In Silico Analysis of NADH-Dependent Nitroreductase-Like Protein from *Aspergillus parasiticus* and *Aspergillus caelatus*

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Abstract. Nitroreductases have potential use in the bioremediation of nitroaromatic compounds and prodrug activation. Reports about this enzyme from bacteria are abundant; however, research on fungal nitroreductase is scarce. *A. parasiticus* nitroreductase (ApNtr) and *A. caelatus* nitroreductase (AcNtr), discovered via whole-genome sequencing, were studied. The physicochemical characteristics showed that ApNtr is cationic and AcNtr is anionic. Both proteins shared a common ancestor. The analysis of ApNtr and AcNtr structures shows that both proteins are comparable to nitroreductase from *Saccharomyces cerevisiae* and *Lactococcus lactis*, suggesting that these enzymes have similar functions, which are NADH-dependent nitroreductases.

Keywords: *Aspergillus*, *In Silico*, Nitroreductase.

1.0 Introduction

Nitroaromatic compounds are components of several chemicals that are widely used in agriculture, for example, herbicides and antibiotics. These compounds are stable in the environment and are toxic to living organisms. Enzymes produced by microorganisms are being developed as a tool to breakdown environmental pollutants. Nitroreductases are a class of enzymes that catalyze the reduction of nitroaromatic compounds such as nitrobenzene, trinitrotoluene, and nitrofurans. Nitro compounds are toxic and mutagenic to living organisms; thus, nitroreductases are potentially used in bioremediating those compounds. The enzymes use FMN or FAD as a cofactor and are homodimers. The nitroreductase monomers forms an □ and β fold motifs Most nitroreductases that have been reported are produced by bacteria. Bacterial nitroreductases are classified as oxygen-insensitive nitroreductases (Type 1), which catalyze two electron transfers, and oxygen-sensitive enzymes (Type 2), which generate one electron transfer. Type I nitroreductase is classified as Group A and Group B. Group A is a group of microorganisms that use only NADPH as the electron donor, and members of Group B use either NADH or NADPH as the reductant during enzyme action. NfsA and NfsB from *E. coli* are examples of Group A and B members, respectively. Reports of these nitroreductases as prodrug activating enzymes in gene

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therapy are abundant [1, 2]. Although work on the discovery of the gene and mechanism of nitroreductases is widely reported, the physiological function of this enzyme is unknown. Nitroreductases from bacteria are widely reported; however, knowledge about this enzyme from fungal species is limited. The whole genome sequencing of *Aspergillus parasiticus* and *Aspergillus caelatus* published reveals the presence of a nitroreductase-like protein gene (https://www.ncbi.nlm.nih.gov) [3]. Studying the protein structure of this gene can give insight into the protein mechanism. This contributes to new knowledge about the newly discovered gene, which is beneficial for the future development of this enzyme for biomedical and biotechnological applications.

The present work explains the function of a nitroreductase-like protein from *A. parasiticus* and *A. caelatus* by reporting the characteristics of the enzyme catalytic region. The similarities between the nitroreductase protein structures predicted from the Aspergillus gene sequence and those of known nitroreductases are discussed. The presence of conserved amino acid sequences that match the NADH or NADPH binding region of a known nitroreductase is detected via structural superposition. This leads to the classification of the two proteins as either using NADH or NADPH as electron donors, thus giving insights into their functions.

2.0 Literature Review

2.1 Nitroreductase

Nitroreductase is a group of enzymes that catalyses the nitroreduction reaction. However, the members of this diverse group also catalyze a range of reactions, e.g., dehydrogenation and dehalogenation [4]. The capability of nitroreductases in degrading nitro compounds and their involvement in the oxidative stress response, biosynthetic pathway, and bioluminescence process show the enzyme has potential in various biotechnology applications. These enzymes have also been used in prodrug applications for cancer treatments to activate the release of anti- cancer drugs [5, 6]. Bacterial type I nitroreductase has been discovered in *Escherichia coli*, *Salmonella typhimurium*, *Lactococcus lactis*, *Vibrio fischeri*, and *Bacillus subtilis.* However, to the authors knowledge, no type II gene has been cloned or published. *Escherichia coli* NfsA and NfsB (nitrofuran-sensitive A and B) are two of the most studied nitroreductases. The genes were discovered in *E. coli* resistant to nitrofuran antibiotics [7[. These enzymes, particularly NfsB, have been developed for cancer gene therapy with nitroaromatic prodrugs [8].

