

**INVESTIGATION OF HOMOCYSTINURIA-ASSOCIATED MUTATIONS IN
THE ACTIVE SITE OF CYSTATHIONINE β -SYNTHASE**

By

Duale Ahmed, B.Sc.H.

A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in partial
fulfilment of the requirements for the degree of

Master of Science

in

Biology

Department of Biology

Ottawa-Carleton Institute of Biology

Carleton University

Ottawa, Ontario

September 2014

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Duale Ahmed

ABSTRACT

Homocystinuria is an inherited autosomal recessive disorder that affects an individual's ability to metabolize the L-methionine derivative L-homocysteine. Cystathionine β -synthase (CBS) catalyzes the condensation of L-homocysteine and L-serine in the first step of the reverse transsulfuration pathway, which shunts excess L-homocysteine to L-cysteine biosynthesis. More than 100 mutations of the CBS gene have been associated with homocystinuria. Treatment for this disease includes supplementation with vitamin B₆ and/or betaine and restriction of dietary L-methionine. Refinement of these therapeutic regimes will be enabled by an enhanced understanding of the distinct effects of homocystinuria-associated mutations as well as detailed characterization of the roles of the residues and hydrogen bonding networks in the enzyme active site. This study: (1) probed the role of active site residues D319, S323 and Y324 in the positioning of the pyridoxal 5'-phosphate cofactor of the model yeast CBS enzyme and (2) investigated a set of 11 homocystinuria-associated mutations in the context of human CBS. In the first study, substitution of D319 with alanine, removing only the carboxylate moiety of the side chain, eliminated activity, while the S323A and Y324F variants, each lacking the side chain hydroxyl group, had negligible effect on the kinetic parameters, compared to the wild-type enzyme. Residue G148 provides the mobile loop the flexibility to transition between the open and closed active site conformations. Residues E176, K384 and G307 are involved in maintaining a catalytically productive active site architecture. Residues T257 and G259 serve critical roles in PLP binding and in the bi-directional communication between the heme and PLP

cofactors. Residues G305 and S349 also participate in positioning of the PLP cofactor. Understanding the structure-function relationships that underlie the molecular basis for each distinct homocystinuria-associated mutation of CBS provides insight that can be used to refine treatment for patients with homocystinuria.

ACKNOWLEDGEMENTS

Dr. Martin Luther King Jr. once said, “The ultimate measure of a man is not where he stands in moments of comfort and convenience, but where he stands at times of challenge and controversy”. The challenge of completing my M.Sc. degree would not have been possible without the rays of light that have guided me through this tunnel. Firstly, I would like to thank Dr. Susan M. Aitken for giving me the opportunity to work in her lab as an undergraduate albeit my grades signified that I did not deserve nor earned that opportunity. Yet, you helped me realize that research is my passion. Without your insight, I would not be in the position that I am in right now. I still have a lot to learn when it comes to being a researcher but you have helped me get this far and I know you will continue to guide me to becoming the researcher I want to be during my tenure as a Ph.D. candidate in your lab.

I would like to extend my appreciation to my committee members, Dr. Amanda J. MacFarlane and Dr. Kenneth B. Storey. Your insights and feedback were essential to the creation of this thesis and I am sincerely thankful for your guidance!

I would not have gotten to this point without those who have already paved the road ahead for me. A special thank you goes to Allison Jaworski, Dr. Dominique Morneau and Edgar Abouassaf. You have been with me since Day 1 in the Aitken Lab and I would not be the researcher that I am today without your expertise. To Ally, thank you for vouching for me to Sue when it came to taking on a fourth year honour student even though my grades did not warrant you going out on a limb for me. Your guidance as my TA, 4908 graduate supervisor, friend and an overall awesome person is something that cannot be replaced and for that, I am forever grateful. To Dom, working in the lab

was never a dull moment when you were around. You always helped me come up with ideas when I wasn't able to do on my own and while your taste in comic book heroes may be questionable, your friendship never was. To Eddy (and past Eddy), thank you for showing me the ropes when it comes to working on the CBS enzyme. You helped me become a better TA and RA. For all the gains I have made as a person, I thank you. My fellow graduate students, Emily Hopwood, Sorin Gustin and Victoria Samaki, were always willing to help me out when I needed it and helped create a great working environment in the lab. I also extend my gratitude to the undergraduate students that I had the pleasure of working with during my time in the lab, Usman Khan, Katrina Farrell and Menachem Loewenthal. You have helped me with my research and you have taught me something even though it was my job to teach you. I will always cherish the friendships I have made with my fellow graduate students within the department, Mohsen Hooshyar, Magdalena Bruno, Bahram Samanfar, Katayoun Omid, Kristina Shostak and Dan Burnside. All of you have made my time here an experience that I will never forget!

Lastly but certainly not least, I would not be the person I am today without the support of my family. Hooyo and Aabo, you brought me into this world, nurtured me and raised me to become someone who believes that I can accomplish anything that I set my mind to do. While life was not always easy for us, you still gave everything you had to make sure that my three brothers and I never felt those hardships. Hopefully, I have and will continue to live up to the name you have bestowed onto me. To the rest of my support system, my brothers Mohamed, Mubarak and Mukhtar; my cousin Kombe; my aunt Muna; my brother from another mother Hussein; thank you for being there for me whenever I needed you. The members of my family that I have not mentioned, I am

grateful for you as I would be here without you. You all are my pillars and without you I would crumble.

I know that I have accomplished a great deal thus far but there is still a ways to go before I have reached my potential. All of you have played a role in getting me to where I am today. I am excited for what the future holds for me knowing that I have all of you to lean on for support. Malcolm X once said, “The future belongs to those who prepare for it today”. The one thing I know is that I am ready for what is next as long as you all have my back!

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	vii
LIST OF TABLES	ix
LIST OF FIGURES	x
ABBREVIATIONS	xii
1. INTRODUCTION	1
1.1. Homocystinuria.....	2
1.2. Sulfur-Containing Amino Acids.....	9
1.3. The Transsulfuration and Reverse Transsulfuration Pathways.....	10
1.3.1. Pyridoxal 5'-Phosphate.....	13
1.4. Cystathionine β -synthase.....	17
1.4.1. Protein Architecture.....	17
1.4.2. Reaction Mechanism.....	22
1.4.3. Role of CBS in Hydrogen Sulfide Production.....	25
1.4.4. Proposed Roles of Heme in hCBS.....	28
1.4.5. SAM Regulation in CBS.....	32
1.4.6. Homocystinuria-associated Mutations of CBS.....	33
1.5. Focus of Study and Objectives.....	35
2. METHODS	37
2.1. Materials.....	37
2.2. Construction of Site-Directed Mutants.....	37
2.3. Expression and Purification of yfCBS Mutants.....	39
2.4. Expression and Purification of htCBS Mutants.....	40
2.5. Spectroscopic Characterization of htCBS.....	40
2.6. Enzyme Assays.....	41
2.6.1. Hydrolysis of L-Cystathionine by yfCBS.....	41
2.6.2. Condensation of L-Serine and L-Homocysteine by htCBS.....	43
3. RESULTS	45
3.1. The Construction and Purification of the Site-Directed Variants.....	45
3.2. Kinetic Characterization of the yfCBS Variants.....	45
3.3. Kinetic Characterization of the htCBS Variants.....	50
3.4. Spectral Characterization of the htCBS Wild-Type Enzyme and Site-Directed Variants.....	50

4. DISCUSSION.....	55
4.1. The yfCBS Variants (D319A, S323A & Y324F).....	55
4.2. The Truncated Human CBS Enzyme.....	59
4.2.1. Residue G148.....	59
4.2.2. Residues E176 and K384.....	62
4.2.3. Residues T257 and G259.....	65
4.2.4. Residue S349.....	69
4.2.5. Residues G305 and G307.....	71
5. CONCLUSION.....	74
6. REFERENCES.....	75

LIST OF TABLES

Table 2.1.	Primers used in the construction of the yfCBS and htCBS site-directed mutants.....	38
Table 3.1.	Protein yield of yfCBS and htCBS variants.....	49
Table 3.2.	Steady-state kinetics parameters of wild-type yfCBS and the D319A, S323A and Y324F variants for the reverse-physiological hydrolysis of L-cystathionine.....	49
Table 3.3.	Steady-state kinetic parameters for the condensation of L-serine (L-Ser) and L-homocysteine (L-Hcys) by wild-type htCBS and homocystinuria-associated variants.....	52

