Denitrification, a Novel Type of Respiratory Metabolism in Fungal Mitochondrion*

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Subcellular localization and coupling to ATP synthesis were investigated with respect to the denitrifying systems of two fungi, Fusarium oxysporum and Cylindrocarpon tonkinense. Dissimilatory nitrate reductase of F. oxysporum or nitrite reductase of C. tonkinense could be detected in the mitochondrial fraction prepared from denitrifying cells of each fungus. Fluorescence immuno-localization, cofractionation with mitochondrial marker enzymes, and cytochromes provided evidence that the denitrifying enzymes are co-purified with mitochondria. Respiratory substrates such as malate plus pyruvate, succinate, and formate were effective donors of electrons to these activities in the mitochondrial fractions. Moreover, nitrite and nitrate reduction were shown to be coupled to the synthesis of ATP with energy yields (P:NO₃⁻ or P:2e ratios) of 0.88 to 1.4, depending upon whether malate/pyruvate or succinate were provided as substrates. Nitrate or nitrite reductase activity was inhibited by inhibitors such as rotenone, antimycin A, and thenoyltrifluoroacetone. Thus, fungal denitrification activities are localized to mitochondria and are coupled to the synthesis of ATP. The existence of these novel respiration systems are discussed with regard to the origin and evolution of mitochondria.

Denitrification is a process whereby nitrate or nitrite is reduced to a gaseous form of nitrogen (N₂ or N₂O). Biological denitrifying activities, which play important roles in the global nitrogen cycle, were believed to be uniquely characteristic of prokaryotes (1–3) until we discovered their presence in several fungi (4–6). Whether the fungal denitrification systems resemble bacterial systems for anaerobic respiration and, if so, whether they are localized to mitochondria has not been established. Bacterial denitrification usually involves four sequential reactions, which are catalyzed by nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide (NO) reductase (Nor), and nitrous oxide (N₂O) reductase, respectively (1–3). Among fungal denitrifying systems, those of Fusarium oxysporum and Cylindrocarpon tonkinense have been best characterized (6–11). Fungal systems usually lack N₂O reductase, and consequently evolve N₂O as the final denitrification product (4–6). Many systems such as that of C. tonkinense also lack Nar. However, many bacterial denitrifying (or nitrate-respiring) systems may also lack part of the reductase chain (1–3). Involvement of cytochrome P450 (P450nor) for Nor is typical in the fungal systems that have been analyzed to date (4–12), whereas bacterial Nor is of the cytochrome bc type (1–3). In contrast, Nir isolated from F. oxysporum is very similar to its copper-containing counterparts of bacteria (11). Further, denitrification by C. tonkinense has been shown to support anaerobic cell growth (6). These findings are highly indicative that fungal denitrification is an energy-yielding process like the bacterial counterpart.

We proposed previously that the reactions catalyzed by Nar in F. oxysporum and by Nir in C. tonkinense might be coupled to the synthesis of ATP (4, 6, 11). We now demonstrate the intracellular localization of the reductases, characterize their electron donors, and show they are coupled to the synthesis of ATP.

EXPERIMENTAL PROCEDURES

Fungal Strains—F. oxysporum MT 811, first identified as a fungal denitrifier (4), and C. tonkinense IFO 30561, that was shown to grow under denitrifying conditions (6), were used throughout this work.

