# Exploring the Dual Role of Dihydroorotate Dehydrogenase in *Toxoplasma gondii*: Beyond Pyrimidine Biosynthesis.

Carolina Urbina Camacho, Cristian C. Barrera Grijalba, and Barbara H. Zimmermann.
Departamento de Ciencias Biológicas, Universidad de los Andes, Bogotá, Colombia.

## Introduction

*Toxoplasma gondii* is the causative agent of toxoplasmosis, a widely prevalent parasitic disease. Limited treatment options and the emergence of drug-resistant strains underscore the urgency for novel anti-infective strategies [1]. Dihydroorotate Dehydrogenase (DHODH), the fourth enzyme in *T. gondii*'s pyrimidine biosynthesis pathway, stands out as a potential drug target [2,3].

Genetic deletion of TgDHODH gene has not been possible; however, substituting the native sequence with a catalytically deficient DHODH results in uracil auxotrophy, unveiling a yet-to-be-characterized essential, pyrimidine-independent function of TgDHODH [4].

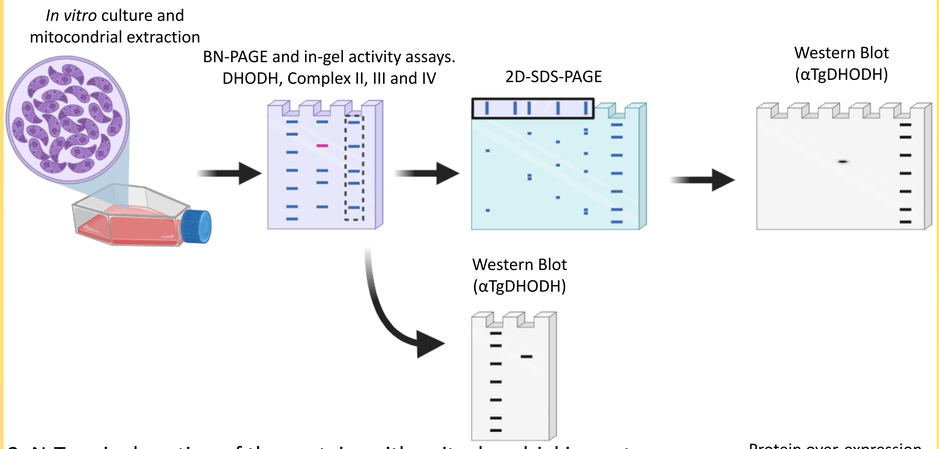
Studies on human DHODH found physical interactions with mitochondrial complexes I and II, suggesting a potential structural role of DHODH [5].

TgDHODH is nuclear-encoded, and initially synthesized as a precursor with a remarkably long 157-residue N-terminal region, which contains a signal sequence for mitochondrial targeting [6]. In mammals, some mitochondrial proteins use cleaved signal peptides as functional proteins within the mitochondria [7,8]. The extended length of the signal peptide in TgDHODH suggests that this sequence might be repurposed with a distinct function, separate from the pyrimidine-related C-terminal of the protein.

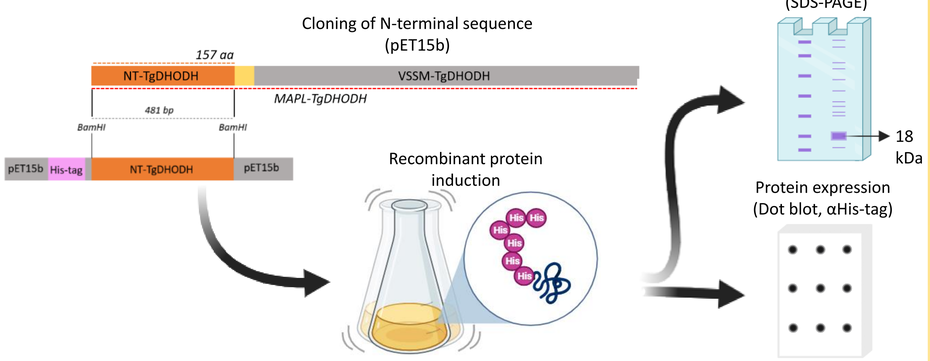
To gain understanding on the pyrimidine-independent essential function of TgDHODH, as well as the specific region involved in this function, this project probes protein-protein interactions of both the N and C-terminal regions with other mitochondrial proteins within the parasite. Here, we present the steps taken and the results obtained thus far.