LIST OF FIGURES

Figure 1.1.	The major pathways of sulfur amino acid metabolism.....	3
Figure 1.2.	The transsulfuration pathway of bacteria and plants, the reverse transsulfuration pathway of mammals and yeast as well as their association to the methionine cycle.....	12
Figure 1.3.	The variety of reactions catalyzed by PLP-dependent enzymes.....	15
Figure 1.4.	A comparison of the organization of domains among a selection of representative fold-type II PLP-dependent enzymes.....	16
Figure 1.5.	Structural differences in the positioning of the regulatory domain in the hCBS (Δ 516-525) and dCBS protein structures.....	21
Figure 1.6.	Reaction mechanism of CBS, showing the PLP-bound intermediates as well as their associated absorption maxima, as observed in yCBS.....	23
Figure 1.7.	The four known β -replacement reactions catalyzed by CBS.....	27
Figure 1.8.	Enolimine and ketoenamine tautomers of the PLP-internal aldimine in human cystathionine β -synthase.....	30
Figure 1.9.	Proposed mechanism for the heme-CO linked inactivation of hCBS.....	31
Figure 1.10.	A 3D model of the hCBS Δ 516-525 variant crystal structure showing both CBS domains (CBS1 and CBS2) and the location of the proposed binding sites of SAM (S1 and S2).....	34
Figure 2.1.	DNTB assay utilized to measure the reverse-physiological hydrolysis of L-cystathionine by CBS.....	42
Figure 2.2.	The assay employed to determine the β -replacement activity of CBS, <i>via</i> the use of CBL and LDH coupling enzymes.....	44
Figure 3.1.	Agarose gel of PCR amplicons produced for the construction of the htCBS site-directed mutants <i>via</i> overlap-extension PCR.....	46
Figure 3.2.	A SDS-PAGE gel, stained with Coomassie Blue, showing 4 μ g each of the wild-type and S323A, Y324F and D319A site-directed variants of yfCBS.....	47
Figure 3.3.	A SDS-PAGE gel stained with Coomassie Blue reagent showing 4 μ g of the wild-type and site-directed variants of htCBS.....	48
Figure 3.4.	UV-Visible spectra of the wild-type htCBS (black) and ytCBS (red) enzymes.....	53
Figure 3.5.	UV-Visible spectra of the purified htCBS variants examined in this study.....	54

Figure 4.1.	Proposed active site contacts in the γ CBS-aminoacrylate complex.....	58
Figure 4.2.	The placement of residue G148 (red) within the CBS active site and its direct and indirect interaction with T146 (pale blue) and the external aldimine (E-Ser; Yellow), respectively.....	61
Figure 4.3.	The location of the K177 hydrogen bonding network to which K384 and E176 are members of said group.....	64
Figure 4.4.	A view of the glycine-rich loop that positions the PLP cofactor <i>via</i> interactions with the phosphate moiety.....	66
Figure 4.5.	Location of key residues and α -helix 8, which extends from the heme cavity to the PLP-binding site, proposed to be involved in bi-directional communication between the two cofactors.....	68
Figure 4.6.	The interaction between residue S349 (red) of hCBS and the N1 position of the PLP cofactor (E-Ser; yellow).....	70
Figure 4.7.	Position of G305 and G307 (red) at the mouth of the hCBS active site.....	73

ABBREVIATIONS

AD	Alzheimer disease
δ-ALA	δ -aminolevulinic acid
ATP	Adenosine triphosphate
BHMT	Betaine:homocysteine methyltransferase
cAMP	Cyclic adenosine monophosphate
CBL	Cystathionine β -lyase
CBS	Cystathionine β -synthase
CGL	Cystathionine γ -lyase
CGS	Cystathionine γ -synthase
CNS	Central nervous system
CO	Carbon monoxide
CT	Computed tomography
D,L-Hcys	D,L-Homocysteine
dCBS	<i>Drosophila melanogaster</i> cystathionine β -synthase
DNA	Deoxyribonucleic acid
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
DTT	Dithiothreitol

<i>E. coli</i>	<i>Escherichia coli</i>
E-Cth	External aldimine of L-cystathionine
E-Ser	External aldimine of L-serine
E.C.	Enzyme Commission
eCBL	<i>Escherichia coli</i> cystathionine β -lyase
EDTA	Ethylenediaminetetraacetic acid
Fe³⁺	Ferric Iron State
Fe²⁺	Ferrous Iron State
GSH	Glutathione
H₂S	Hydrogen sulfide
hCBS	Human cystathionine β -synthase
htCBS	Human truncated cystathionine β -synthase
IPTG	Isopropyl- β -D-thiogalactopyranoside
ITC	Isothermal titration calorimetry
L-Cth	L-Cystathionine
L-Cys	L-Cysteine
L-Hcys	L-Homocysteine

L-Met	L-Methionine
L-Ser	L-Serine
LB	Luria Broth
LDH	L-lactate dehydrogenase
NADH	Nicotinamide adenine dinucleotide, reduced form
NaCl	Sodium Chloride
NH₃	Ammonia
Ni-NTA	Nickel-nitrilo triacetic acid
NMDA	<i>N</i> -methyl-D-Aspartate
NO	Nitric oxide
MS	Methionine synthase
MTHFR	5,10-methylene-tetrahydrofolate reductase
OASS	<i>O</i> -acetyl- L-serine sulfhydrylase
OD	Optical density
OE-PCR	Overlap-extension polymerase chain reaction
PCR	Polymerase chain reaction
PLP	Pyridoxal 5'-phosphate

RNA	Ribonucleic acid
SAH	<i>S</i> -adenosylhomocysteine
SAHH	<i>S</i> -adenosylhomocysteine hydrolase
SAM	<i>S</i> -adenosylmethionine
SAMS	<i>S</i> -adenosylmethionine synthetase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TD	Threonine deaminase
THF	Tetrahydrofolate
TNB⁻	3-thio-6-nitrobenzoate
TrpS	Tryptophan synthase
TS	Threonine synthase
UV	Ultraviolet
yCBS	Yeast cystathionine β -synthase
yfCBS	Yeast full-length cystathionine β -synthase
ytCBS	Yeast truncated cystathionine β -synthase

1. INTRODUCTION

Inborn errors of metabolism, as first described by Archibald Garrod in 1908, are a class of disorders in which genetic defects affect the function of the encoded enzymes (Scriver, 2008). The presentation of symptoms is commonly correlated with the accumulation of metabolite(s), resulting from the reduced function of the associated enzyme, which impedes cellular and/or physiological processes (Pasternak, 2005). Treatment of these disorders is complicated by the ability of distinct mutations of a gene to result in clinical manifestations which may vary in presentation and/or severity and with respect to treatment options, depending on the specific allele(s) an individual possesses. This presents researchers with the challenge of understanding the structure-function relationships that underlie each distinct disease-associated allele and how these are manifested *in vivo*, as well as clinically. This study explores these structure-function relationships, focusing on homocystinuria-associated mutations and hydrogen bonding networks in the active site of the enzyme cystathionine β -synthase.

Cystathionine β -synthase (CBS) catalyzes the condensation of L-serine (L-Ser) and L-homocysteine (L-Hcys), producing L-cystathionine (L-Cth), in first committed step of the reverse transsulfuration pathway, which transfers the sulfur atom of L-Hcys, a product of L-methionine (L-Met) metabolism, to L-cysteine (L-Cys) (Figure 1.1) (Stipanuk, 2004). Typical of enzymes catalyzing transformations of amino acid substrates, CBS is reliant on the catalytically versatile pyridoxal 5'-phosphate (PLP) cofactor. Higher eukaryotic forms of CBS (e.g. *Drosophila melanogaster* and human, but not yeast) bind heme in addition to PLP (Kery *et al.*, 1994). Mutations in the gene encoding CBS are the primary cause of homocystinuria, a condition characterized by high levels of L-Hcys and L-Met in the plasma and urine (Mudd, *et al.*, 1985). Worldwide

prevalence of homocystinuria, the most common of sulfur amino acid metabolic disorders, is 1 in 344,000 births but recent reports suggest the incidence rate may be higher (Mudd *et al.*, 2001; Yap, 2012). An enhanced understanding of the effect of homocystinuria-associated mutations on the biochemical properties of CBS will aid in the refinement of therapeutic approaches for patients with homocystinuria.

1.1. Homocystinuria

Homocystinuria was first described in 1962 by independent groups in Northern Ireland and in the United States examining the amino acid content of the urine of children with mental retardation, and was linked to CBS deficiency in 1964 (Carson *et al.*, 1962; Gerritsen *et al.*, 1962; Mudd *et al.*, 1964). It is biochemically characterized by elevated levels of plasma and urine L-Hcys ($>100 \mu\text{M}$) and L-Met. Prevalence of homocystinuria varies from 1:65,000 in Northern Ireland to 1:900,000 in Japan (Mudd *et al.*, 2001; Naughten *et al.*, 1998). Recent reports suggest the incidence rate may be higher as genetic screening of specific CBS mutations has resulted in estimates ranging from 1:6,500 to 1:20,000 in European countries, including Denmark, Germany, Norway and the Czech Republic, and a study in Qatar reported an incidence rate of 1:1200 based on the biochemical screening of newborns (Yap, 2012; Kozich *et al.*, 2010; Zschocke *et al.*, 2009).

