Toward a Comprehensive Framework for Investigating Copper Dyshomeostasis: The Role of His-Tag Modularity and Alternative Expression Systems in Evaluating Dopamine β-Hydroxylase

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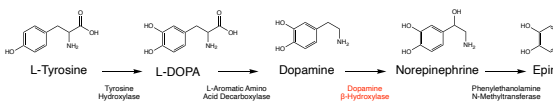
Background and Significance

Norepinephrine is a catecholamine with roles in many central nervous system functions, including memory, movement, learning, attention, and stress response (**Scheme 1**). However, deficiencies in the noradrenergic system are tied to neurodegeneration, such as in Alzheimer's (AD) and Parkinson's disease (PD).[1] Dopamine β-Hydroxylase (DBH) is a copper-containing monooxygenase responsible for the benzylic hydroxylation of dopamine to norepinephrine (**Figure 1**).[2] A marked decrease in DBH activity has been clinically reported in both AD and PD, providing evidence for DBH as a disease-causing or modifying gene.[3]



Scheme 1. Catecholamine Synthesis Pathway. Catecholamines dopamine, norepinephrine, and epinephrine are derived from the amino acid L-tyrosine. A different enzyme catalyzes each reaction. The enzyme of interest, Dopamine β-Hydroxylase, catalyzes the benzylic hydroxylation of dopamine to norepinephrine and is highlighted in red

Hallmarks of AD and PD include the buildup of plaques from noradrenergic regions such as the locus coeruleus (LC), where copper is highly localized.[3] Under normal conditions, bioavailable metals like copper play a critical role in many cellular processes, such as cofactors for enzymes like DBH. However, extensive data shows irregular levels of metal ions in disease states compared to healthy brains, which has led to a prominent view that impaired metal homeostasis is involved in the pathology of neurodegeneration.[4] Despite the correlational evidence that DBH is involved in the etiology of disease, the mechanisms behind how DBH activity becomes depleted and impacts neurodegeneration has yet to be understood. This preliminary work details progress toward elucidating the role of copper ion dyshomeostasis on the structure and function of Dopamine β-Hydroxylase while establishing a new lab.

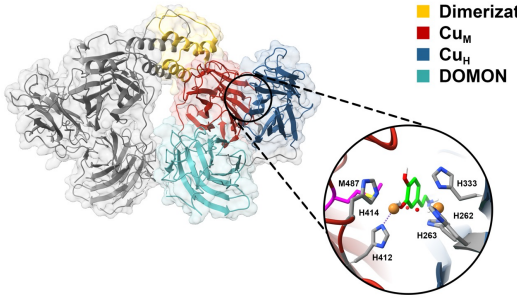
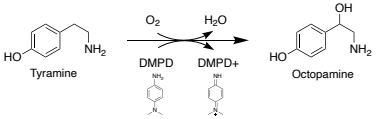


Figure 1: Crystallographic model of Dopamine β-Hydroxylase. The surface structure of the DBH dimer is overlayed with the ribbon model. Subdomains of one monomer are highlighted according to the key. The Cu_M-Cu_H copper coordination residues are labeled in the active site, shown alongside 2 copper ions (orange) and dopamine (green). Dopamine was docked into the active site using AutoDock4. Adapted from PDB 4ZEL.[2]

Research Question

Scheme 2. Hydroxylation reaction catalyzed by DBH for Steady-State Assay. DBH displays substrate promiscuity; in practice, tyramine is more stable than dopamine and is used regularly in DBH activity assessment. N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) is an alternative reducing agent to co-factor ascorbate.



What role does copper ion dyshomeostasis have on the structure and activity of Dopamine β-Hydroxylase?

Approach:

- Establish a bacterial expression system for DBH using SHuffle T7 *E. coli* engineered for disulfide bond formation, optimizing to maximize soluble enzyme generated.
- Assess for activity perturbations by copper using high-performance liquid chromatography and continuous UV-vis spectroscopy, where DMPD oxidation for the real-time assay is monitored at 515 nm.
- Determine bound copper:DBH stoichiometry using X-Ray Fluorescence.
- Evaluate DBH structure for site-specific amino acid oxidation by mass spectrometry

Bacterial Expression System Yields Soluble but Enzymatically Inactive DBH

IPTG Concentration Does Not Impact Cell Density

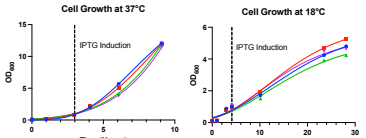


Figure 2. Growth of SHuffle T7 *E. coli* during DBH-His expression. 1 mL aliquots were obtained from each culture and read at 600 nm to obtain optical density prior to pelleting.

