Modeling the N-Terminal Domain of Cystathionine β-Synthase to Elucidate the Role of CBS in Vascular Defects Associated with Homocystinuria

Brookfield Academy Upper School SMART Team

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INTRODUCTION

Homocystinuria is an autosomal recessive disorder affecting approximately 1 in 350,000 people worldwide. It is characterized by skeletal, nervous system, and vascular abnormalities, such as delayed developmental milestones, myopia, dislocation of the eye lens, osteoporosis, mental retardation, and increased risk of blood clotting. Major causes of homocystinuria are mutations in the enzyme cystathionine ß-synthase (CBS), which catalyzes the condensation of serine and homocysteine to cystathionine, an intermediate in cysteine synthesis (Figure 1).

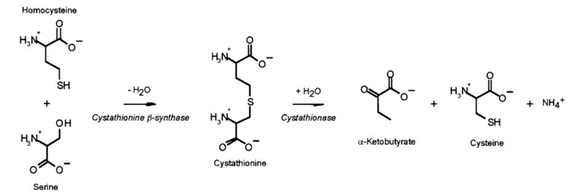


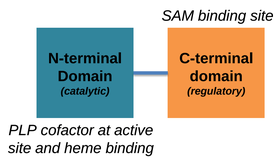
Figure 1. Reaction pathway for the synthesis of the amino acid cysteine. Synthesis of cysteine begins with CBS-catalyzed condensation of homocysteine and serine. Figure from Meier *et al.*

Homocystinuria can lead to several cardiovascular defects such as increased carotid plaque thickness, known as atheroma, in artery walls and intravascular thrombosis, the formation of a blood clot that obstructs blood flow through the circulatory system. A primary research goal is to understand how mutations in CBS, an enzyme found in muscle tissue, may lead to vascular abnormalities by identifying changes in embryonic vascular development in zebrafish (*Danio rerio*) lacking expression of the Cbs-b isoform. Zebrafish are used as a model to study vascular development because of their small size, closed circulatory system, and rapid development. In addition, the vascular system of the zebrafish closely mimics that of humans. Thus, scientists hope to draw connections between the change in vascular development in zebrafish to the changes in physical and chemical changes in human vascular development seen in homocystinuria. Using 3D printing techniques, the Brookfield Academy SMART (Students Modeling A Research Topic) Team modeled the N-Terminal domain of the CBS enzyme, highlighting the heme and PLP coenzymes, along with various mutations associated with homocystinuria.

STRUCTURE AND REGULATION OF CBS

The CBS gene, found on chromosome 21, provides instructions for the enzyme cystathionine β-synthase. This enzyme contains both a vitamin B6, or PLP, and heme cofactor and catalyzes the synthesis of cystathionine (see Figure 1). Only PLP directly partiicipates in the reaction chemistry. The heme cofactor may paly in role in proper folding of CBS, as CBS aggregrates when expressed in the absence of heme.

Figure 2. Each subunit of CBS contains two domains. CBS is a pyridoxal 5'-phosphate (PLP) and heme-dependent enzyme regulated by S-adenosylmethionine (SAM). The N-terminal domain is the catalytic region, containing both the PLP and the heme cofactor, while the C-terminal domain is the regulatory region, binding SAM.



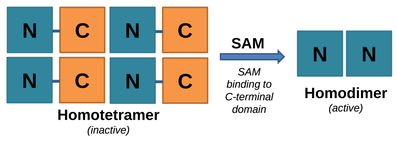


Figure 3. CBS is converted from an inactive homotetramer to an active homodimer upon SAM binding. CBS is a homotetramer, each subunit consisting of distinct N and C-terminal domains. SAM is a cofactor involved in methyl group transfers such as transmethylation, transsulfuration, aminopropylation and other anabolic reactions in the body. SAM is synthesized by methionine adenosyltransferase from ATP and methionine. Upon binding with SAM, the C-terminal domain is removed, and the enzyme functions as a homodimer.

