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Confirmation of Frm2 as a novel nitroreductase in *Saccharomyces cerevisiae*

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ABSTRACT

Nitroreductases comprise a group of FMN- or FAD-dependent enzymes that reduce nitrosubstituted compounds by using NAD(P)H, and are found in bacterial species and yeast. Although there is little information on the biological functions of nitroreductases, some studies suggest their possible involvement in oxidative stress responses. In the yeast *Saccharomyces cerevisiae*, a putative nitroreductase protein, Frm2, has been identified based on its sequence similarity with known bacterial nitroreductases. Frm2 has been reported to function in the lipid signaling pathway. To study the functions of Frm2, we measured the nitroreductase activity of purified Frm2 on 4-nitroquinoline-N-oxide (4-NQO) using NADH. LC-MS analysis of the reaction products revealed that Frm2 reduced NQO into 4-aminoquinoline-N-oxide (4-AQO) via 4-hydroxyaminoquinoline (4-HAQO). An Frm2 deletion mutant exhibited growth inhibition in the presence of 4-NQO. Thus, in this study, we demonstrate a novel nitroreductase activity of Frm2 and its involvement in the oxidative stress defense system.

1. Introduction

Nitroreductases are a family of proteins with conserved sequences initially found in Eubacteria and grouped together based on their sequence similarity. These enzymes can catalyze the reduction of nitrosubstituted compounds, using FMN or FAD as a cofactor and NADH or NADPH as a reducing agent. Bacterial nitroreductases are classified into 2 groups based on their biochemical characteristics. Type I nitroreductases (oxygen-insensitive) catalyze the reduction of nitro groups via sequential two-electron reductions to nitroso and hydroxylamine intermediates, and finally to primary amines [1–3]. Type II nitroreductases (oxygen-sensitive) catalyze a one-electron reduction of the nitro group to produce a nitro-anion radical that subsequently reacts with oxygen to form a superoxide radical and the original nitroaromatic compound. This "futile cycle" can cause oxidative stress by producing large amounts of superoxides [4]. Type I nitroreductases are known to catalyze the reduction of organic nitroaromatic and nitroheterocyclic compounds, such as nitrobenzene, 2,4,6-trinitrotoluene (TNT), nitrofurazone, metronidazole, and nitrofurantoin [5–8]. Type I genes have been reported and studied in *Escherichia coli* [9], *Salmonella typhimurium* [10], *Enterobactercloacae* [11], and *Helicobacter pylori* [12]. Genes encoding bacterial type II nitroreductases have not been cloned till date [13]. The distribution of nitroreductase-like sequences is not as common in eukaryotic cells as it is in prokaryotic cells.

In the yeast *Saccharomyces cerevisiae*, 2 genes, *frm2* (YCL026c-A) and *hbn1* (YCL026c-B), encoding putative nitroreductase-like proteins were identified by *in silico* analysis [14]. The biological functions of the nitroreductase family of proteins are not well studied; however, their possible involvement in oxidative stress responses has been suggested [15]. Although the function of the Frm2 protein is not fully understood, experimental data from McHale et al. indicate that Frm2 may be involved in the lipid signaling pathway and cellular homeostasis [16].

The functionally closest relative of Frm2 is *Lactococcuslactis* CinD, which is a component of a regulon in the copper-responsive system. Purified CinD is a flavoprotein and reduces 2,6-dichlorophenolindophenol and 4-nitroquinoline-N-oxide (4-NQO) using NADH as a reductant. The next closest relative of Frm2, NfsA of *E. coli*, exhibits broad substrate specificity typical of most nitroreductases. The structure of CinD has been resolved at a resolution of 1.7 Å [17] and closely resembles the structures of other enzymes belonging to the oxygen-insensitive nitroreductase family. NfsA has been shown to participate in the degradation of TNT [18]. This

Abbreviations: 4-NOSO, 4-nitrosoquinoline-N-oxide; 4-NQO, 4-nitroquinoline-Noxide; 4-AQO, 4-aminoquinoline-N-oxide; LC–MS, liquid chromatography–mass spectrometry.

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suggests that nitroreductases may play an important role in xenobiotic metabolism.