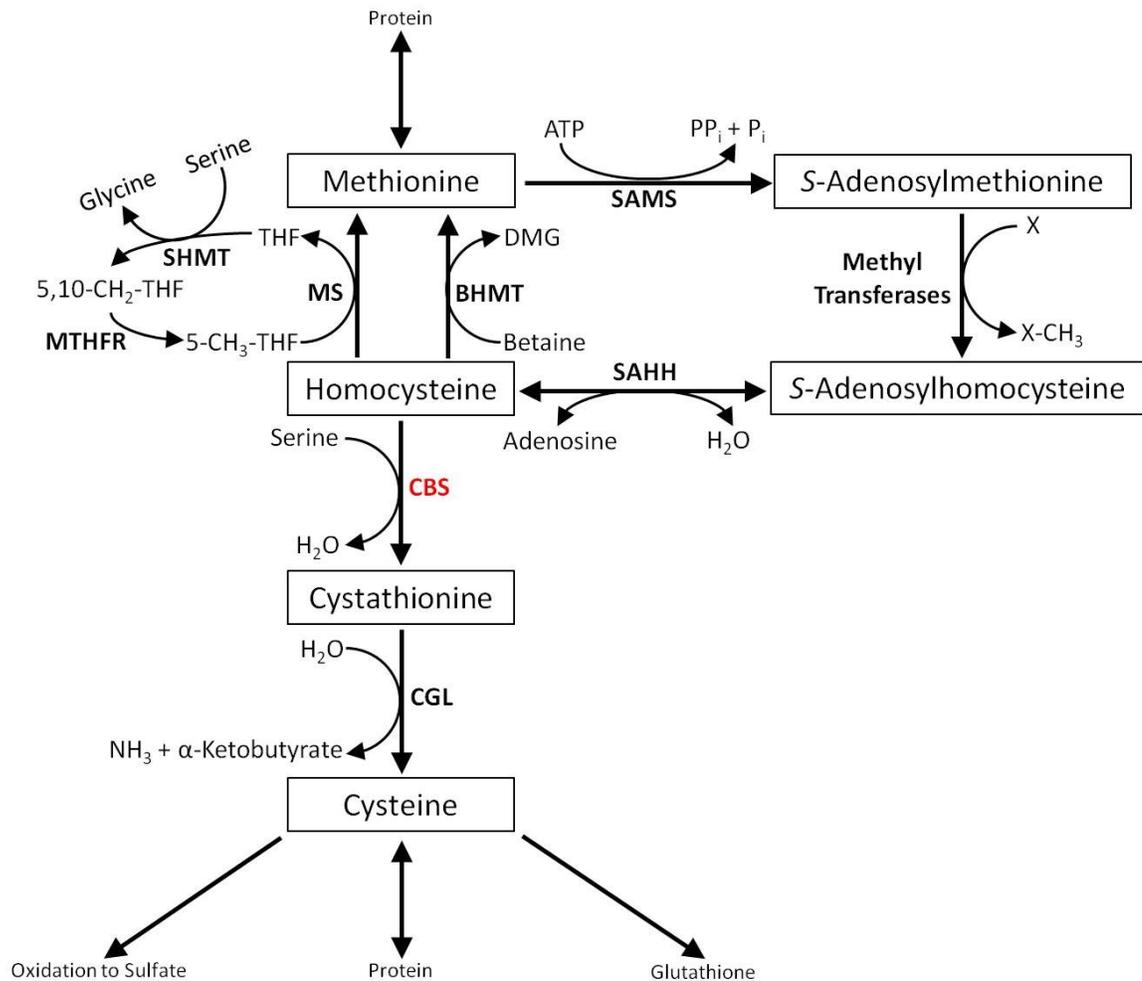


Figure 1.1: The major pathways of human sulfur amino acid metabolism (adapted from Brosnan & Brosnan, 2006). SAMS: *S*-adenosylmethionine synthetase, ATP: Adenosine triphosphate, PP_i: Pyrophosphate, P_i: Inorganic phosphate, SAHH: *S*-adenosylhomocysteine hydrolyze, BHMT: Betaine:homocysteine methyltransferase, DMG: Dimethylglycine, MS: Methionine synthase, THF: Tetrahydrofolate, SHMT: Serine hydroxymethyltransferase, 5,10-CH₂-THF: 5,10-methylene-THF, MTHFR: 5,10-methylene-THF reductase, 5-CH₃-THF: 5-methyl-THF, CBS: Cystathionine β-synthase, CGL: Cystathionine γ-lyase.

Homocystinuria resulting from CBS enzyme deficiency is inherited as an autosomal recessive trait. The *CBS* gene, which encodes the enzyme, is located on the 21st human chromosome (Munke *et al.*, 1988). The severity of the observed symptoms ranges from nearsightedness and limited bone demineralization, to blockage of blood vessels due to thromboembolisms, and their severity is dependent on a number of factors including the individual's environment, diet, and the specific CBS mutation (Mudd *et al.*, 2001). As of 2011, 164 distinct CBS mutations associated with the disorder have been reported (<http://cbs.lfl.cuni.cz/mutations.php>). Homocystinuria-associated mutations can be categorized according to patients' responsiveness to therapeutic doses of the PLP precursor, pyridoxine (vitamin B₆), which may be linked to the degree of reduced PLP affinity associated with each diseased CBS enzyme. Factors affecting the enzyme's affinity (K_d) for PLP include enzyme misfolding and alterations in active site architecture (Miles and Kraus, 2004). Elevated *in vivo* PLP concentration over the K_d of a particular variant can allow for proper enzyme formation and designation as a pyridoxine responsive variant.

The A144V, R266K, I278T, R336H, K384E or L539S variants have been shown to be among the 50% of variants that are responsive to pyridoxine treatment *in vivo*. Conversely, patients with the R121L, R125Q, C165Y, E176K, T191M, T257M, T262M or G307S mutations have been shown to be nonresponsive to pyridoxine treatment (Mudd *et al.*, 2001). Although the majority of the mutations are identified as private missense mutations, single base substitutions found in one or a small number of individuals, more than half (51%) of homocystinuria diagnoses correspond to four

prevalent variants of CBS: I278T, T191M, G307S and R336C, which all but the I278T substitution are nonresponsive to pyridoxine (Kraus *et al.*, 1999; Urreizti *et al.*, 2003).

The clinical presentation of homocystinuria is primarily associated with four organ systems: the vascular system, the central nervous system (CNS), the eye and the skeletal system. The most frequent cause of death in patients with CBS deficiency is the development of vascular thromboembolisms, which have been reported to develop in patients as young as 19 months (Kerrin *et al.*, 1996). An international study conducted by Mudd and colleagues (1985) demonstrated that thromboembolic development is not restricted to a particular section of the vascular system. In this study, 253 distinct thromboembolic events were reported for 158 of the 629 patients examined. Of these, 51% involved the peripheral veins while 32% were found to be cerebrovascular, 11% occurred in the peripheral arteries, and 4% were associated with myocardial infarctions (Mudd *et al.*, 1985). Several hypotheses attempting to explain the association between elevated plasma L-Hcys and increased prevalence of thromboembolic events have been put forward. For example, Majors and colleagues (2002) provided evidence that L-Hcys binding to fibronectin, a cysteine-rich protein involved in cell migration and wound healing, may result in abnormal thrombosis formation by impairing fibronectin-fibrin binding. Other reported hypotheses suggest that attacks by the metabolite's cyclic thioester form, L-Hcys thiolactone, on nucleophilic amino acid side chains of proteins lining the vascular endothelium or reactive oxygen species produced by the reduced and cyclic forms of L-Hcys may damage the vascular endothelium resulting in the development of thromboembolisms (Perna *et al.*, 2003; Stamler and Slivka, 1996; Jakubowski, 2008). However, while these *in vitro* studies assumed a causal role of L-

Hcys in the development of vascular occlusions, recent clinical studies suggest the relationship may be correlational.

The presence of hyperhomocysteinaemia, generally described as total L-Hcys levels greater than 15 μM , independent of any genetic disorder were proposed to be an independent risk factor for vascular diseases (Clarke *et al.*, 1991; Saposnik *et al.*, 2009). However, studies have revealed that lowering the total L-Hcys concentration *via* B-vitamin supplementation does not decrease the risk of vascular disease. A large study analyzing the stroke outcomes of 5522 adults diagnosed with cardiovascular disease were given folic acid, vitamins B₆ and B₁₂ (cobalamin), or a matching placebo for an average of 5 years, found that B-vitamin supplementation lowered total L-Hcys concentration, but it did not reduce the risk of major vascular events (Bosnaa *et al.*, 2006). Zhang *et al.* (2013) conducted a meta-analysis of 18 randomized controlled trials consisting of 57,143 individuals and found that the combination of folic acid and Vitamins B₆ and B₁₂ was not associated with a significant reduction in stroke risk. As a result, there is some uncertainty about the role of L-Hcys, whether causal or incidental, in vascular disease.

Neurological abnormalities such as seizures and mental retardation are often the first recognized sign of CBS deficiency (Mudd *et al.*, 1985). Neurological disorders such as neural tube defects can be attributed to L-Hcys, acting as an *N*-methyl-D-aspartate (NMDA) receptor agonist, disrupting neural tube development through an influx of calcium ions and reactive oxygen species, resulting in neuronal damage (Lipton *et al.*, 1997). Studies have also suggested a link between elevated plasma L-Hcys levels and Alzheimer disease (AD). For example, Clarke *et al.* (1998) found that plasma L-Hcys levels were significantly higher in patients with AD than the controls. Among patients

with AD, the study observed a higher degree of disease progression over a 3-year period, as determined by computed tomography (CT) scans, in patients with a higher total plasma L-Hcys concentration (Clarke *et al.*, 1998). A recent study found that L-Hcys exacerbates the three major pathological features of AD in a mouse model: memory loss, and β -amyloid and tau protein accumulation (Li *et al.*, 2014).