Subcellular Fractionation of F. oxysporum—Denitrifying cells (5 g, wet weight), cultivated in a glycerol-containing medium under microaerobic condition (4, 11), were disrupted by grinding in a mortar on ice with quartz sand (12.5 g) and 10 ml of sucrose buffer (0.8 M sucrose, 2 mM EDTA, 0.1% bovine serum albumin, and 10 mM Tris-HCl, pH 7.2) that contained 0.3 mM phenethylsulfonyl fluoride (PMSF). After addition of another 10 ml of the same buffer, the slurry was centrifuged 3 times at 1,500 g for 15 min to remove the quartz sand and undisrupted cells. The subsequent pellet obtained by two sequential centrifugations at 10,000 × g for 40 min was suspended in the sucrose buffer and then subjected to ultracentrifugation in a discontinuous sucrose density (43.1, 50.4, and 68.9%) gradient, at 105,000 × g for 180 min. The 10,000 × g supernatant was further fractionated into the soluble and microsomal fractions by centrifugation at 150,000 × g for 60 min. Each fraction obtained was incubated under aerobic conditions for 60 min at room temperature in the sucrose buffer containing 4 mM ADP, 4 mM MgCl₂, and 10 mM sodium phosphate to deplete endogenous electron donors. Finally, each fraction was pelleted by centrifugation at 150,000 × g and subsequently resuspended in the sucrose buffer.

Subcellular Fractionation of C. tonkinense—Denitrifying cells were cultured under initial aerobic condition (6). Subsequently, glucose in the reported culture medium was replaced with an equivalent level of glycerol. Cells were disrupted as above except that the buffer was 10 mM Tris-HCl, pH 7.4, containing 0.6 M mannitol, 0.1 mM EDTA, 0.1% bovine serum albumin, 0.3 mM PMSF, 0.3 mM leupeptin, and 0.5 mM taspigenylalanil chloride and 3-methyl ketone. Subcellular fractions were prepared as in the case of F. oxysporum above except that the 10,000 × g pellet was fractionated in a sucrose density gradient of 25, 35, 42, and 53%.

Fluorescence Microscopy—All manipulations were performed at room temperature. A drop of the putative mitochondrial fraction from F. oxysporum was smeared on a glass slide, air-dried, and fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline (10 mM sodium

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1 The abbreviations use are: Nar, nitrate reductase; Nir, nitrite reductase; Nor, nitric oxide reductase; P450, cytochrome P450; P450nor and P450nor2, cytochrome P450 nitrite reductase and its isozyme; PMSF, phenethylsulfonyl fluoride; PMS, phenazine methosulfate; TMPD, N,N,N',N'-tetramethylphenylenediamine; MVH, reduced methylvioleogen; TFA, thenoyltrifluoroacetone; Mes, 4-morpholinethanesulfonic acid.
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phosphate, pH 7.2, 150 mM NaCl). The sample was washed for 10 min in phosphate-buffered saline and blocked with BLOT BlockAce (Dainihonseiyaku Inc., Osaka, Japan) at 0.1% Triton X-100 for 30 min. Rabbit antiserum against Nir was diluted 1:200 with BLOT BlockAce and incubated with the sample for 2 h. The sample was then washed for 15 min in TBS-TX (10 mM Tris- HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100) and further incubated for 1 h with a drop of Texas Red-Linked antibodies against rabbit IgG raised in a donkey (Amersham Corp., Bucks, UK) and diluted 1:100 with BLOT Block containing 1 μg/ml Hoechst 33342 (Hoechst, Germany). The sample was washed for 15 min in TBS-TX, rinsed in TBS (TBS-TX without Triton X-100), and mounted in anti-fade solution (0.1% para-phenylenediamine, 10 mM Tris- HCl, pH 7.4, 150 mM NaCl, 90% glycerol). Confocal fluorescence images were obtained with a laser scanning confocal system (MRC-500; Bio-Rad) attached to a microscope with epifluorescence optics (BHS-RF; Olympus, Tokyo, Japan). Fluorescence due to Hoechst 33342 was recorded with a CD camera (model XC-73CE; Sony, Tokyo, Japan) and the MRC-500 system. The antiserum against Nir was produced by immunizing a rabbit with purified Nir from F. oxysporum (12) (Biologica, Nagoya, Japan). The specificity of the antiserum was confirmed by Western blot analysis (see Fig. 4C). A negative control was also prepared in which antiserum against Nir was replaced by preimmune rabbit serum. No staining with Texas Red-conjugated secondary antibody was observed (data not shown).