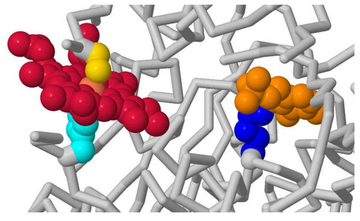


Figure 4. Coordination of PLP and heme cofactors in the N-terminal domain of CBS. The PLP cofactor (*orange*) is located within the active site of the enzyme and is covalently attached to CBS by Lysine-119 (*blue*). The heme cofactor (*red*) is reversibly bound to CBS by coordination with Cysteine-52 (*yellow*) and Histidine-65 (*cyan*). The iron (*peach*) rests in the center of the heme ring. Figure generated JMol and PDB 1JBQ from Meier *et al.*

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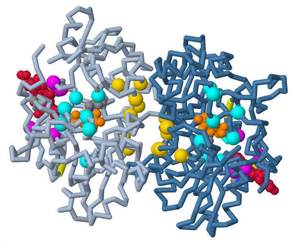
MUTATIONS IN CBS ASSOCIATED WITH HOMOCYSTINURIA, CLUSTER IN THREE REGIONS OF CBS, RESULTING IN DIFFERENTIAL EFFICACY OF VITAMIN B6 TREATMENT

Over 150 mutations have been identified affecting CBS. These mutations cluster around three areas of the enzyme: the heme binding site, the PLP and substrate binding active site, and the dimer interface . At locations other than the PLP binding site, vitamin B6 treatment is ineffective and homocystinuria can not be managed. Thus, research is being conducted to find other treatments for CBS related vascular abnormalities due to mutations at other areas of the CBS enzyme.



At the dimer interface, the most prevalent mutation is A114V. Mutations at the dimer interface, prevent proper protein folding or dimer formation, and appear non-responsive to PLP treatment.

Approximately 50% of CBS mutations occur in the PLP binding site region, and can be treated with vitamin B6 therapy. The two most prevalent mutations at the PLP active site are G305R and G307S. The G305R mutation decreases the ability of PLP to bind to the active site, and the G307S mutation changes the shape of the entry lining of the active site.

The most common mutation at the heme binding site is the H65R mutation. H65 is directly involved in heme binding. Patients with H65R mutation do not respond to PLP treatment. The H65R protein does not fold properly, suggesting a role of the heme cofactor during CBS folding.

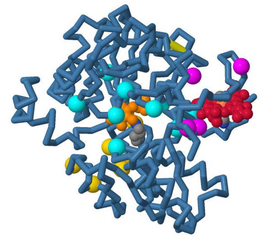


Figure 5. Wireframe models of the CBS homodimer, illustrating mutations associated with homocystinuria. Mutations primarily cluster around three areas of the enzyme: the heme binding site (*magenta*), the PLP and substrate binding active site (*cyan*), and the dimer interface (*yellow*). Only the C_α of each amino acid residue has been highlighted. Figure generated using JMol and PDB file 1JBQ from Meier *et al.*

CBS DEFICIENT ZEBRAFISH PROVIDE A MODEL FOR UNDERSTANDING THE ROLE OF CBS IN VASCULAR DEFECTS ASSOCIATED WITH HOMOCYSTINURIA

In zebrafish, following the initial formation of the dorsal artery and posterior cardinal vein, new blood vessels extend from these preexisting vessels. These budding vessels, the intersegmental vessels (ISVs), are among the first angiogenic vessels to form. Since zebrafish are a well-established model for vascular development, scientists may be able to observe the vascular development in zebrafish lacking functional CBS enzyme. Blood vessel formation can be monitored in developing embryos by staining for the presence of the VEGF receptor 2 (VEGFR2) mRNA. VEGFR2 is expressed on the surface of endothelial cells to direct vessel formation in response to the growth factor, VEGF, secreted by neighboring somites.