## Methodology

1. C-Terminal, catalytically active portion of the protein




2. N-Terminal portion of the protein, with mitochondrial import sequence




## Results

1. C-Terminal, catalytically active portion of the protein

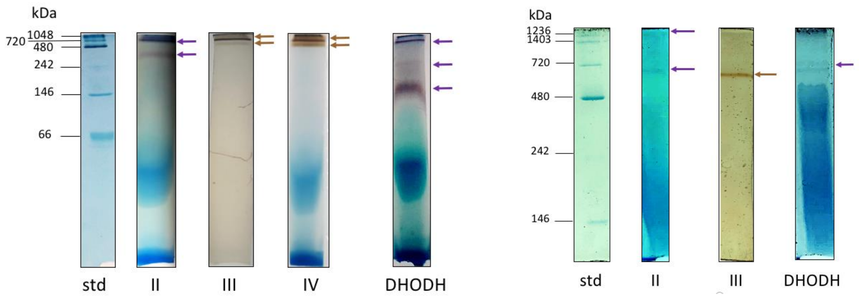



**Figure 1.** BN-PAGE and activity assays for DHODH, Complexes II, III, and IV in mitochondrial samples extracted from 2x10$^7$ tachyzoites. Acrylamide content and running conditions were optimized for high (A) and low (B) molecular weight resolution. Notably, two DHODH activity bands migrated around 720 kDa, exceeding the expected 48 kDa for the protein alone. Activity bands with similar migration patterns were observed for Complexes II and III.

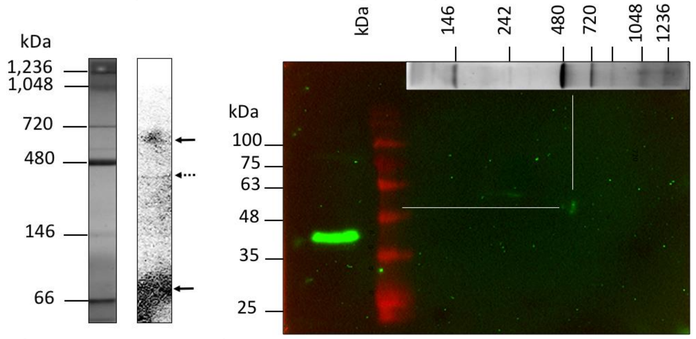



**Figure 2.** Western Blots using antibodies against recombinant, N-terminally truncated TgDHODH. (A) BN-PAGE fractionation of mitochondrial samples. The dashed line indicates an artifact present across the membrane. (B) 2D-SDS-PAGE fractionation. 16 ng of pure recombinant TgDHODH were used as positive control (left). For both cases, a band indicating presence of DHODH appears at higher molecular weight than anticipated for the protein alone.

2. N-Terminal portion of the protein, with mitochondrial import sequence

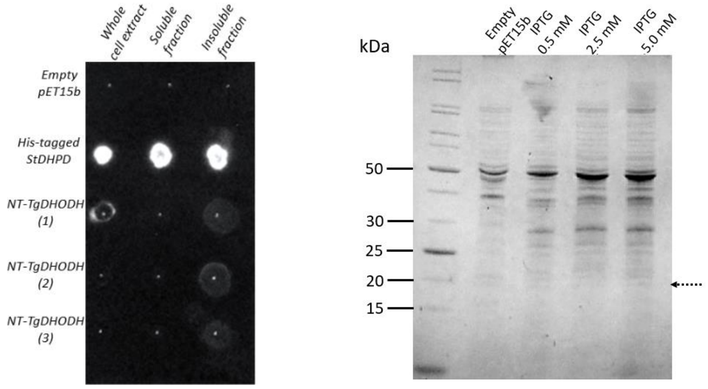



**Figure 3.** Dot blot (A) employing α6x His-tag and SDS-PAGE (B) of bacterial lysates containing pET15b plasmid with the N-terminal sequence of TgDHODH as an insert (confirmed by Sanger sequencing). Despite low protein expression indicated by dot blot results, the overexpressed protein band is not evident in SDS-PAGE (expected protein size 18 kDa, dashed line). Efforts to purify the protein using Cobalt and Nickel affinity chromatography columns were unsuccessful.

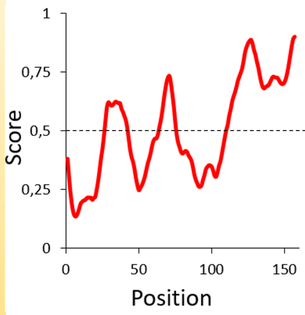



**Figure 4.** Disorder prediction for the N-terminal sequence of TgDHODH. Two small peaks indicating local, small disordered regions, along with a highly disordered terminal region, are observed. High disorder may explain the challenges in re-combinant expression. Prediction generated using the IUPred3 (https://iupred.elte.hu/).

## Conclusions

1.BN-PAGE and activity assays revealed DHODH activity bands migrating at high molecular weight, suggesting its association with other mitochondrial protein(s).

2.Activity bands indicated potential co-migration of DHODH with complexes II and III, aligning with findings from human DHODH studies [4].

3.Lack of higher molecular weight complex formation of TgDHODH observed in previous proteomic surveys [9] may be explained by differences in detergent selection and concentration in mitochondrial protein solubilization.

4.Initial attempts to recombinantly produce the N-terminal sequence of TgDHODH were unsuccessful, likely due to high protein disorder. Future efforts will focus on producing the first 133 residues to reduce protease sensitivity and enhance solubility.

## References

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