A consistent observation in patients with homocystinuria is the appearance of ectopia lentis, the dislocation of the ocular lens due to a disruption of the zonular fibres, which are responsible for maintaining the lens' position within the eye (Mudd *et al.*, 2001; Cruysberg *et al.*, 1996). It is believed that excess L-Hcys in the aqueous humour forms disulfide bonds with the L-Cys residues of the glycoprotein fibrillin, which plays a key role in maintaining the strength and elasticity of the ocular fibers, thus compromising its structural integrity (Sakai, 1990; Mudd *et al.*, 2001). The resulting loosening of the ocular lens leads to the development of myopia (nearsightedness) (Mudd *et al.*, 1985).

The development of osteoporosis (decrease in bone mass and density) in the spine and the long bones (femur, tibia and humerus) is the most common skeletal change found in patients with homocystinuria. At least 50% of patients are reported to have varying degrees of osteoporosis by the age of 20, with earlier onset in those with the pyridoxine-nonresponsive disease variants such as the E176K, T257M and G307S variants (Mudd *et al.*, 1985). It has been suggested that excess L-Hcys results in defective crosslinking of collagen within the bone (Harris and Sjoerdsma, 1966). The crosslinking of collagen molecules involves the formation of aldehyde groups *via* the oxidation of terminal amino groups of lysine or hydroxylysine residues. L-homocysteine is capable of reacting with the exposed aldehyde groups of immature collagen molecules, resulting in the formation

of thiazine rings that subsequently reduce the structural integrity of the bone (Jackson, 1973; Kang and Trelsand, 1973). Taken together, these findings demonstrate the importance of understanding the role L-Hcys plays in the pathophysiology of homocystinuria is critical to finding new therapeutic approaches.

Three different modes of treatment are currently available to patients with homocystinuria. The treatment of choice consists of high doses of pyridoxine. In order to clinically determine a patient's responsiveness to pyridoxine, physicians conduct the pyridoxine challenge test, consisting of an initial daily intake of 100mg pyridoxine followed by an increase in dosage to 200mg and then 500mg, if a 30% reduction in the total plasma L-Hcys is not observed. If no significant change in plasma L-Hcys is observed, the patient is considered B₆-non-responsive (Pagon *et al.*, 2011). Methionine synthase (MS) activity is dependent on cobalamin while the folate cycle provides the methyl moiety transferred to L-Hcys by methionine synthase (Figure 1.1) (Morrow and Barness, 1972). Therefore, folic acid and cobalamin are added to supplement pyridoxine treatment to prevent their depletion in the remethylation of L-Hcys (Morrow and Barness, 1972). A methionine-restricted/cysteine-supplemented diet which includes a combination of natural and synthetic proteins, as well as fruits and vegetables can be used alone or in conjunction with other treatments of CBS deficiency (Yap, 2012). Supplementation with betaine provides an alternative treatment and is chiefly employed for pyridoxine-nonresponsive patients and in cases where dietary compliance is an issue (Singh *et al.*, 2004). Betaine serves as a methyl donor for betaine:homocysteine methyltransferase (BHMT) in the remethylation of L-Hcys observed primarily in liver and kidney tissues (Singh *et al.*, 2004).

1.2. Sulfur-Containing Amino Acids

The presence of excess L-Hcys in plasma and urine is an indicator of an imbalance in the metabolism of sulfur-containing amino acids L-Met and L-Cys. This has implications for cellular homeostasis because, in addition to their roles in the formation of proteins, L-Met and L-Cys are precursors of metabolites central to processes including maintenance of the redox status and methyl transfer reactions (Wu, 2009). Cysteine is the limiting amino acid for the synthesis of glutathione (GSH), key to cellular redox regulation *via* the scavenging of free radicals and other reactive oxygen species (Wu *et al.*, 2004; Banerjee and Zou, 2005; Carballal *et al.*, 2013). The methionine cycle begins with the transfer of an adenosyl moiety, from ATP, to L-methionine by S-adenosylmethionine synthetase (SAMS) to create S-adenosylmethionine (SAM) (Brosnan and Brosnan, 2006). SAM is the primary methyl donor in the biosynthesis of compounds including DNA, RNA, small molecules and amino acids (Finkelstein and Martin, 1984; Stipanuk, 2004; Brosnan and Brosnan, 2006). Transfer of the methyl group of SAM to the various methyl acceptors results in the formation of S-adenosylhomocysteine (SAH), which is subsequently hydrolyzed to L-Hcys and adenosine by SAH-hydrolase (SAHH) (Figure 1.1). L-homocysteine is situated at a crucial metabolic branch point between L-Hcys remethylation, either by MS or BHMT, and the reverse transsulfuration pathway in which the sequential action of the enzymes CBS and cystathionine γ -lyase (CGL) convert L-Hcys to L-Cys (Stipanuk, 2004) (Figure 1.1).

1.3. The Transsulfuration and Reverse Transsulfuration Pathways

The transsulfuration and reverse transsulfuration pathways interconvert L-Cys and L-Hcys, with the direction of the pathway (L-Cys to L-Hcys or *vice versa*) dependent on the evolutionary lineage of the organism (Figure 1.2). Plants and microorganisms have the ability to reduce inorganic sulfur for the *de novo* production of sulfur-containing amino acids, while animals cannot and must obtain L-Met through in their diet (Atmaca, 2004; Aitken and Kirsch, 2005). L-methionine is an essential amino acid but L-Cys, which is produced from L-Met, is considered a conditionally essential amino acid since it can derive either from diet or from the breakdown of L-Met (Stipanuk, 2004; Wu, 2009).

The transsulfuration pathway of bacteria and plants converts L-Cys to L-Hcys by the action of cystathionine γ -synthase (CGS) and cystathionine β -lyase (CBL) (Steegborn *et al.*, 1999). Subsequent methylation of L-Hcys by MS yields L-Met (Figure 1.2). In mammals and yeast, the reverse transsulfuration pathway serves as the only catabolic route for the removal of excess L-Hcys, a product of the methionine cycle (Yadav and Banerjee, 2012; Smith *et al.*, 2012). CBS catalyzes the condensation of L-Hcys and L-Ser, forming L-Cth, subsequently hydrolyzed by CGL, producing L-Cys, α -ketobutyrate and ammonia (NH₃) (Figure 1.2) (Janosik *et al.*, 2001a; Banerjee *et al.*, 2003).

Comparison of the K_m values of the competing branch-point enzymes metabolizing L-Hcys demonstrates that the remethylation of L-Hcys is favoured over transsulfuration, as MS and BHMT have a higher affinity for L-Hcys ($K_m(\text{MS}) = 0.06$ mM; $K_m(\text{BHMT}) = 0.012\text{-}0.06$ mM) than CBS ($K_m = 0.17$ mM) (Finkelstein, 1998; Janosik *et al.*, 2001a). As the concentration of L-Hcys exceeds the capacity of MS and BHMT, and approaches the K_m of CBS, flux through the reverse transsulfuration pathway

is increased, thereby shunting excess L-Hcys irreversibly to L-Cys biosynthesis. This shift is enhanced by the presence of a concentration of SAM sufficient for cellular metabolism, as SAM both hinders L-Hcys remethylation, by inhibiting 5,10-methylene-THF reductase (MTHFR) and BHMT, and allosterically activates CBS (Daubner and Matthews, 1982; Finkelstein and Martin, 1984; Finkelstein *et al.*, 1975). Therefore, the reverse transsulfuration pathway serves the dual role of removing excess L-Hcys and producing L-Cys (Banerjee *et al.*, 2003; Kraus *et al.*, 1998).

