Denitrification by the Fungus *Fusarium oxysporum* and Involvement of Cytochrome P-450 in the Respiratory Nitrite Reduction* 

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Hirofumi Shoun† and Tatsuo Tanimoto

From the Institute of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305, Japan

From conditions for production in *Fusarium oxysporum* of the unique nitrate/nitrite-inducible cytochrome P-450, tentatively called P-450\textsubscript{Nit}, it was expected that the fungus is capable of metabolizing nitrate or nitrite dissimilatively. Here we report that *F. oxysporum* exhibits a distinct denitrifying ability which results in the anaerobic evolution of nitrous oxide (N\textsubscript{2}O) from nitrate or nitrite. Comparison of the cell growth during denitriﬁcation indicated that the dissimilatory reduction of nitrate to nitrite is an energetically favorable process in *F. oxysporum*; however, further reduction of nitrite to N\textsubscript{2}O might be energy-exhausting and may function as a detoxiﬁcation mechanism. A potent nitrite reductase activity to form N\textsubscript{2}O could be reconstituted by combination of the cell-free extract prepared from the denitrifying cells and an NADH-phenadinemethosulfate-dependent reducing system. The activity was strongly inhibited by carbon monoxide, cyanide, oxygen (O\textsubscript{2}), and the antibody against P-450\textsubscript{Nit}. The results, along with those concerning inducing conditions of P-450\textsubscript{Nit}, were highly indicative that the cytochrome is involved in the denitrifying nitrite reduction. This work has thus presented not only the first demonstration that an eukaryotic cell contains a cytochrome P-450 that is involved in a reducing reaction with a distinct physiological significance against a hydrophilic, inorganic substrate.

Denitrification is a process in which nitrate and/or nitrite is reduced to a gaseous form of nitrogen, generally to dinitrogen (N\textsubscript{2}) or nitrous oxide (N\textsubscript{2}O), and plays an important role in the global nitrogen cycle. A number of bacteria perform this process in which nitrate and/or nitrite acts as the ultimate electron acceptor for anaerobic respiration (1–5). However, this activity has not been detected in eukaryotic cells. The activity was strongly inhibited by oxygen and by the antibody against P-450\textsubscript{Nit}. The results, along with those concerning inducing conditions of P-450\textsubscript{Nit}, were highly indicative that the cytochrome is involved in the denitrifying nitrite reduction. This work has thus presented not only the first demonstration that an eukaryotic cell contains a cytochrome P-450 that is involved in a reducing reaction with a distinct physiological significance against a hydrophilic, inorganic substrate.

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* MATERIALS AND METHODS

**Fungal Strain**—*Fusarium oxysporum* MT-811, previously isolated from soil and identified (10), was used throughout this work. Prior to following experiments the fungal strain was purified by a single spore isolation followed by a submerged culture in the presence of antibiotics (tetracycline, streptomycin, and ampicillin; 10 \( \mu \text{g/ml} \) each).

**Cultivation**—*F. oxysporum* was cultivated (incubated) under conditions with limited aeration to examine denitrifying ability. The fungus was incubated in 500- or 300-mi volume Erlenmeyer flasks with two sides arms containing 150 or 90 ml (final) of a medium that consisted of 3% glycerol, 0.2% peptone, 10 mM sodium nitrate (or nitrite), and inorganic salts (6, 8), on a rotary shaker (150 rpm) at 26.5°C. The seed was prepared by cultivation for 5 days in 5-liter Erlenmeyer flasks containing 2 liters of the same medium without nitrate/nitrite and inoculated by one-fifth of the final volume (30 ml of the 150-ml culture). The top and side arms of the flask were sealed with a butyl rubber stopper and a double butyl rubber stoppers, respectively, after inoculation. When necessary, the air in flasks was substituted just after inoculation by helium or carbon monoxide (CO) gas that was passed through a sterilized cotton filter.

**Sampling**—At each indicated time during the time course of cultivation, three flasks were harvested. Mycelia were collected by filtration (filter paper) and washed with distilled water. A portion of mycelia was dried by heating at 90°C for 1 day and weighed. Other portions were disrupted and fractionated (see below), and assayed for P-450\textsubscript{Nit} (soluble fraction) or nitrate and nitrite reductases. The first filtrate (broth) was further filtrated with a microfiter (Millipore, UFC 30GV00) to remove proteins, and residual nitrate or nitrite was determined.