Temperature Exerts a Pronounced Effect on DBH-His Expression

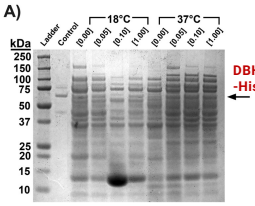
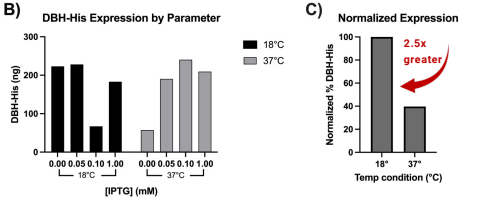
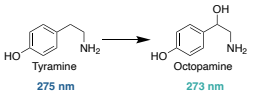


Figure 3. Quantifying soluble DBH-His. Equivalent volumes of CFE from each expression condition at 24 hours post-induction (18°C, OD_{600} = ~4.7) and 6 hours post-induction (37°C, OD_{600} = ~12). (**A**) SDS-PAGE gel stained by colloidal Coomassie. The control band is purified protein at a known concentration. The arrow indicates the location of DBH-His (67 kDa). (**B**) Soluble DBH-His present in gel quantified by ImageJ compared to the control. (**C**) Normalized expression by OD_{600} readings.



Absorption Spectra Maxima for HPLC

Scheme 3. Reaction for the Hydroxylation of Tyramine to Octopamine. Reactions were performed in 125 mM NaOAc buffer. A diode array monitored separation by HPLC to detect maxima at 275 nm and 273 nm. In practice, octopamine elutes first ~ 2.366 min. and tyramine second ~ 3.069 min.



Product Formation is Detected from DBH-His Expressed in HEK293, but *not* DBH from *E. coli*

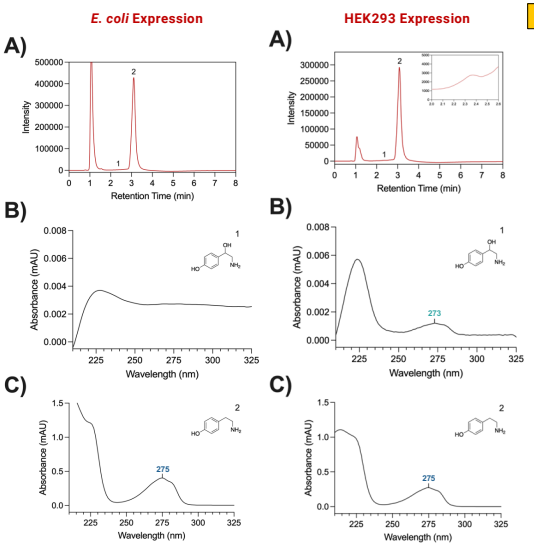


Figure 4. HPLC chromatogram and diode array spectra of DBH expressed in *E. coli*. (A) Corresponding chromatogram of the reaction run with 0.01 mg/mL DBH containing 1 mM tyramine, 0.5 mM ascorbate, and 1.0 μM $CuSO_4$. (B) Spectrum of peak 1 at 2.366 minutes corresponds with the anticipated location of octopamine, but no apparent peak can be distinguished. (C) Spectrum of peak 2 at 3.078 minutes is tyramine.

Figure 5. HPLC chromatogram and diode array spectra of DBH-His expressed in HEK293 cells. The reaction ran as previously mentioned. (**A**) Corresponding chromatogram of the reaction run with 0.01 mg/mL DBH-His containing 1 mM tyramine, 0.5 mM ascorbate, and 1.0 μM $CuSO_4$. Insert: zoomed-in peak 1 for evidence of octopamine formation. (**B**) Spectrum at 2.380 minutes reveals the presence of octopamine at peak 1. (**C**) Peak 2 spectrum at 3.043 minutes is tyramine.