Determination of ATP—ATP was quantitated by the luciferin-luciferase bioluminescence technique with a Lucifer-LU kit (Kikkoman, Tokyo, Japan) and the MRC-500 system. The antiserum against Nir was produced by immunizing a rabbit with purified Nir from F. oxysporum (12) (Biologica, Nagoya, Japan). The specificity of the antiserum was confirmed by Western blot analysis (see Fig. 4C). A negative control was also prepared in which antiserum against Nir was replaced by preimmune rabbit serum. No staining with Texas Red-conjugated secondary antibody was observed (data not shown).

Assays of Nar and Nir—Nir activity was detected by quantitation of its product, nitrite, by the method of Nicholas and Nason (13). The reaction mixture for Nar (final volume, 2 ml) contained 10 mM sodium nitrate, 10 mM potassium phosphate, 1.5 mM sodium azide (to inhibit aerobic respiration), 5 mM electron donor, 0.8 μM P450nor, and an electron donor (5 mM) (total volume, 2 ml). ATP was quantitated by the luciferin-lucif-luciferase bioluminescence technique with a Lucifer-LU kit (Kikkoman, Tokyo, Japan) and the MRC-500 system. The antiserum against Nir was produced by immunizing a rabbit with purified Nir from F. oxysporum (12) (Biologica, Nagoya, Japan). The specificity of the antiserum was confirmed by Western blot analysis (see Fig. 4C). A negative control was also prepared in which antiserum against Nir was replaced by preimmune rabbit serum. No staining with Texas Red-conjugated secondary antibody was observed (data not shown).

RESULTS

Subcellular Localization of Nitrate Reductase and Nitrite Reductase—Denitrifying cells of F. oxysporum and C. tonkinense were disrupted and fractionated, and the distribution in the various subcellular fractions of Nar of F. oxysporum and Nir of C. tonkinense was investigated (Fig. 1). Fig. 1A shows distribution of Nar activity in fractions from F. oxysporum. In addition to an artificial electron donor (reduced methylviologen (MVH)), respiratory substrates, malate plus pyruvate, were effective donors of electrons for the activity. Although MVH-dependent Nar and the marker enzyme (cytochrome oxidase) activities were recovered in various fractions, Nar activity supported by malate plus pyruvate was enriched in fraction II (50.4% sucrose). The result is highly indicative that the fraction contained intact mitochondria. Nir activity was also detected in fraction II of F. oxysporum (data not shown). This fraction additionally contained cytochromes a and b (Fig. 2), as well as cytochrome oxidase activity (Fig. 1A).

Fig. 1B shows distribution of cytochrome oxidase and Nir among various subcellular fractions of C. tonkinense. Most of the NADH-PMS-dependent Nir activity was recovered in the 35% sucrose fraction (fraction 2). Most of cytochrome oxidase activity was also recovered in this fraction, whereas catalase (a marker enzyme of peroxisome) activity was primarily in the 42% sucrose fraction (fraction 3) (data not shown). Apparently, mitochondria of C. tonkinense grown under micro-aerobic con-
ditions are less dense and/or have a smaller sedimentation coefficient than those of *F. oxysporum*.

**Electron Donors**—As shown above, malate plus pyruvate were effective donors of electrons to the Nar activity of the mitochondrial fraction of *F. oxysporum*. This suggests that electrons were supplied to Nar from these respiratory substrates via the mitochondrial electron transport system. As shown in Fig. 3, formate and succinate were also effective electron donors for Nar. In contrast, NADH is ineffective in donating electrons to the activity, consistent with the fact that NADH does not permeate into the mitochondrial matrix. Formate is a physiological electron donor for the reduction of nitrate in the nitrate respiration system of *Escherichia coli* (14, 15) and the fungal Nar system resembles the bacterial system in this regard. Malate plus pyruvate and succinate were also effective donors of electrons to the mitochondrial Nir activity of *C. tonkinense* (see Figs. 6 and 8).