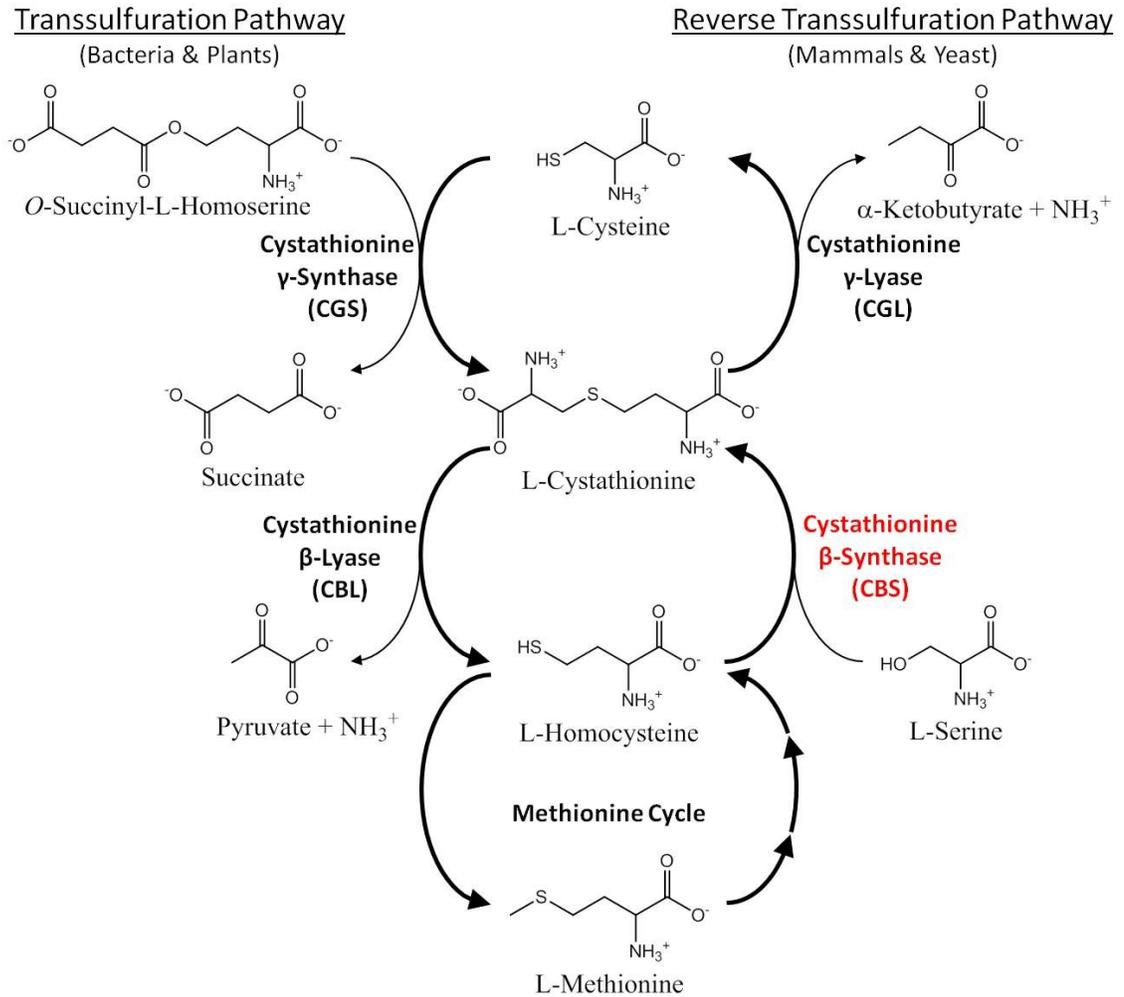


Figure 1.2: The transsulfuration pathway of bacteria and plants, the reverse transsulfuration pathway of mammals and yeast as well as their relationship with the methionine cycle (Steegborn *et al.*, 1999).

1.3.1. Pyridoxal 5'-Phosphate

Many of the enzymes involved in the metabolism of amino acids and their derivatives rely on PLP. This catalytically versatile cofactor is used in diverse reactions including decarboxylation, transamination, racemisation, and various side chain eliminations and rearrangements (Percudani and Peracchi, 2003; Clayton, 2006). Consequently, PLP-dependent enzymes are associated with 121 distinct reactions and have been assigned more than 100 Enzyme Commission (E.C.) numbers that span five of the six different enzymes classes as defined by the International Union of Biochemistry and Molecular Biology (Aitken *et al.*, 2011; Aitken and Kirsch, 2005).

The versatility of PLP originates from its ability to function as an electrophilic catalyst, once covalently bound to an amino acid substrate *via* Schiff base linkage (Percudani and Peracchi, 2003). In its resting state, PLP exists in its internal aldimine form; a Schiff-base linkage with the ϵ -amino group of an active site lysine residue. Once an amino acid substrate enters the catalytic cavity, the active-site lysine residue is exchanged, *via* a transaldimination reaction, for the amino group of the substrate, forming the external aldimine (Hayashi, 1995; Schneider *et al.*, 2000). The various transformations of amino acid substrates catalyzed by PLP-dependent enzymes diverge from this point (Figure 1.3). The specific reaction catalyzed by a PLP-dependent enzyme is in part determined by the orientation of the bonds at the C_{α} atom of the substrate moiety of the external aldimine, with respect to the ring of the cofactor, which determines the C_{α} -bond to be broken (Dunathan, 1966; Christen and Metha, 2001). The pyridinium nitrogen also plays a role in reaction specificity as its electron-withdrawing properties are modulated by electrostatic and hydrogen bonding interactions with nearby residues. The

pyridinium ring functions as an electron sink, weakening the bond of interest enabling cleavage, and subsequently stabilizes the resulting carbanion *via* resonance (Schneider *et al.*, 2000; Christen and Metha, 2001).

The PLP-dependent enzymes catalyzing transformation of amino acids belong to four distinct fold types and are classified on the basis of protein sequence comparisons, available 3D structural data as well as predicted secondary structure elements (Percudani and Peracchi, 2003; Schneider *et al.*, 2000). It has been proposed that each of the four fold types corresponds to a separate independent evolutionary lineage of PLP-dependent enzymes (Percudani and Peracchi, 2003). The largest and most thoroughly characterized of the enzyme families is Fold-type I, which includes aminotransferases, decarboxylases, and enzymes catalyzing α -, β -, and γ -elimination reactions (e.g. CGL and the enzymes of the bacterial and plant transsulfuration pathways). Fold-type II enzymes primarily catalyze β -elimination and replacement reactions and often possess a regulatory domain as well as a catalytic domain (Schneider *et al.*, 2000) (Figure 1.4). Examples of Fold-type II enzymes include *O*-acetylserine sulfhydrylase (OASS), the β -subunit of tryptophan synthase (TrpS), threonine deaminase (TD), threonine synthase (TS), and CBS. Although the overall architecture of enzymes in this fold type may vary depending on the position of regulatory domain(s), each catalytic core shares the same overall fold. The catalytic domain consists of 2 subdomains of approximately equal size, with the active site situated in the cleft formed at their interface (Schneider *et al.*, 2000; Eliot and Kirsch, 2004). Fold types III and IV encompass relatively few members, compared to fold types I and II, and are exemplified by alanine racemase and D-Alanine aminotransferase, respectively (Percudani and Peracchi, 2003).

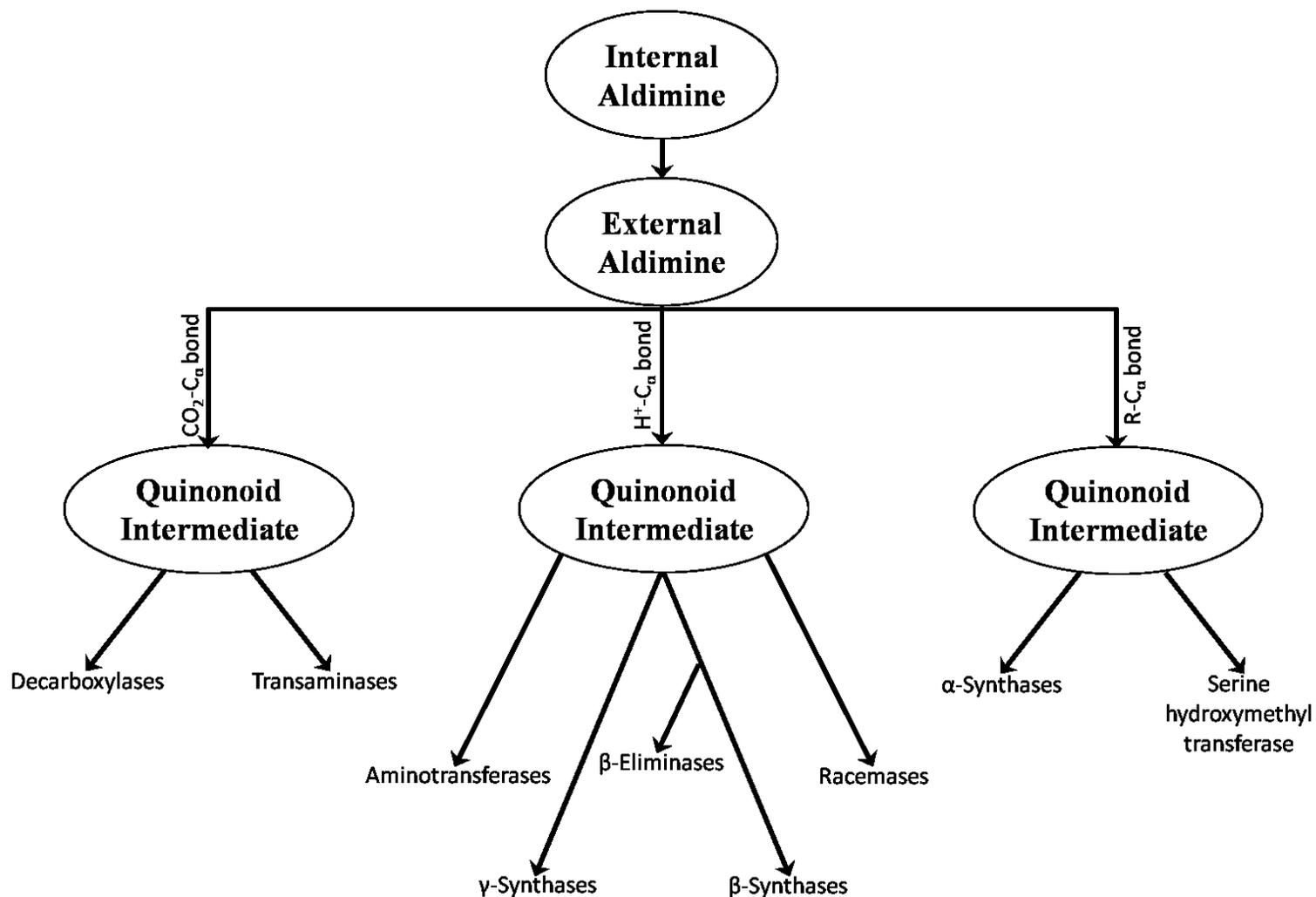


Figure 1.3: The variety of reactions catalyzed by PLP-dependent enzymes. The orientation of the substrate moiety of the external aldimine with respect to the plane of the cofactor ring determines which C_{α} -bond is to be cleaved (Dunathan, 1966; adapted from Schneider *et al.*, 2000).