DBH-His Deviation from Steady State Kinetics

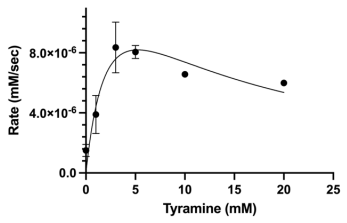


Figure 6. DBH-His initial rate kinetics. Steady-state initial rates were recorded for HEK293-expressed DBH-His, tracking DMPD oxidation at 515 nm where tyramine was the variable substrate. Tyramine concentrations (mM) were 0.0, 1.0, 3.0, 5.0, 10.0, and 20.0. Non-linear regression analysis was performed using GraphPad Prism 9.

Table 1. Steady-state kinetic parameters for DBH-His as defined by [tyramine]

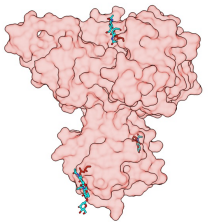
Variable Substrate	K_M (mM)	V_{max} (mM/sec)	k_{cat} (sec^{-1})	k_{cat}/K_M ($sec^{-1}mM^{-1}$)	K_i (mM)
Tyramine	2.689*	1.661×10^{-5}*	2.232*	0.830*	10.17*

*Confidence interval could not be defined

- A 10-fold decrease in V_{max} and catalytic efficiency is observed compared to literature reporting similar conditions for DBH without a His-tag.[5]
- The apparent K_M is also greater.
- Our data are indicative of mixed inhibition and substrate inhibition when a His-tag is present
- Additional experiments are required to validate these claims.

Conclusions on the Consequences of DBH Expression Conditions on Activity

Figure 7. Structure highlighting DBH N-glycosylation. Glycans are shown in cyan. Monomer adapted from PDB 4ZEL. Disulfide bonds are not the sole post-translational modification of DBH. Indeed, DBH is a glycoprotein consisting of 3 N-glycosylated residues per monomer at Asn64, Asn184, and Asn566.[2] N-linked glycosylation is believed to have a wide variety of functions, including effects on enzymatic activity. Though the precise role of glycols is largely unknown, studies suggest they contribute to the proper folding, dynamics, and stability of native mammalian proteins, expanding beyond the localized region of glycosylation.[6] Experiments have also demonstrated that deglycosylation of a variety of human enzymes leads to a complete loss of activity.[6] Provided this, it appears that N-glycosylation could be necessary for formal hydroxylation activity in DBH.



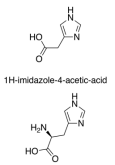


Figure 8. Structural comparison between 1H-imidazole-4-acetic-acid (IAA) and histidine. IAA is a potent mixed-type allosteric inhibitor of DBH activity.[7] The initial rate data suggest a similar phenomenon. Because mixed inhibitors may bind unequally to the enzyme and enzyme-substrate complex, the His-tag may preferentially coordinate with the copper residues near the exposed active site cleft only after substrate tyramine is bound and induces the closed conformation. This can also account for the substrate inhibition demonstrated; in a case where the tag has a greater affinity for the closed conformation, the perturbed activity is thereby substrate dependent.

Future Work: Develop a Mammalian Expression System Employing an Alternative Tag in Lieu of His-Tag

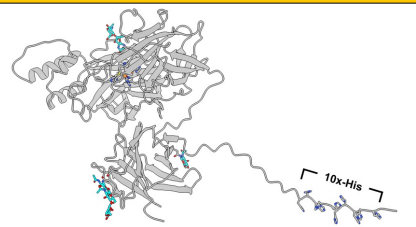


Figure 8. Predicted Structure of DBH-His from HEK293. AlphaFold was used to generate a monomeric structure given the sequence received for DBH-His. A highly flexible N-terminus is evident, with potential for interactions that span the subunit. Thus, it is in our best interest to establish a mammalian system for DBH with an alternative affinity tag (i.e. FLAG) to minimize modulation of the metalloenzyme and provide the post-translational modification machinery necessary for glycosylation. Once there is a functional enzyme system, investigating activity assay best practices and follow-up experiments like those established under *Approach* provide further grounds to elucidate the impact of copper ion dyshomeostasis on the structure and activity of DBH.

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