2.2 Nitroreductase from *S. cerevisiae*

Literature searches show *Saccharomyces cerevisiae* and *Taiwanofungus camphorata* are the only fungi that have been described as exhibiting nitroreductase activity [9, 10]. The Frm2 gene of *S. cerevisiae* has been identified based on its

sequence similarity with known bacterial nitroreductases [12]. The expression of this gene in *E. coli* resulted in recombinant nitroreductase, and the purified enzyme demonstrated significantly higher activity in the presence of substrate and NADH than in the presence of NADPH as the reductant [9]. A CinD gene codes a copper-induced oxygen-insensitive nitroreductase that functions in defense against oxidative stress. This nitroreductase produced by *Lactococcus lactis* showed a high reduction of DCPIP and 4-nitroquinoline-N-oxide (4-NQO) with NADH as the electron donor. The enzyme also showed significant catalase activity [13]. It was discovered that Frm2 catalyzes 4-NQO primarily with NADH and has minor activity with NADPH. The comparison of the nitroreductase Frm2 (PDB ID: 4URP) crystal structure with that of bacterial nitroreductases showed the Frm2 protein lacks the helical domain arrangement that is typically observed in the NADPH binding region within Group A or B enzymes.

2.3 Nitroreductase from *T. camphorata*

The nitroreductase gene of *T. camphorata* (TcNr) was cloned into the *E. coli* expression system, and the enzyme was purified. The protein sequence alignments of TcNr with well-studied nitroreductases revealed conserved amino acids in the catalytic motif, LQHY, and the FMN binding domain, RR. This showed that TcNr belongs to the nitroreductase family. Enzyme assays of the purified TcNr exhibited nitroreductase activity when reductions of nitroaromatic compounds were determined in the presence of NADPH and NADH, respectively, thus confirming the enzyme function. However, in the presence of NADH, the reduction rate of DCPIP and potassium ferricyanide was higher than in the presence of NADPH, showing that the former is a better reductant for TcNr [10].

3.0 Methodology

3.1 Nitroreductase Sequence Data Mining

The nitroreductase protein sequences of *Aspergillus parasiticus* and *Aspergillus caelatus* translated from the genome sequence were retrieved from UniProt (https://www.uniprot.org/) (Table 1) and used as the targets in this study. The nitroreductase sequences of *Saccharomyces cerevisiae* and *Lactococcus lactis* as references were also downloaded. Table 1 summarizes information on the nitroreductase sequences used in the present work. The proteins were named as follows: ApNtr (*A. parasiticus* nitroreductase), AcNtr (*A. caelatus* nitroreductase), Frm2 (*S. cerevisiae* Frm2 nitroreductase), and LiNtr (*L. lactis* nitroreductase).

Table 1. Nitroreductase and nitroreductase-like proteins used in this study.

Organism	Protein Name	GenBank Accession Number	UniProt ID	PDB ID	References
Aspergillus parasiticus CBS117618	ApNtr	KAB8206525.1	A0A5N6DN37	Not Available	[3]
Aspergillus caelatus CBS763.97	AcNtr	KAE8370794.1	A0A5N7ALK3	Not Available	
Saccharomyces cerevisiae ATCC204508	Frm2	DAA07459.1	P37261	4URP	[11]
Lactococcus lactis IL1403	LiNtr	AAK06011.1	Q9CED0	4BNB	[13]

3.2 Nitroreductases Sequence Analysis

Comparisons of ApNtr, AcNtr, Frm2, and LiNtr protein sequences were performed using the Clustal Omega Program (https://www.ebi.ac.uk/Tools/msa/clustalo/). The conserved amino acids located in the catalytic sites were analyzed in order to predict the ApNtr and AcNtr functions. The molecular weight and isoelectric point (pI) of the target protein were computed using the ProtParam (https://web.expasy.org/protparam/) web server.

3.3 Protein Structure Analysis

The UniProt ID in Table 1 was used to retrieve the predicted protein structure of ApNtr and AcNtr from the AlphaFold Protein Structure Database (AlphaFold DB; https://alphafold.ebi.ac.uk/) (Varadi, *et al*., 2022) [14], whereas the structure of Frm2 and LiNtr was downloaded from the Protein Data Bank (https://www.rcsb.org/). The structures were visualized using PyMOL (http://www.pymol.org/). The target proteins ApNtr and AcNtr were used in global structural superimposition onto Frm2 and LiNtr as reference structures (http://www.pymol.org/). The superimposition was performed to determine the similarity between the listed nitroreductase structures and to predict ligand-binding residues at the ApNtr and AcNtr catalytic sites.