In this study, we measured the nitroreductase activity of Frm2 on 4-NQO. Based on its ability to use 4-NQO as a substrate and mass analysis of the reaction products, we confirm for the first time that Frm2 is a novel nitroreductase.

2. Materials and methods

2.1. Cloning of Frm2

The *Frm2* gene (YCL026C-A) was cloned by PCR using the following primers: Frm2-F, 5′-CGCGGATCC ATG GGC AAT ATC CTT CGG AAA GG-3′, and Frm2-R, 5′-ATAAGAATGCGGCCGC CAG GAG CTG TCT AAC CAG AGC-3′. The Frm2-F and Frm2-R primers were designed to contain an *Eco*RIsite and a *Not*I site. The PCR product containing the *Frm2* gene was cloned into the yeast expression vector pECS-URA digested with *Eco*RI and *Not*I.

2.2. Protein expression and purification

Escherichia coli BL21 DE3 transformed with the expression plasmid pET28a was cultured to mid-log phase at 37 °C in Luria–Bertani broth containing 30 μg/mLof kanamycin, cooled to 20 °C, and induced with 1 mM isopropyl-D-thiogalactoside. Growth was continued for 9 h, and the cells were harvested by centrifugation at 3000×*g* for 10 min at 4 °C. Cell pellets were washed with extraction buffer (50 mM HEPES at pH 7.9, 500 mM NaCl, 1 mM EDTA, 1% NP40, and 10% glycerol). The final pellet was resuspended in extraction buffer, and a 1/100 volume of a protease inhibitor cocktail and 1 mM DTT were added. The cells were disrupted using a Sonicator (Vibra-cell VCX600; Sonics and Materials Inc., USA) and centrifuged at 10,000×*g* for 20 min at 4 °C. Recombinant his6-tagged FRM2s were purified by nickel-affinity chromatography (Peptron, Korea). Enzyme purity was confirmed by SDS–PAGE. Purified proteins were stored at −70 °C. Cleavage of the N-terminal his6 tag with thrombin did not significantly affect the activity of any of the Frm2 proteins. Protein concentrations were determined with the Bio-Rad assay kit (Bio-Rad, CA, USA) using BSA as a standard.

2.3. Enzyme assay

Steady-state enzyme kinetics of purified Frm2 was spectrophotometrically assessed at 340 nm. Assays were performed in 1 mL of 50 mM Tris–HCl (pH 7.5) containing 10 μM FMN, 0.2 mM NAD(P)H, and 0.05 mM 4-NQO. Reactions were started by adding 500 nM enzyme and changes in absorbance were measured for 20 min.

2.4. MS/MS analysis of the reaction products

After the enzymatic reaction, 2 μL of supernatant was analyzed by liquid chromatography followed by mass spectrometry (LC–MS) in parallel with a control reaction sample. LC–MS was performed using a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Electron Co., USA) equipped with an electrospray ionization (ESI) source. Separation by UHPLC was performed on a Thermo Accelar System (Thermo Electron Co.), using a ACQUITY BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters, Ireland). Water and acetonitrile were used as mobile phase A and mobile phase B, respectively, and both contained 0.1% formic acid. Gradient elution was carried out at a flow rate of 0.45 mL/min as follows: 0–4 min with 10–80% B (linear gradient) and 4–5 min with 80–100% B (linear gradient). Full-scan mass spectra were obtained in the positive ion modes at an *m/z* range of 100–1000. All experiments were controlled by the menu-driven software provided with the system and were performed under automatic gain control conditions.

3. Results

3.1. Frm2 reduces 4-NQO using NADH as a reducing agent

Because Frm2 exhibited sequence similarity with known bacterial nitroreductases, such as NfrA and CinD, we first tested whether the Frm2 protein reduced nitrosubstituted compounds. By monitoring the decay of NADH or NADPH at 340 nm, we measured the enzymatic activity of purified Frm2 in the presence of a nitrosubstituted compound. We tested 10 different nitrosubstituted compounds, including TNT. As a control for the nitroreductase reaction, we used NfsA that was cloned from *E. coli* and purified using Ni column. NfsA exhibited normal nitroreductase activity on 4-NQO with NADPH as a reducing agent but relatively weak activity with NADH. NfsA is known to use NADPH as a reductant. Frm2 reduced 4-NQO with NADH as a reducing agent, but the activity was relatively weak compared to that of NfsA. Frm2 preferred NADH to NADPH (Fig. 1).

