-nitropropane was added to the enzyme under anaerobic conditions, an absorption peak at 440 nm and a shoulder at 470 nm disappeared.

Stoichiometry of the reaction. After the standard reaction mixture, containing 2-nitropropane as a substrate, was incubated in a Warburg manometer flask at 30 C for 60 min with shaking, oxygen consumption was measured and residual substrate and products were determined. This enzymatic oxidative denitrification was found to proceed stoichiometrically (Table 2). The formation of acetone and nitrite and the oxygen uptake were not observed in the absence of enzyme or 2-nitropropane. Hydrogen peroxide was not detected at all, and the reaction was never influenced by the addition of catalase. Neither nitrite nor acetone was produced from 2-nitropropane under anaerobic conditions as described previously for the crude enzyme (5). In addition to 2-nitropropane, 1-nitropropane and nitroethane were enzymatically denitrified to yield nitrite and the corresponding carbonyl compounds, although the stoichiometry was not determined.

When the reaction mixture containing nitromethane, D-alanine, or D-methionine and 5 μg of enzyme was incubated for 40 min, formaldehyde and nitrite, and α-keto acids and ammonia, were not produced from nitromethane, and D-alanine and D-methionine, respectively. Thus, nitromethane, D-alanine and D-methionine are not substrates.

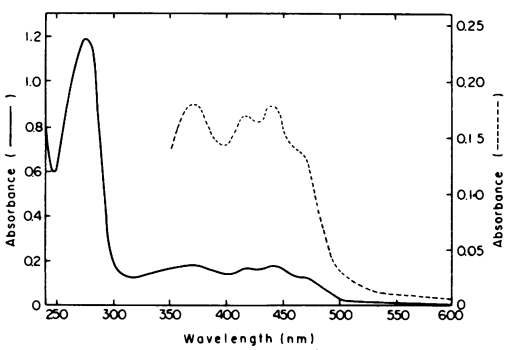

FIG. 2. *Absorption spectrum of nitroalkane-oxidizing enzyme (1.22 mg/ml) in 0.01 M potassium phosphate buffer (pH 7.0). Absorption spectra were taken with a Shimadzu recording spectrophotometer, MPS-50L, with a 1.0-cm light path.*

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

Expt	2-Nitropropane consumed (μmol)	Oxygen consumed (μmol)	Nitrite formed (μmol)	Acetone formed (μmol)
1	1.06	0.51	1.03	1.06
2	0.86	0.44	0.85	0.86

[a] In experiments 1 and 2, 6 and 4 μg of enzyme were used, respectively.

DISCUSSION

Since Little (7, 9) demonstrated the oxidative degradation of nitroethane and 2-nitropropane by extracts of *Neurospora crassa* and pea seedlings, respectively, little attention has been given to the metabolism of nitro compounds, especially the enzymological aspects. Recently, Porter et al. (14, 15) reported that D-amino acid oxidase (EC 1.4.3.3) catalyzes the oxidation of nitromethane and nitroethane as follows:

$$CH_3NO_2 \text{ (or } CH_3CH_2NO_2) + O_2 + H_2O \rightarrow HCHO \text{ (or } CH_3CHO) + HNO_2 + H_2O_2$$

and investigated the reaction mechanism in detail. Habig et al. (3, 4) demonstrated that glutathione S-transferase catalyzes the formation of nitrite from organic nitrate esters and nitro compounds.

The studies described here deal with the purification and some fundamental properties of nitroalkane-oxidizing enzyme from *H. mrakii*, which assimilates nitroethane as a nitrogen source, and also the stoichiometry of enzyme reaction. The enzyme has been purified approximately 1,300-fold, with an overall yield of about 22%, and is homogeneous by the criteria of ultracentrifugation and disc-gel electrophoresis. The visible absorption spectrum of the enzyme is characterized by maxima at 370, 415, and 440 nm and a shoulder at 470 nm. This spectrum suggests that the enzyme probably contains flavine adenine dinucleotide (or flavine mononucleotide) and iron as prosthetic groups, as observed for dihydroorotic dehydrogenase (2) and xanthine oxidase (12). The stoichiometric study of the enzymatic oxidation of 2-nitropropane shows that the enzyme cata-