4.0 Results and Discussion

4.1 Sequence Alignment of ApNtr, AcNtr, Frm2, and LiNtr

Aspergillus parasiticus (ApNtr) and *Aspergillus caelatus* (AcNtr) nitroreductase have 206 amino acid residues and a molecular weight of 23.2 kDa. The size of the open reading frame (ORF) is 621 bp. However, nitroreductase forms

homodimer to be functional. The computed pI values of ApNtr and AcNtr were 7.89 and 6.98, suggesting that ApNtr is cationic and AcNtr is anionic at physiological pH. Multiple sequence alignment (MSA) results show that ApNtr shares 95.63% sequence similarity with AcNtr, indicating that the two nitroreductases have a common ancestor. Both protein sequences showed presence of the conserved amino acids in the FMN binding domain (R15, R16) and in the catalytic domain (P44, W138, L150, Q151, H152). These amino acids are conserved in LiNtr and Frm2, thus their existence indicates that ApNtr and AcNtr are nitroreductases (Figure 1). Therefore, ApNtr and AcNtr have identical functions with LiNtr and Frm2. Earlier work described LiNtr and Frm2 as functional enzymes in nitroaromatic compound metabolism, and they protect the organism against oxidative stress [13, 9].

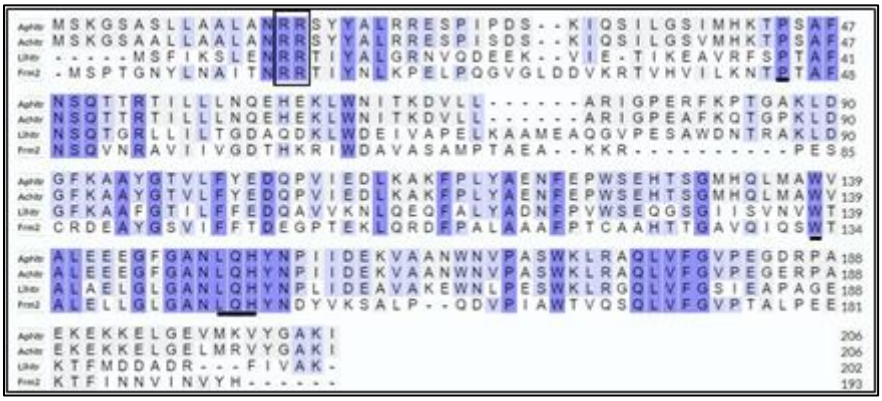

Fig. 1. Multiple sequence alignment of the AcNtr, LiNtr, and Frm2 protein sequences. The sequences in shaded blue, light blue, and grey denote 100, 75, and 50% sequence conservation. The box shows the two arginines that are conserved in the FMN binding domain. The underlines show amino acids that are conserved in the nitroreductase catalytic domain [15].

4.2 ApNtr and AcNtr 3D Models

Predicted protein models of ApNtr and AcNtr were retrieved from the AlphaFold DB. AlphaFold uses deep learning AI code to forecast a protein 3D structure from its amino acid sequence. Both models show a high per-residue confidence score (90 > pLDDT > 70), showing the prediction is highly accurate. The low predicted aligned error (PAE) value shown by the shade of dark green indicates that the expected distance error in residues position is low (Figure 2A and 2B). Therefore, the structure of ApNtr and AcNtr generated by the AlphaFold program shows a good protein backbone prediction. The ApNtr and AcNtr structures possessed a typical nitroreductase fold that has been observed in most studied nitroreductases, for example, the NfsA (PDB ID: 7Q0O) and NfsB (PDB ID: 7X32). Figure 2 shows the predicted structural models of ApNtr and AcNtr.

4.3 Comparative Analysis of the Nitroreductase Models

Structural comparisons were performed to evaluate the similarity between the models. Structures are relatively more conserved than sequence, and related proteins generally maintain similar structural folds [16]. The structural conservation of certain regions within the compared protein models is a strong indication that the region is essential for the protein's function. Song et al. [17] have suggested that Frm2 and its homologues are distinct from those of Group A and Group B of Type I nitroreductase. Frm2 primarily uses NADH during the reduction of 4- nitroquinoline-N-oxide (4-NQO), which indicates that Frm2 favours NADH over NADPH as an electron donor during its catalysis.