3.2. Frm2 is a novel nitroreductase

To further confirm the Frm2-mediated reduction of 4-NQO, we analyzed the products of the enzymatic reaction using LC–MS. When 4-NQO (*m/z* 190, $[M–H]^-$) was incubated without the enzyme, the mass peak for 4-NQO appeared at the expected position. When Frm2 was added to the reaction mixture, a new peak appeared in the chromatogram. Mass analysis of that peak revealed the Frm2-mediated production of 4-hydroxyaminoquinoline-N-oxide (4-HAQO) (*m/z* 351, $[M–H]^-$), the two-electron reduction product of 4-NQO (Fig. 2). A dimer peak was detected for the final reduction product, 4-aminoquinoline-N-oxide (4-AQO) (*m/z* 320, $[M–H]^-$) in the chromatogram. Furthermore, 4-HAQO was also detected as a dimer peak (Fig. 2). These results from the LC–MS analysis clearly support our conclusion that Frm2 is indeed a nitroreductase that can reduce 4-NQO to 4-AQO via 4-HAQO as a two-electron reduction intermediate (Fig. 4).

3.3. Frm2 defends yeast cells from oxidative stress

To determine whether Frm2 participated in the defense mechanism against 4-NQO, we measured the effect of Frm2 on the growth of yeast cells in a medium containing 4-NQO. Under these conditions, growth of the wild-type yeast cells decreased slightly, whereas growth of the deletion mutant was reduced (Fig. 3). The reduction of 4-NQO by Frm2 inhibits the futile oxidation of 4-NQO, which produces reactive oxygen species (ROS), and relieves the toxic effect of 4-NQO on the cell. Furthermore, western blotting analysis revealed that 4-NQO induced the expression of Frm2 (data not shown). Collectively, these results led us to conclude that Frm2 facilitates the reduction of 4-NQO to 4-AQO and confers resistance to oxidative stress.

4. Discussion

Frm2 was initially identified as a repressor of a regulon controlled by exogenous fatty acids. A number of potential response elements important for regulating gene expression in response to a carbon source were identified in the upstream region of *frm2*. Furthermore, *lacZ* activity driven by Frm2 or *Frm2* promoters was induced two- to three-fold by fatty acids. These observations led to the conclusion that Frm2 functions in the fatty acid signaling pathway and is regulated by fatty acids [16].

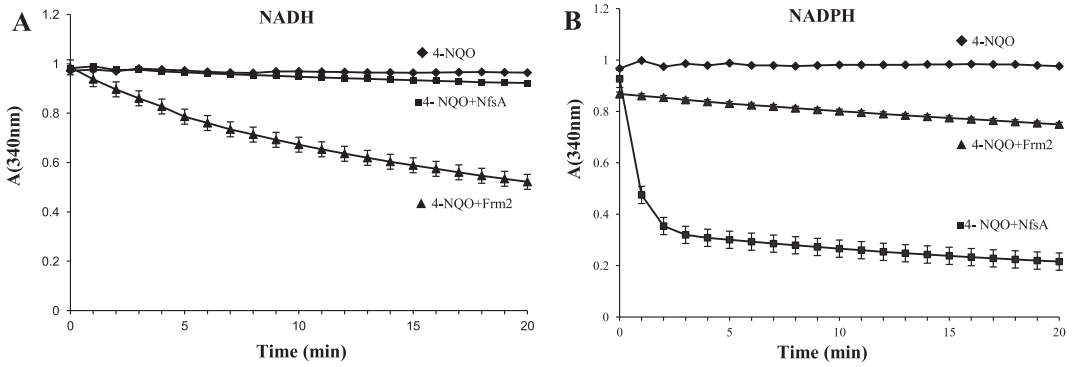

Fig. 1. Acceleration of 4-nitroquinoline-N-oxide (4-NQO) decomposition by purified Frm2. The Frm2 reaction was indicated by a decrease in absorbance at 340 nm. Before the addition of Frm2, the reaction mixture was pre-incubated with 4-NQO for 1 min, and then Frm2 was added to the solution to start the reaction.