**Gas Analyses**—The upper space gas of cultivation flasks or of the reaction vessel for reductase assays was analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) with a Shimadzu gas chromatograph GC 12A and with a Shimadzu gas chromatograph-mass spectrometer GCMS-9000C, both of which were equipped with a Porapack Q column (3 mm × 2 m inner diameter) for analysis of N\textsubscript{2}O and a molecular sieve 5A column (same size, for N\textsubscript{2} and O\textsubscript{2}), a thermal conductivity detector, and helium carrier, respectively. Each sample gas was taken off through the double butyl rubber stopper with a syringe and applied to analyses. Each peak was identified and quantified by comparison with standard gases (GL Sciences, Tokyo).

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† To whom correspondence should be addressed: Inst. of Applied Biochemistry, University of Tsukuba, Tsukuba, Ibaraki 305, Japan.

‡ The abbreviations used are: P-450, cytochrome P-450; GC, gas chromatograph; MS, mass spectrometry; MVH, reduced methyl viologen; PMS, phenadinemethosulfate; dNiR, dissimilatory nitrite reductase; dNaR, dissimilatory nitrate reductase; HPLC, high performance liquid chromatography.
Fractionation of the Cell-free Extract—The fungal cells were disrupted as reported (8, 11) by grinding with alumina, and the extract was fractionated by differential centrifugation (first at 10,000 × g, and then at 105,000 × g). Each precipitate was washed once, filled up to the same volume as the supernatant with the buffer used for disruption, and utilized for each assay.

Nitrate and Nitrite Reductase Assays—Cell-free nitrate and nitrite reductase activities were determined as follows employing an NADH-phenadinemethosulfate (PMS) or reduced methylviolegen (MVH) system as an electron donor. The reaction mixture (final, 1 ml) containing 1 ml of a subcellular fraction, 2.0 mM sodium nitrite, 5% glycerol, 0.02 mM PMS, or 0.1 mM methylviolegen, in 60 mM potassium phosphate buffer (pH 7.5), was put in a vial (27-ml volume) and degassed. The vial was sealed with a butyl rubber stopper under flush of helium gas. The reaction was initiated by adding with a syringe 1 ml of a solution containing 80 mM NADH or 1% sodium hydrosulfite, 1% sodium bicarbonate, at 30 °C. The increase in the gas phase of evolved gas such as N2O and/or the decrease of nitrate or nitrite in the reaction mixture was determined.

Determination of Nitrate, Nitrite, and Ammonium Ions—The concentration of nitrate or nitrite in culture media or the reaction mixture was determined with a high performance liquid chromatograph (HPLC; Tosoh CCP and 8010 Series) equipped with a TSK gel IC-Anion PW column. Eluting solution was 50 mM boric acid containing 0.7 mM sodium 1-octanesulfonate. The anions were monitored with a UV detector at 210 nm. Ammonium ions were determined colorimetrically (12).

Results

N2O Evolution by Intact Cells from Nitrate—Since dissimilatory nitrate reduction is usually an anaerobic process, F. oxysporum was cultivated (incubated) in a nitrate-containing medium under the condition where oxygen supply was discontinued, and its denitrifying ability was examined. After oxygen that was derived from air and initially present in the flask was consumed, a rapid N2O evolution was observed, as shown in Fig. 1. Added nitrate was stoichiometrically (i.e. with 100% yield) converted to N2O. Slight (as compared with that due to oxygen) but distinct cell growth was observed during the incubation period in which the N2O evolution was continuing, suggesting that the dissimilatory nitrate reductase activity (dNIR) in the fungus is connected with the activity. The N2O evolution was preceded by the onset of nitrate uptake and induction of both P-450dNIR and the cell-free N2O-forming nitrite reductase (dNIR) activity (cf. Fig. 3) in the cells. The concomitant induction of P-450dNIR and dNIR activity suggested that the P-450 is connected with the activity. The N2O evolution was not observed at all when nitrate was omitted from the medium (not shown). The results clearly showed that the fungus exhibits a potent denitrifying ability.

N2O Evolution by Intact Cells from Nitrite—A similar result was obtained upon incubation of the fungal cells with a nitrite-containing medium (Fig. 2), although N2O-evolving activity was somewhat lower. Nitrogen atoms in N2O was shown derived from nitrite by the GC-MS analysis (cf. Fig. 4). More potent activity (i.e. 100% conversion) was observed when air in the flask was replaced by helium before incubation (not shown). In contrast to the growth on the nitrate medium above, however, dry cell matter began to decrease when the N2O evolution was initiated. It seems therefore that the denitrifying process from nitrite to N2O is not an energy-yielding but an energy-exhausting process. The N2O evolution was strongly inhibited or repressed by CO or by intermittent supply of oxygen. The inhibition by CO is a unique feature of the fungal denitrification, which is consistent with the hypothesis that P-450dNIR is involved in the process.