lyzes the following reaction:

$$2\ CH_3CH(NO_2)CH_3 + O_2 \rightarrow 2\ CH_3COCH_3 + 2\ HNO_2$$

It is likely that 1-nitropropane and nitroethane also are oxidized to nitrite and the corresponding carbonyl compounds in the same manner by analogy with 2-nitropropane. Hydrogen peroxide is not produced in the enzyme reaction, and nitromethane and D-amino acids are not oxidized by the enzyme. Therefore, the enzyme is unambiguously different from D-amino acid oxidase. The stoichiometry of the reaction implies that the nitroalkane-oxidizing enzyme is an oxygenase rather than an oxidase, although further work with $^{18}O_2$ is needed to elucidate this problem. More detailed investigations on the prosthetic groups and the physicochemical properties of the enzyme are currently in progress.

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LITERATURE CITED

1. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404-427.
2. **Friedmann, H. C., and B. Vennesland.** 1958. Purification and properties of dihydroorotic dehydrogenase. J. Biol. Chem. 233:1398-1406.
3. **Habig, W. H., J. H. Keen, and W. B. Jakoby.** 1975. Glutathione S-transferase in the formation of cyanide from organic thiocyanates and as an organic nitrate reductase. Biochem. Biophys. Res. Commun. 64:501-507.
4. **Habig, W. H., M. J. Pobst, and W. B. Jakoby.** 1974. Glutathione S-transferase. J. Biol. Chem. 249:7130-7139.
5. **Kido, T., T. Yamamoto, and K. Soda.** 1975. Microbial assimilation of alkyl nitro compounds and formation of nitrite. Arch. Microbiol. 106:165-169.
6. **Levin, O.** 1962. Column chromatography of protein: calcium phosphate, p. 27-28. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
7. **Little, H. N.** 1951. Oxidation of nitroethane by extracts from *Neurospora crassa*. J. Biol. Chem. 193:347-358.
8. **Little, H. N.** 1955. Nitroethane oxidase, p. 400-402. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 2. Academic Press Inc., New York.
9. **Little, H. N.** 1957. The oxidation of 2-nitropropane by extracts of pea plants. J. Biol. Chem. 229:231-238.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
11. **Malmstadt, H. V., and H. A. Djiioannou.** 1963. Specific enzymatic determination of some alpha-amino acids by an automatic spectrophotometric reaction rate method. Anal. Chem. 35:14-16.
12. **Massey, V., P. E. Brumby, H. Komai, and G. Palmer.** 1969. Studies on milk xanthine oxidase: some spectral and kinetic properties. J. Biol. Chem. 244:1682-1691.
13. **Paz, M. A., O. O. Blumenfeld, M. Rojkind, E. F. Henson, C. Fureine, and P. M. Gallop.** 1965. Determination of carbonyl compounds with N-methyl benzothiazolone hydrazone. Arch. Biochem. Biophys. 109:548-559.
14. **Porter, D. J. T., J. G. Voet, and H. J. Bright.** 1972. Nitromethane: a novel substrate for D-amino acid oxidase. J. Biol. Chem. 247:1951-1953.
15. **Porter, D. J. T., J. G. Voet, and H. J. Bright.** 1973. Direct evidence for carbanions and covalent N^5-flavin-carbanion adducts as catalytic intermediates in the oxidation of nitroethane by D-amino acid oxidase. J. Biol. Chem. 248:4400-4416.
16. **Soda, K.** 1968. Microdetermination of D-amino acids and D-amino acid oxidase activity with 3-methyl-2-benzothiazolone hydrazone hydrochloride. Anal. Biochem. 25:228-235.
17. **Turner, W. B.** 1971. Fungal metabolites, p. 303. Academic Press Inc., New York.
18. **Venulet, J., and R. L. VanEtten.** 1970. Biochemistry and pharmacology of the nitro and nitroso groups, p. 201-287. *In* H. Feuer (ed.), The chemistry of the nitro and nitroso groups, part 2. Interscience Press, New York.