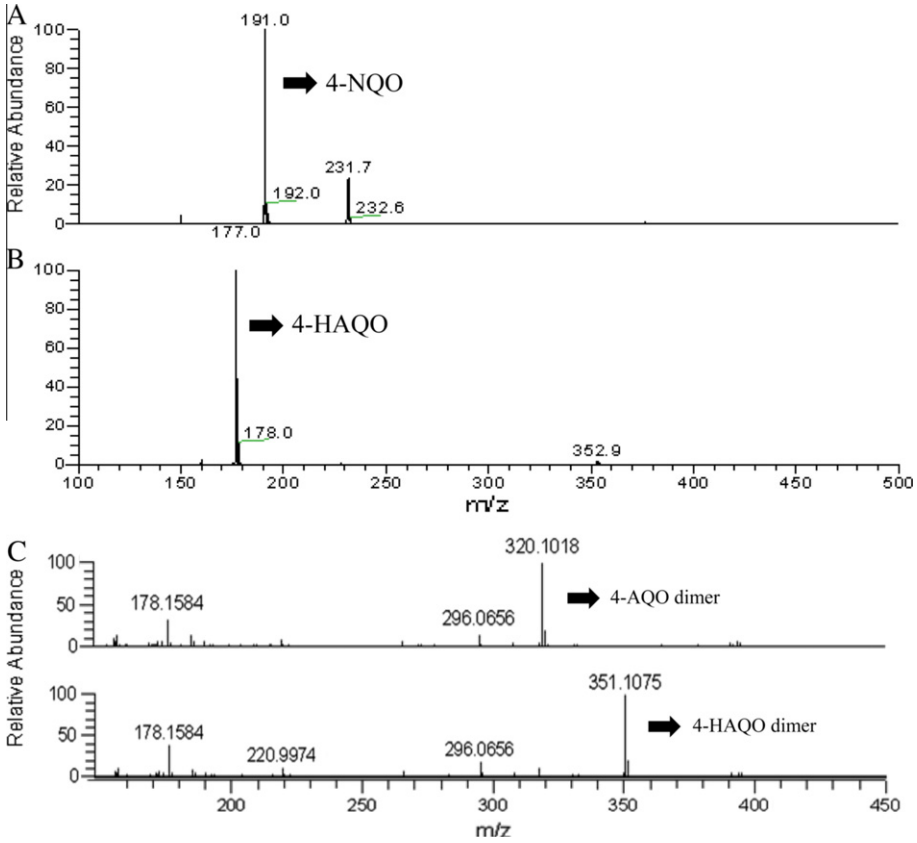

Fig. 2. LC–MS analysis of the products derived from the reaction mixture containing Frm2. (A) Mass peaks obtained from 4-NQO. (B and C) Mass peaks from the reaction mixture of 4-NQO plus Frm2. Samples were immersed in a water bath at 30 °C. After 20 min, a liquots were withdrawn for analysis. All mass peaks were obtained in the positive ion mode.

Recently, Frm2 was identified as a nitroreductase based on *in silico* screening. Although Frm2 is known to participate in the lipid signaling pathway, its exact function is unknown. An in-depth phylogenetic analysis suggested a function for Frm2 and the presence of homologous sequences in other prokaryotic and eukaryotic species. The results showed that bacterial cells have Frm2/Hbn1-like-sequences and form a distinct clade within the fungal Frm2p/Hbn1p family. Hydrophobic cluster analysis and three-dimensional protein modeling clearly revealed that Frm2 is a putative nitroreductase [17].

Evaluation of Frm2 functions revealed reduced basal activity of superoxide dismutase (SOD), ROS production, lipid peroxidation, and higher sensitivity to 4-nitroquinoline-oxide (4-NQO), as well as higher basal activity of catalase (CAT) and glutathione peroxidase (GPx) and reduced glutathione (GSH) content in the single- and double-mutant strains *frm2Δ* and *frm2Δ hbn1Δ*. These strains also exhibited less ROS accumulation and lipid peroxidation when exposed to the peroxides H_2O_2 and *t*-BOOH. Frm2 and Hbn1 influence the oxidative stress response in *S. cerevisiae* by modulating the GSH content and activities of antioxidant enzymes, such as SOD, CAT, and GPx [19].