These results (Figs. 1 and 2) unequivocally demonstrated that the fungus F. oxysporum can metabolize both nitrate and nitrite in a dissimilative manner, resulting in denitrification.
This is the first demonstration that eukaryotic cells exhibit a distinct denitrifying activity. It is also clear that the activity is inducible with nitrate or nitrite only when oxygen applicable to aerobic respiration is exhausted or not present.

**Cell-free Nitrite Reductase Activity**—Since a potent denitrifying ability of *F. oxysporum* was demonstrated above, we then sought to detect a cell-free dNiR activity. As shown in Fig. 3, the cell-free extract (10,000 × g supernatant; cf. Table I) obtained from the denitrifying cells exhibited a potent, NADH-PM-dependent nitrite reductase activity. The substrate nitrite was stoichiometrically converted to N₂O within 20–30 min. The initial rate was approximately 1,000 min⁻¹ (reduction of nitrite) or 500 min⁻¹ (N₂O evolution) with respect to involved P-450dNR. MVH could also serve as an electron donor (not shown). Fig. 4 shows results of identification of the product. Nitrogen atoms in the product N₂O were proved to be derived from nitrite by GC-MS (m/z = 46), employing a heavy isotope ([¹⁵N]nitrite) as substrate. Other possible intermediates or products, such as nitric oxide (NO) or N₂, could not be detected. This was also the case with the denitrification by intact cells above (Figs. 1 and 2; data not shown). The activity did not appear in the absence of the artificial electron donor. The cell-free extract, obtained from non-denitrifying cells (the seed culture) and thus containing no P-450dNR, did not possess N₂O-forming activity at all (Fig. 4A). As was expected, the cell-free activity did not produce detectable amounts of ammonium ions, the product of assimilatory reduction of nitrite (not shown).

The activity was strongly inhibited by CO, cyanide, or O₂ (Fig. 3). These substances usually interact with heme-containing terminal oxidases or reductases. When purified P-450dNR was incubated with the NADH-PM-reducing system in the same manner, a slight but distinct amount of N₂O was formed (Fig. 3). In addition, the rate of N₂O evolution was somewhat increased by combination of the active extract and purified P-450dNR (not shown). It is therefore highly possible that P-450dNR is the nitrite reductase itself. The low recovery of product by the purified P-450dNR system is probably due to lack of supporting component(s).

**Cytochromes in the Extract**—Since the soluble fraction above (Fig. 3; cf. Table I) was fully active in forming N₂O, cytochrome components in the fraction seemed worth determining (Fig. 5). It is rather surprising that the soluble fraction from denitrifying cells contained a large amount of cytochromes. The spectra for non-denitrifying cells were typical of cytochrome b₅ (15), suggesting that the cytochrome is not involved in the denitrification. The spectra for denitrifying cells indicated that the cells contained higher amounts of b₅ or c-type cytochromes and CO-binding heme protein (P-420) in addition to P-450. This would suggest the possible involvement of cytochrome b₅ or c in the nitrite reductase system in addition to P-450.

**Dissimilatory Nitrate Reductase (dNaR) Activity**—Along with dNiR activity above, a distinct NADH-PM- or MVH-dependent dNaR activity could be detected only in the large particle fraction (10,000 × g precipitate; cf. Table I), as shown in Fig. 6. Although the rate of N₂O evolution was lower than that due to the nitrite reductase system (Fig. 3), a distinct N₂O evolution could be observed when the NADH-PM-reducing system was employed. In contrast, only a minor amount of N₂O was evolved as compared with decreased nitrate when MVH was used. The discrepancy in stoichiometry between substrate and product might depend on trapping of intermediates or products by some nonenzymatic reactions caused by the potent reducing force of dithionite in the system. Nitrite, the expected product due to dNaR reaction, could also be detected to form in the MVH-dependent system.
The nitrite level was lower than that of utilized nitrate. The discrepancy seems reasonable, however, because a portion of the formed nitrite might have been utilized for the subsequent dNiR reaction. The N₂O-evolving activity of the particulate fraction indicated that all components essential for reduction of nitrate to N₂O are connected with the large particles.

Localization of Nitrate and Nitrite Reductase Activities—The highly active cell-free extract was fractionated by centrifugation, and both reductase activities were determined with each subcellular fraction (Table I). As noted above, nitrate reductase activity was recovered only in the large particle fraction. In contrast, nitrite reductase activity was recovered in both the soluble (105,000 x g supernatant) and the large particle fractions. This suggests that components corresponding to the activity are connected to or have interactions with particles and that they are easily released into the soluble fraction. The cell-free activity shown above (Figs. 1-4) thus depended on these soluble (or solubilized) components; however, the reconstituted activity was highly active (Fig. 3).

Inhibition of Nitrite Reductase by Antibody—The involvement of P-450dNIK in the dissimilatory nitrite reductase activity was conclusively shown by the inhibition experiment with the antibody that was raised against purified P-450dNIK (Fig. 7).