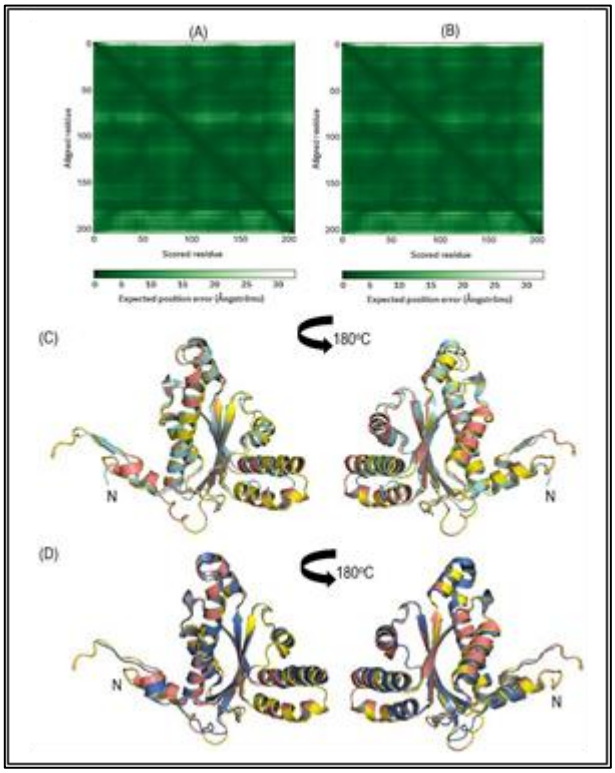

Fig. 2. The predicted aligned error (PAE) plot and the structures of ApNtr and AcNtr. (A) and (B) are the PAEs of ApNtr and AcNtr. A dark green area was observed for both of the models. (C) and (D) are the superimposition of ApNtr (in pink) and AcNtr (in yellow) structures with Frm2 (in cyan) and LiNtr (dark blue). No significant structural differences were observed between models of ApNtr and AcNtr with Frm2 and LiNtr. The N terminal is showed.

The analysis of ApNtr and AcNtr structures shows that both proteins are comparable to Frm2. The pairwise alignment of the ApNtr and AcNtr models with Frm2

showed a RMSD value of 0.819 Å for 142 aligned atoms and 0.859 Å over 141 aligned atoms, respectively. In comparison to Frm2, no noticeable contrast of helical and beta-sheet domains was observed in the ApNtr and AcNtr structures (Figure 2C). Similarly, ApNtr and AcNtr have the same structure fold as LiNtr, with pairwise structural comparison yielding RMSD values of 0.522 and 0.493, respectively, over 132 aligned atoms (Figure 2D). Via structure superposition, the catalytic sites of ApNtr and AcNtr and the specificity of NADH and NADPH as the electron donors in the enzyme activity were postulated. In comparison to the wild-type Frm2, a single mutation at R82A and R82E, respectively, produced low enzyme activity in the presence of NADH, suggesting R82 is a significant amino acid in the Frm2 interaction during enzyme catalysis [17]. Within ApNtr and AcNtr, the R is replaced by K, and this amino acid corresponds to K88 of LiNtr, which is located near the NO2 moiety of 4-NQO. (Figure 3). Therefore, this research implies that ApNtr and AcNtr serve a similar role as Frm2 and LiNtr in the degradation of 4-NQO. Furthermore, these Aspergillus nitroreductase-like protein architectures are identical to Frm2, which lacks prominent helical domains that can bind to NADPH. Because the folding of ApNtr and AcNtr's catalytic domains is identical to that of Frm2, it is presumed that these proteins have the same properties as Frm2, which prefers NADH over NADPH as an electron donor during catalysis. The enzyme assays of nitroreductase from *Saccharomyces cerevisiae*, *Lactococcus lactis*, and *Taiwanofungus camphorate* [10, 13, 17] showed that the enzyme favored NADH over NADPH during the reduction of 4-NQO.

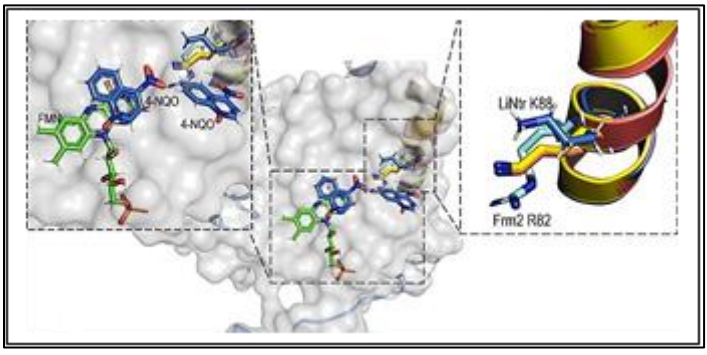

Fig. 3. The active site of the nitroreductase. (A) shows the location of the substrate 4-NQO (in blue) and FMN (in green); (B) is the K88 sidechain of ApNtr (in pink) and AcNtr (in yellow) that is enlarged. The conformation is in a similar direction with K88 of LiNtr (in blue) and R82 of Frm2 (in cyan). This suggests that the characteristics of ApNtr and AcNtr binding to 4-NQO are the same with LiNtr and Frm2 Interactions.

5.0 Conclusion

As a conclusion, analysis using in silico approaches predicted that ApNtr and AcNtr have the same biochemical function as Frm2 and LiNtr, whereby these enzymes

can reduce 4-NQO as substrate using NADH as a reducing agent. Therefore, these enzymes can be classified under the same classification group as Frm2. However, experimental work needs to be conducted to confirm this finding. Through cloning of the ApNtr and AcNtr genes and evaluation of the recombinant enzyme activity, the preferences for NADH as a reductant and the enzymes functional characteristics can be determined. Furthermore, analysis of ApNtr and AcNtr protein structures using X-ray crystallography will give answers to the involvement of amino acid K88 in the enzyme-substrate interaction.

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