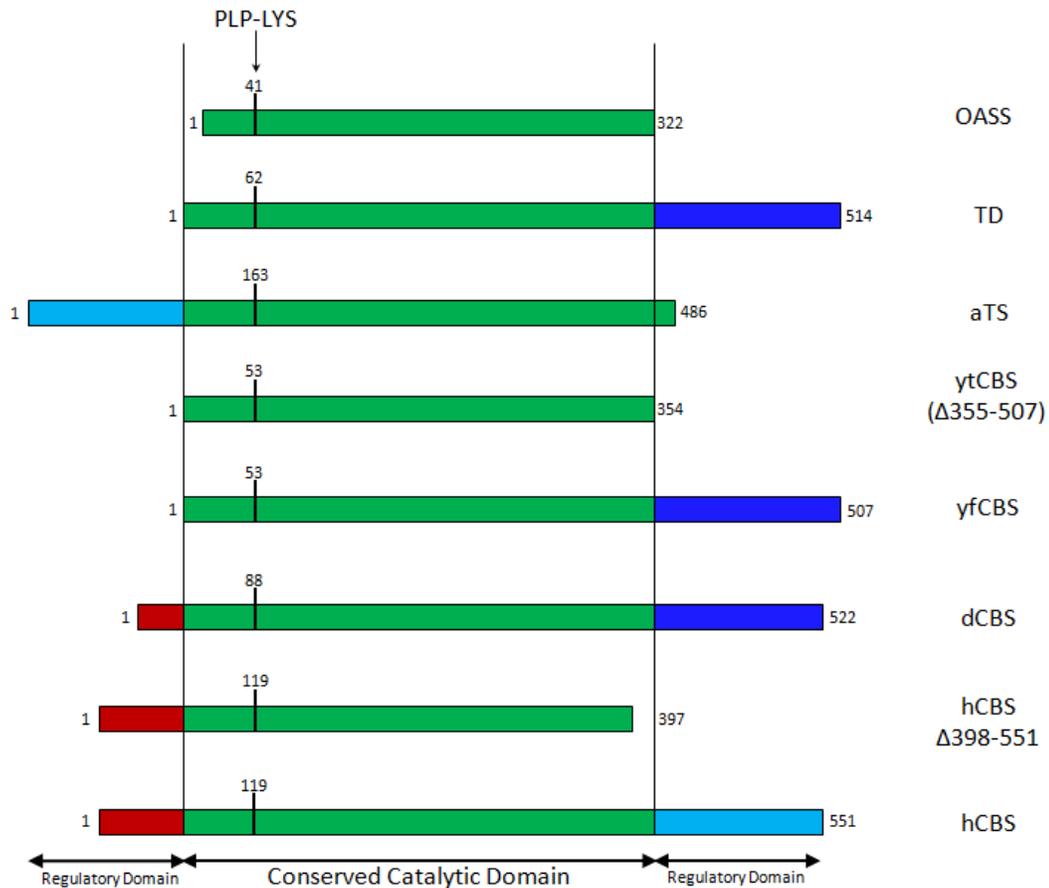


Figure 1.4: A comparison of the organization of domains among a selection of representative fold-type II PLP-dependent enzymes. Domains coloured in **dark red** represent the heme-binding region that is specific to CBS from higher eukaryotes, **green** represents the catalytic, PLP-binding region, **light blue** domains are those activated by S-adenosylmethionine (SAM) while **dark blue** domains of some CBS enzymes possess sequence and structural similarity with those activated by SAM, but have been reported to be nonresponsive to SAM (Adapted from Miles and Kraus, 2004). OASS: *O*-Acetylserine sulphydrylase, TD: Threonine deaminase, aTS: *Arabidopsis thaliana* Threonine synthase (Mas-Droux *et al.*, 2006), ytCBS: Yeast truncated Cystathionine β -synthase (residues 1-354), yfCBS: Yeast full-length Cystathionine β -synthase, dCBS: *Drosophila melanogaster* Cystathionine β -synthase, hCBS: Human Cystathionine β -synthase, hCBS Δ 398-551: truncated form of hCBS employed in this study.

1.4. Cystathionine β -synthase

1.4.1. Protein Architecture

Higher eukaryotic CBS enzymes such as the human (hCBS) and *Drosophila melanogaster* (dCBS) homologues are unique in that they are the only PLP-dependent enzymes that also possess a heme cofactor. In contrast, CBS of lower eukaryotic forms such as yeast (yCBS) does not possess the heme-binding domain. (Kery *et al.*, 1994; Jhee *et al.*, 2000a; MacLean *et al.*, 2000). The human CBS enzyme, spanning 551 residues, is a homotetramer of 63 kDa subunits. Each subunit comprises 3 distinct domains: a N-terminal, heme-binding domain spanning ~ 70 residues, a central catalytic core of ~ 340 residues, which binds one PLP molecule in Schiff base linkage with active-site lysine (K119), and a ~ 140 residue C-terminal, regulatory domain (Kery *et al.*, 1999; Meier *et al.*, 2001). Due to the tendency of the full-length CBS enzyme to aggregate, the only crystal structure available for CBS for many years was the truncated form of hCBS, which lacks the C-terminal domain (Meier *et al.*, 2001). Recently, the structures of the full-length *Drosophila melanogaster* CBS (dCBS) enzyme and a deletion variant of full-length hCBS, lacking residues 516-525 (hCBS $\Delta 516-525$), were reported (Koutmos *et al.*, 2010; Oyenarte *et al.*, 2012; Ereno-Orbea *et al.*, 2013a). The 10-residue (516-525) segment of hCBS has been proposed to participate in higher order oligomerization of the enzyme and their removal enabled the successful crystallization of full-length hCBS (Ereno-Orbea *et al.*, 2013a).

In higher eukaryotes, the heme-binding domain lacks secondary structure, with the exception of a short 3_{10} helix. Notwithstanding the lack of secondary structure, this domain is able to tightly bind the heme molecule *via* the axial ligands C52 and H65

(hCBS numbering) (Ojha *et al.*, 2000; Meier *et al.*, 2001). Substitutions to the heme axial ligands lead to a decrease of activity to as low as ~10% of the wild-type enzyme while maintaining a similar PLP content to the wild-type suggesting that this domain modulates enzyme activity (Ojha *et al.*, 2002; Singh *et al.*, 2009a; Smith *et al.*, 2012). Removal of the heme-binding domain of hCBS results in a hemeless variant that retains ~40% of its activity compared to the wild-type enzyme thus providing proof that heme is not vital to activity (Evande *et al.*, 2004). The fact that lower eukaryotic CBS enzymes, such as yCBS, do not possess a heme-binding domain and yet perform the same overall reaction further indicates that heme is not required for catalysis in hCBS (Jhee *et al.*, 2000a; Jhee *et al.*, 2000b).

Similar to other Fold-type II enzymes, CBS interconverts between an open, solvent-accessible active site where both substrate and product can freely enter the cavity, and a more restrictive closed conformation where the catalytic intermediates are shielded from the solvent (Aitken and Kirsch, 2005). A crystal structure of the L-Met complex of the K41A variant of the closely related OASS first established a model of the closed conformation. It revealed a “mobile loop” that undergoes a conformational change, upon substrate binding, that triggers the closing of the active site (Burkhard *et al.*, 1999). The shift of the mobile loop was also evident in the comparison of the crystal structure of full-length dCBS in its open and closed conformations (Koutmos *et al.*, 2010). Once in the closed state, residues of the mobile loop form a hydrogen bonding network with the carboxylate and hydroxyl moieties of the L-Ser substrate. Substitution of residues within this hydrogen bonding network in yCBS leads to a competing β -elimination reaction, not observed for the wild-type enzyme, in which L-Ser is hydrolyzed to produce pyruvate and

NH₃. This suggests a shift in protein equilibrium towards the open conformation and increased exposure of reaction intermediates to solvent, thus demonstrating the subtle structural features that regulate catalysis and reaction specificity (Aitken and Kirsch, 2004; Aitken *et al.* 2011).