Other Inhibitors—It has been shown from the above results that the cell-free nitrite reductase activity was inhibited by CO, O₂, and cyanide, and that the N₂O evolution by intact cells was inhibited or repressed by CO and O₂.

The effects of other inhibitors were also examined (Table II). Antimycin A, azide, salicylhydroxamic acid (an inhibitor of cyanide-insensitive respiration; Ref. 16), metyrapone (a P-450 inhibitor), and menadione (an inhibitor against NADPH-cytochrome P-450 reductase system) showed little inhibition of the cell-free activity. The lack of inhibition by metyrapone or menadione is not surprising, considering the uniqueness of the electron transport system of P-450dNIK. Although antimycin did not inhibit the cell-free activity at all, it inhibited to a considerable extent the N₂O evolution from nitrite by intact cells. The results suggest that the nitrite reduction is connected to some respiratory chain, although the process does not seem energetically favorable. This is consistent with the above observation that nitrite reductase was connected to large particles.

Cyanide, CO, and oxygen strongly inhibited the cell-free activity, in contrast to the inhibition by antimycin. This can be explained by that P-450dNIK is the nitrite reductase itself, as noted above. Inhibition by cyanide is one of unique features of P-450dNIK. This is consistent with our results that cyanide induced a spectral change in the P-450 which closely resembled that caused by cyanide in cytochrome oxidase. Oxygen might exhibit both repression (Figs. 1 and 2) and inhibitory effects (Fig. 3) on the reductase system.

A higher concentration of cyanide was required to inhibit
the intact cell activity than the cell-free activity. This may depend on rather high cell population in the culture.

**DISCUSSION**

The results reported above provide evidence that the fungus *F. oxysporum* is capable of reducing nitrate and nitrite to a gaseous form of N\(_2\O\) when oxygen supply is limited or discontinued and that P-450\(_{\text{DNR}}\) might be involved in this process. It is also likely that the denitrification from nitrite is for a dissimilatory purpose and thus is, as a whole, an energy-yielding process. On the other hand, the denitrification did not seem energetically favorable when nitrite was employed as substrate. It may function as a detoxification mechanism. These features somewhat resemble those of the denitrifying bacteria, *Propionibacterium acnes*, an obligate anaerobe (17). It has been reported that several yeasts and fungi can form N\(_2\)O from nitrate (18). The rate (requiring more than 10 days) and recovery (below 0.2\%) of nitrate-form nitrogen are, however, far from physiologically significant in these cases. Therefore, *F. oxysporum* is the first eukaryote that has been shown to catalyze denitrification, an anaerobic process.

We did not find significant evolution of N\(_2\) from that of the traditional P-450 system; the substrate 450, which has been known as a group of hemoproteins catalyzing a variety of monooxygenation reactions (19-21). Whether the fungus can further reduce N\(_2\O\) to N\(_2\) or not, the substrate nitrite is a hydrophilic and inorganic substance that is quite unique with many respects. For example, it appears to be connected to the respiratory chain and thus composed of quite different electron transport system from that of the traditional P-450 system; the substrate nitrite is a hydrophilic and inorganic substance that is quite different from so far known, hydrophobic, organic substrates.

We have recently succeeded in obtaining a cDNA clone for P-450\(_{\text{DNR}}\). The deduced amino acid sequence revealed many interesting properties of the P-450, i.e. that P-450\(_{\text{DNR}}\) exhibited higher sequence homology against bacterial P-450s (in particular those of *Streptomyces* (Ref. 25) rather than against eukaryotic P-450s, and that the N terminus region contained neither the signal peptide-like, hydrophobic domain that is characteristic of microsomal P-450s nor the tagging sequence that is essential for the localization of mitochondrial P-450s. These results strongly support our previous claim that P-450\(_{\text{DNR}}\) is the first soluble P-450 of eukaryotes (6). The result along with the present data have promised us unique features of the P-450 to be clarified in many respects with high potential interests. It remains to be elucidated how the soluble P-450 interacts with organella such as mitochondria, although some interaction has been demonstrated above (Table I, Fig. 6).

We have recently found that several strains of *F. oxysporum* and related genera produce similar, nitrate/nitrite-inducible P-450 and that these P-450 species show properties immunologically and spectrally similar to those of P-450\(_{\text{DNR}}\) (7). It seems highly possible that at least these fungal strains also exhibit a similar denitrifying ability. It would be also ecologically important to study the distribution among eukaryotic microorganisms of such an ability to evolve the greenhouse effect gas (N\(_2\O\)), since the recent tendency toward acidification in soil will potentiate the activity of fungi.

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