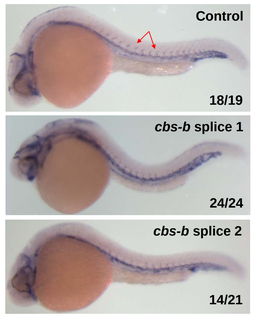


Figure 6. In situ hybridization for *vegfr2* probe in control, *cbs-b* splice 1, or *cbs-b* splice 2 MO injected 24 hpf embryos. In the control embryo you can see intersegmental vessels (ISVs) forming in the trunk of the embryo indicated by red arrow. In *cbs-b* splice 1 or 2 MO injected embryos, the ISVs appear to be stunted. The number in the lower right of each picture indicates the number of embryos that exhibited this phenotype out of the total number of embryos injected.

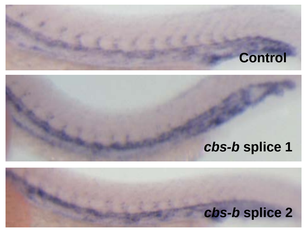


Figure 7. Magnified images of the three trunk regions of the three MO injection conditions.

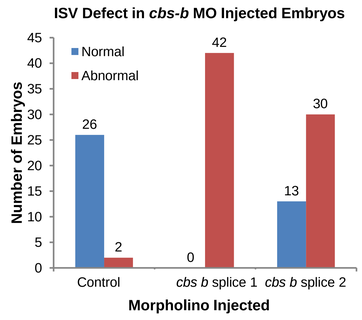


Figure 8. Quantification of two different injection experiments for the ISV phenotype observed in Figures 6 and 7.

To investigate the role of CBS enzyme in vascular development, morhpholino technology was used to reduce the expression of the Cbs-b isoform in zebrafish. Morpholino oligos (MO) are synthetic oligonucleotides that use DNA bases on a phosphorodiamidate backbone. Once injected into the fertilized egg, an MO specifically binds to its target mRNA, blocking mRNA splicing or translation of the mRNA into its respective protein product. The MO's were synthesized to be complementary to different regions of the *cbs-b* mRNA. Injection of MO's into fertilized eggs prevent proper splicing, processing, or translation of mRNA in the cell, significantly reducing the amount of functional Cbs-b protein in zebrafish embryos. Embryos were harvested 24 hours post fertilization (hpf) and blood vessels were visualized by staining for *vegfr2* mRNA. *Cbs-b* knockdown embryos appeared to exhibit a diminution in ISV trunk length, as summarized in Figure 8.

CONCLUSION

In this study, morpholino technology was used to prevent the expression of the *cbs-b* gene in zebrafish and vascular development was assessed in embryos to determine if any phenotype might arise as a result of the loss of Cbs-b. A morpholino oligonucleotide, complementary to the mRNA of *cbs-b* was injected into fertilized eggs of the zebrafish, inhibiting proper splicing and/or translation of the CBS gene. As shown in Figure 9, Zebrafish embryos lacking the Cbs-b enzyme exhibited a decrease the length of ISVs in the trunks of the developing embryo. No apparent effect on the number of ISV segments or vessel sprouting was apparent. This disruption in the development of the ISVs suggests that mutations in the CBS gene has an affect on vascular growth and function. The continuation of this project will hopefully lead to more information regarding vascular abnormalities caused by mutations in CBS. As research continues, scientists hope to create a model system to study the effects of CBS in vascular development, in order to better understand CBS deficiencies in humans. Eventually, researchers hope to develop multiple treatments for various CBS related diseases such as homocystinuria.

ISV Defect Model

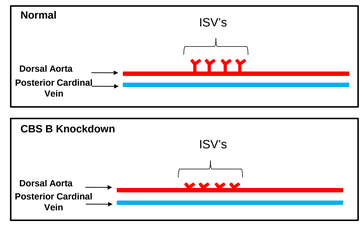


Figure 9. Model illustrating what is hypothesized to be occurring in the *cbs-b* MO injected embryos causing the stunted ISVs.

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