**Mitochondrial Fluorescence Microscopy**—Further evidence that fraction II from *F. oxysporum* really contained mitochondria was obtained by fluorescence microscopy (Fig. 4). Staining by the indirect fluorescent antibody technique showed that particles in fraction II contained Nir (Fig. 4A). These also reacted with the DNA-specific dye (Fig. 4B) showing that they were mitochondria. The results of immunostaining (Fig. 4A) further supported our previous assertion that the Nir of this fungus is located in the intermembrane space of the mitochondrial (11).

**Inhibition of the Mitochondrial Nar and Nir Activities by Detergent and Respiratory Inhibitors**—The effectiveness of various respiratory substrates as electron donors for the mitochondrial activities of Nar and Nir are strong evidence that the fungal denitrifying system was associated with the mitochondrial respiratory chain. This was confirmed by the effects of treatments with a detergent or respiratory inhibitors on the mitochondrial Nar and Nir activities. The malate plus pyruvate-dependent mitochondrial Nar activity of *F. oxysporum* was lost upon treatment with deoxycholate, while the MVH-dependent activity was unaffected (Fig. 5). Inhibitors of respiration, namely rotenone, antimycin A, and thenoyltrifluoroacetone (TTFA), strongly inhibited the mitochondrial Nir activity of *C. tonkinense* (Fig. 6, A and B), while they did not affect the ascorbate-TMPD-dependent activity (Fig. 6C). Deoxycholate (Fig. 5) apparently did not inhibit Nar activity itself but destroyed the aligned electron transport system for oxidation of pyruvate and malate. Thus, pyruvate- and malate-dependent Nir activity was inhibited by rotenone and antimycin but was not inhibited by TTFA, while succinate-dependent Nir activity was inhibited by antimycin and TTFA but not by rotenone (Figs. 6, A and B). Therefore, the fungal Nir shares the mitochondrial electron transport system for aerobic respiration and requires electrons from complex III as do the bacterial nitrite reductases (1).
Synthesis of ATP Coupled to the Mitochondrial Nar and Nir Activities—

The mitochondrial reactions catalyzed by Nar and Nir were coupled to the synthesis of ATP (Figs. 7 and 8). The energy yield (P:NO$_3^-$ or P:2e ratio) (1, 16) was 1.4 for the pyruvate/malate-dependent reaction catalyzed by Nar (at 3 min), and it was 1.0 and 0.88 for the pyruvate- and malate-dependent and the succinate-dependent reaction catalyzed by Nir (between 2 and 6 min), respectively. The synthesis of ATP was also inhibited by antimycin A (data not shown). No synthesis of ATP was observed in replicate experiments in which the mixture of pyruvate and malate together or succinate was replaced by Nar or Nir artificial electron donors, even though formation of the reaction products, namely nitrite or NO, were detected (data not shown).

**DISCUSSION**

Our results clearly demonstrate the presence of novel forms of respiratory metabolism in mitochondria of denitrifying fungi. While the mitochondrion has been regarded as the aerobic organelle of eukaryotes, the occurrence of anaerobic respiration in fungal mitochondria evokes interesting questions. First, what is the evolutionary relationship between the fungal and bacterial denitrifying systems? We previously determined that Nir of F. oxysporum bears close resemblance to the copper-
The morphology of mitochondria during the anaerobic growth also deserves attention. The mitochondria of yeasts exist in a "pseudomitochondrial" form when cells are growing under anaerobic, fermenting conditions (21). In contrast, fungal mitochondria are only moderately modified during exposure to denitrifying conditions (Fig. 4). The fungal cells used in this study were grown under a micro-aerobic or an initially aerobic (4, 6) condition and cytochrome oxidase was also detected (Figs. 1 and 2). However, the denitrifying systems of F. oxysporum and C. tonkinense differ in response to oxygen tensions. The system of F. oxysporum seems to be induced to supplement the aerobic respiration (22), while that of C. tonkinense acts more positively to support the growth even under further restricted aeration (6). The morphological and electron transport capabilities of mitochondria in C. tonkinense that grow under denitrification, restricted aeration conditions deserves further study.

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