While analysis of the crystal structure of the OASS-K41A variant was critical in determining the active site residues involved in L-Ser binding in CBS, as it was the only ligand complex available for a fold-type II enzyme at the time, uncovering the active site residues that bind the L-Hcys substrate has proven more difficult. The OASS-K41A-L-Met complex has provided very little insight into this matter as the second substrate in the OASS-catalyzed reaction is hydrogen sulfide (H₂S), which lacks the α -carboxylate and amino groups of L-Hcys, and subsequent CBS structures have not included ligands binding at this substrate site. Lodha and colleagues (2009) probed the role of conserved polar residues in the vicinity of the proposed L-Hcys binding site and determined that although these residues play a significant role in sustaining proper active-site conformation, their side chains are not direct determinants of L-Hcys binding (Lodha *et al.*, 2009).

The recent report of the full-length hCBS (Δ 516-525) structure, which exhibits similar basal enzyme activity to native hCBS and is activated by SAM, provides the structural basis for studies probing the mechanism of hCBS regulation (Oyenarte *et al.*, 2012; Ereno-Orbea *et al.*, 2013a). The pairing of the catalytic core domains of the Δ 516-525 variant is similar to that of dCBS but the relative positioning of the C-terminal domain of the two CBS enzymes is distinct (Figure 1.5). The regulatory domain of dCBS interacts with the catalytic core only through the linker. As a result, substrates have

unrestricted entry to the dCBS active site (Koutmos *et al.*, 2010; Ereno-Orbea *et al.*, 2013b). Conversely, the SAM-binding domain of the hCBS (Δ 516-525) dimer is at the head of the active site of the complementary subunit, hindering substrate access to the cavity (Ereno-Orbea *et al.*, 2013a; Ereno-Orbea *et al.*, 2013b) (Figure 1.5). As a result, the hCBS (Δ 516-525) dimer forms a basket-shaped symmetrical dimer where the catalytic core of each subunit interacts with both the catalytic and regulatory domain of the neighboring subunit (Figure 1.5). The auto-inhibitory feature of the SAM-binding domain is due to the presence of four loops at the mouth of the active site. SAM binding is believed to rotate the position of the CBS motifs causing the relaxation of the four loops and provide easier substrate entry into the active site (Ereno-Orbea *et al.*, 2013a). Insights into the crystal structure have led to a proposal wherein hCBS tetramerization occurs as an assembly of 2 dimers stabilized by a loop containing residues 513-519 and the absence of residues 516-525 in hCBS may have allowed for this particular structure to be crystallized (Ereno-Orbea *et al.*, 2013a).

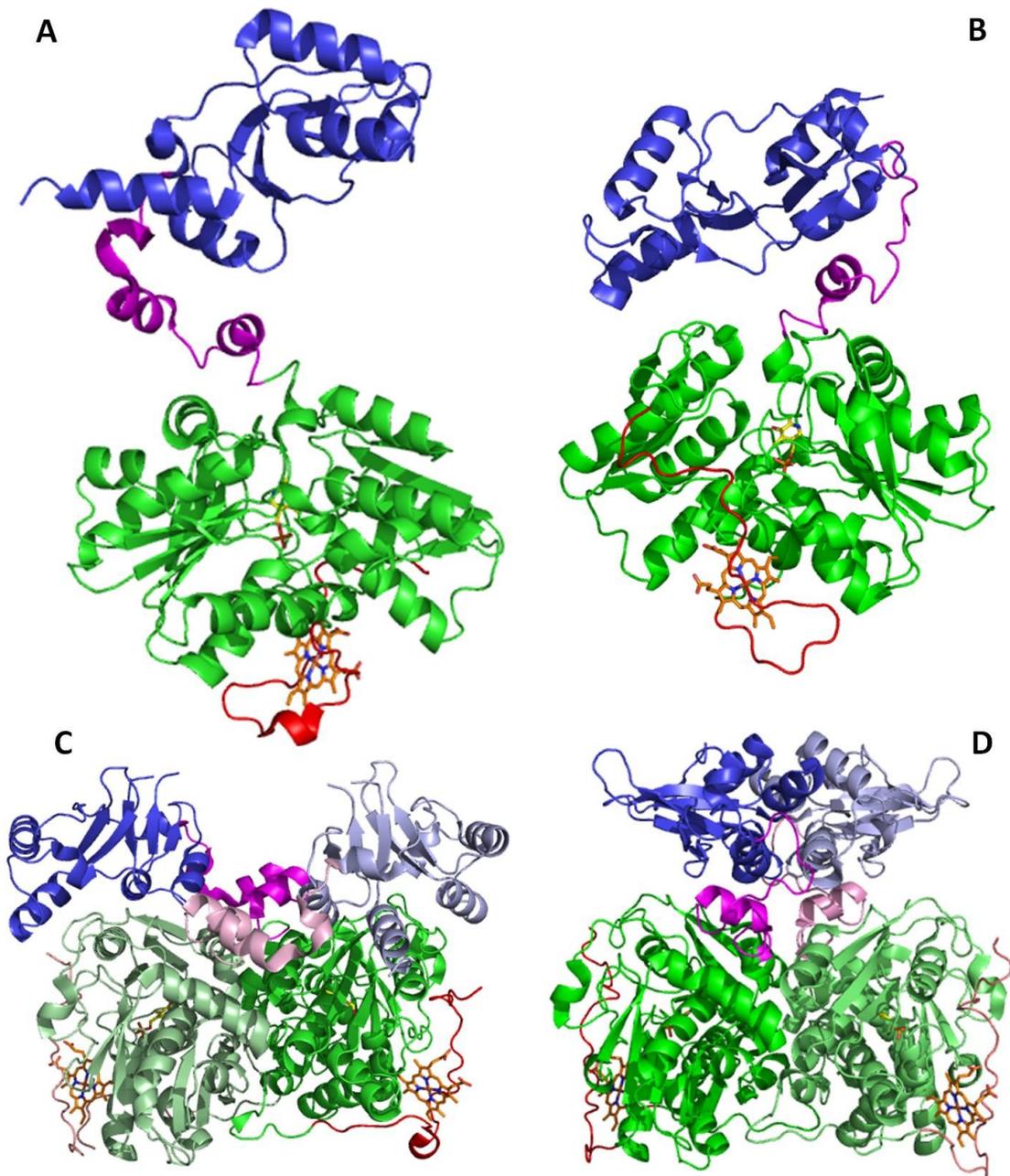


Figure 1.5: Structural differences in the positioning of the regulatory domain in the hCBS(Δ 516-525) and dCBS protein structures. (A and B): hCBS(Δ 516-525) (A) and dCBS (B) monomers showing the heme-binding domain (red), catalytic core (green), C-terminal domain (blue), and the linker (magenta) that connects the catalytic and C-terminal domains. (C and D): the dimeric structures of hCBS (C) and dCBS (D) demonstrating the placement of one subunit (bright colours) relative to the complementary subunit (pale colours) (Koutmos *et al.*, 2010; Ereno-Orbea *et al.*, 2013a). Heme: Orange, PLP: Yellow

1.4.2. Reaction Mechanism

CBS catalyzes the condensation of L-Ser and L-Hcys to form the transsulfuration intermediate L-Cth. The presence of heme within hCBS masks the spectroscopic detection of PLP intermediates, and so the hemeless yCBS homologue has been used in studies investigating the reaction mechanism and the effect of site-directed substitutions of active-site residues. Yeast CBS is a suitable model system for these studies as it catalyzes the same overall reaction and shares 47% amino acid identity with the catalytic core of hCBS (Jhee *et al.*, 2000a; Jhee *et al.*, 2000b).

The α -amino group of the L-Ser substrate nucleophilically attacks the electron-poor carbon (C4') atom of the internal aldimine leading to the formation of the first transient geminal diamine, followed by formation of the external aldimine (E-Ser) *via* the displacement of ϵ -amino group of the active-site lysine (hCBS-K119, yCBS-K53) (Figure 1.6). Abstraction of the C α proton of the L-Ser substrate by the ϵ -amino of the released lysine residue leads to the displacement of the β -hydroxyl group of the substrate and the formation of the aminoacrylate intermediate (Figure 1.6). The free thiol group of L-Hcys then nucleophilically attacks the aminoacrylate intermediate leading to the formation of the external aldimine of L-Cth (E-Cth) (Figure 1.6). The rate-limiting step of this reaction is the release of the L-Cth product, *via* nucleophilic attack on the electron-deficient C4' atom by the catalytic lysine, regenerating the internal aldimine (Figure 1.6) (Jhee *et al.*, 2001). Both yCBS and hCBS have demonstrated the ability to catalyze the reverse-physiological hydrolysis of L-Cth, producing L-Ser and L-Hcys (Jhee *et al.*, 2001; Belew *et al.*, 2009).