In *L. lactis* IL1403, 14 genes are controlled by the copper-inducible CopR repressor. One of these genes, *ytjD*, which we renamed *cinD* (copper-induced nitroreductase) is induced by copper, cadmium, and silver *in vivo*. A knockout mutant of *cinD* is more sensitive to oxidative stress exerted by 4-NQO and copper. The physiological properties of CinD are very similar to those of Frm2. Purified CinD is a flavoprotein and reduces 2,6-dichlorophe-

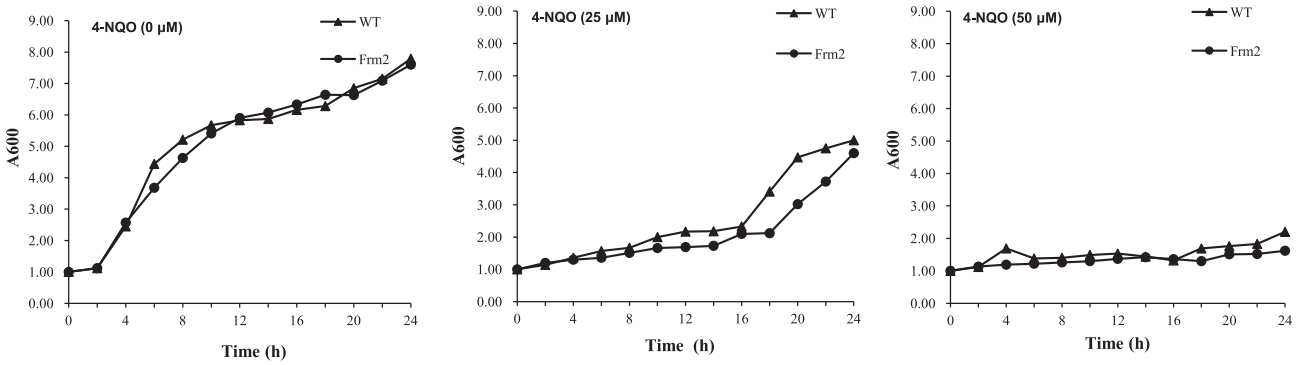

Fig. 3. Growth curves of wild-type (WT) and Frm2-deletion strains. One-day cultures of the strains at 30 °C in medium containing 4-NQO.

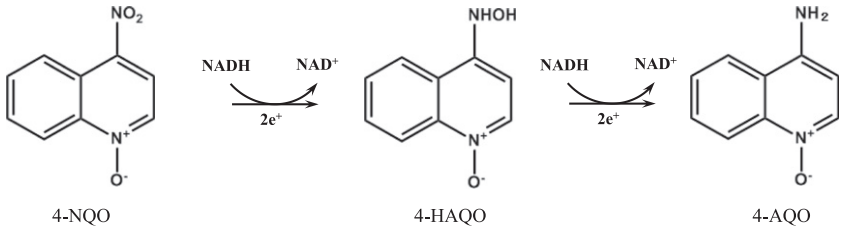

Fig. 4. Schematic representation of the Frm2 pathway. Reduction of 4-NQO is mediated by Frm2 via a two-step two-electron reaction using NADH as the reducing agent.

nolindophenol and 4-NQO with *k*cat values of 27 and 11 s^{-1}, respectively, using NADH as a reductant. The X-ray structure of CinD resembles those of other nitroreductases. Our preliminary X-ray data of Frm2 are also very similar to that of CinD. CinD is a nitroreductase that can protect *L. lactis* against oxidative stress exerted by nitroaromatic compounds and copper [17]. Frm2 may play a similar role in yeast cells.

Although further studies are required to identify the true substrate of Frm2 in cells, Frm2 can serve as a functional enzyme in nitroaromatic compound metabolism. Based on our current findings, we propose that Frm2 is a novel nitroreductase that catalyzes an important step in the nitroaromatic compound catabolic pathway.

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