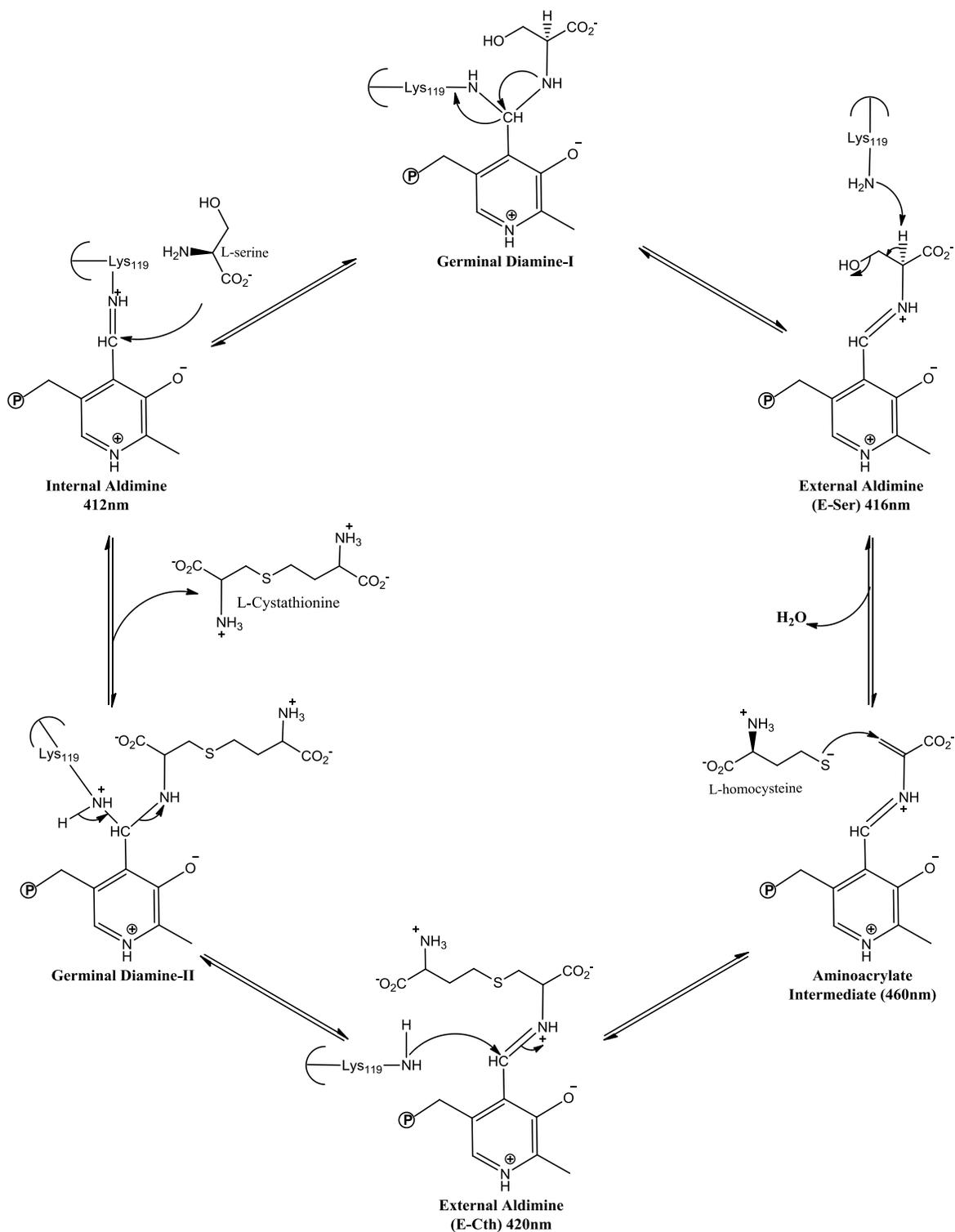


Figure 1.6: Reaction mechanism of CBS, showing the PLP-bound intermediates as well as their associated absorption maxima, as observed in γ CBS (Jhee *et al.*, 2001). E-Ser: external aldimine of L-Ser, E-Cth: external aldimine of L-Cth.

Both yCBS and hCBS are susceptible to substrate inhibition (Jhee *et al.*, 2000a; Belew *et al.*, 2009). At physiological concentrations (L-Ser: ~560 μ M; L-Hcys: ~10 μ M), L-Ser out-competes L-Hcys for access to the free enzyme (Singh *et al.*, 2009b; Jhee *et al.*, 2000a). At elevated L-Hcys concentration, it occupies the L-Ser binding site, effectively reducing the pool of available free enzyme to which L-Ser binds (Jhee *et al.*, 2000a). A second mode of inhibition arises from the premature binding of L-Hcys to E-Ser before the release of H₂O leading to the formation of an inactive enzyme-serine-homocysteine (E-Ser-Hcys) complex (Jhee *et al.*, 2000a).

There are many interactions between the PLP cofactor and the active site residues of hCBS that regulate the orientation and electronic properties of the cofactor as well as define the active site architecture and dynamics, which collectively regulate substrate and reaction specificity as well as the rate of catalysis. For example, interactions with the pyridinium nitrogen (N1), hydroxyl group (O3') and the phosphate moiety of the cofactor can manipulate its electronic properties (Lodha *et al.*, 2010). Similar to other Fold-type II PLP-dependent enzymes, hCBS-S349 (yCBS-S289) is essential for modulating the electron withdrawing properties and orientation of the pyridine ring of PLP *via* a hydrogen bond with the N1 atom of pyridinium ring (Eliot and Kirsch, 2004; Quazi and Aitken, 2009). Residue N149 of hCBS (yCBS-N84) forms a hydrogen bond with the hydroxyl moiety (O3') of PLP and, like hCBS-S349, is believed to maintain the orientation of the pyridine ring while playing a crucial role in preserving PLP in its active ketoenamine state (Lodha *et al.*, 2010; Weeks *et al.*, 2009). Residue G305 (hCBS numbering) may maintain the orientation of the pyridine ring through hydrophobic interactions between the C _{α} of G305 and the pi system of the cofactor's aromatic ring

(Meier *et al.*, 2003). The mobile loop (residues T146-S147-G148-N149-T150 of hCBS, conserved as T81-S82-G83-N84-T85 in yCBS) plays an important role in the coordination of the α -carboxyl group of the L-Ser substrate while the backbone of G307, positioned at the mouth of the active site, is thought to play a role in L-Hcys binding (Aitken and Kirsch, 2004; Meier *et al.*, 2003). Residues S147, K177, E304, K384, E176 and T383 of hCBS are part of a hydrogen bonding network that bridges the mobile loop and residues of the C-terminus of the catalytic domain (D376, R379, S380, Y381) (Lodha *et al.*, 2009; Lodha *et al.*, 2010). A glycine-rich loop (hCBS-G256-T257-G258-G259-T260) wraps around the phosphate moiety with the two threonine residues (T257 and T260) providing hydrogen bonding linkage to PLP (Banerjee *et al.*, 2003; Yadav *et al.*, 2012). Understanding the correlation between the electrostatic and hydrogen bonding properties of residues within the active site and enzymatic function is critical to explaining how modifications to these interactions, caused by homocystinuria-associated mutations, may alter the ability of the enzyme to perform its canonical role and/or its secondary role as a H₂S producer.

1.4.3. *Role of CBS in Hydrogen Sulfide Production*

Recent studies have indicated that H₂S mediates a wide range of physiological responses such as muscle relaxation, the induction of the intracellular signal transducer cyclic adenosine monophosphate (cAMP) and enhancing the flow of cations into the cell by way of NMDA receptors (Abe and Kimura, 1996; Kimura, 2013; Kimura, 2000). The concentration of H₂S *in vivo* is in the tens to hundreds micromolar range (Singh and Banerjee, 2011; Evans, 1967). In mammalian tissues, H₂S is chiefly produced by CBS

and CGL with the majority of H₂S production in the brain attributed to CBS, while in other tissues such as the small intestine and the thoracic aorta H₂S is produced by CGL (Hosoki *et al.*, 1997). H₂S increases GSH production in reactive astrocytes, suggesting a role in the protection of neurons from oxidative stress (Kimura and Kimura, 2004; Kimura, 2013).

Three distinct β -replacement reactions resulting in the production of H₂S have been reported for CBS (Figure 1.7): the condensation of L-Cys (replacing L-Ser) with L-Hcys, leading to the production of L-Cth and H₂S [2], the condensation of two molecules of L-Hcys to form L-lanthionine and H₂S [3] and exchange of the sulfhydryl moiety of L-Cys with a hydroxyl group, from water, to form L-Ser and H₂S [4] (Yadav and Banerjee, 2012). Reaction [2] (the condensation of L-Ser and L-Hcys) is the principal *in vivo* H₂S generator, encompassing ~96% of the total amount of H₂S produced in the brain (Singh *et al.*, 2009b). The generation of H₂S is generally believed to depend on the availability of L-Cys rather than L-Hcys, since L-Cys is capable of functioning as both the first and second substrates in a CBS-catalyzed reaction while L-Hcys can only be the second substrate, and because H₂S production by CBS is relatively insensitive to increasing L-Hcys concentration (Singh and Banerjee, 2011; Singh *et al.*, 2009b). This is further supported by the ~1-2-fold order-of-magnitude difference between the expected intracellular concentration of L-Cys (~200 μ M) and the K_m values of L-Cys with respect to the two substrate binding sites (6.8 mM and 27 mM, respectively) (Singh and Banerjee, 2011; Özkan *et al.*, 2002). Consequently, elucidating the roles of active site residues of CBS may provide insight into its diverse physiological roles.

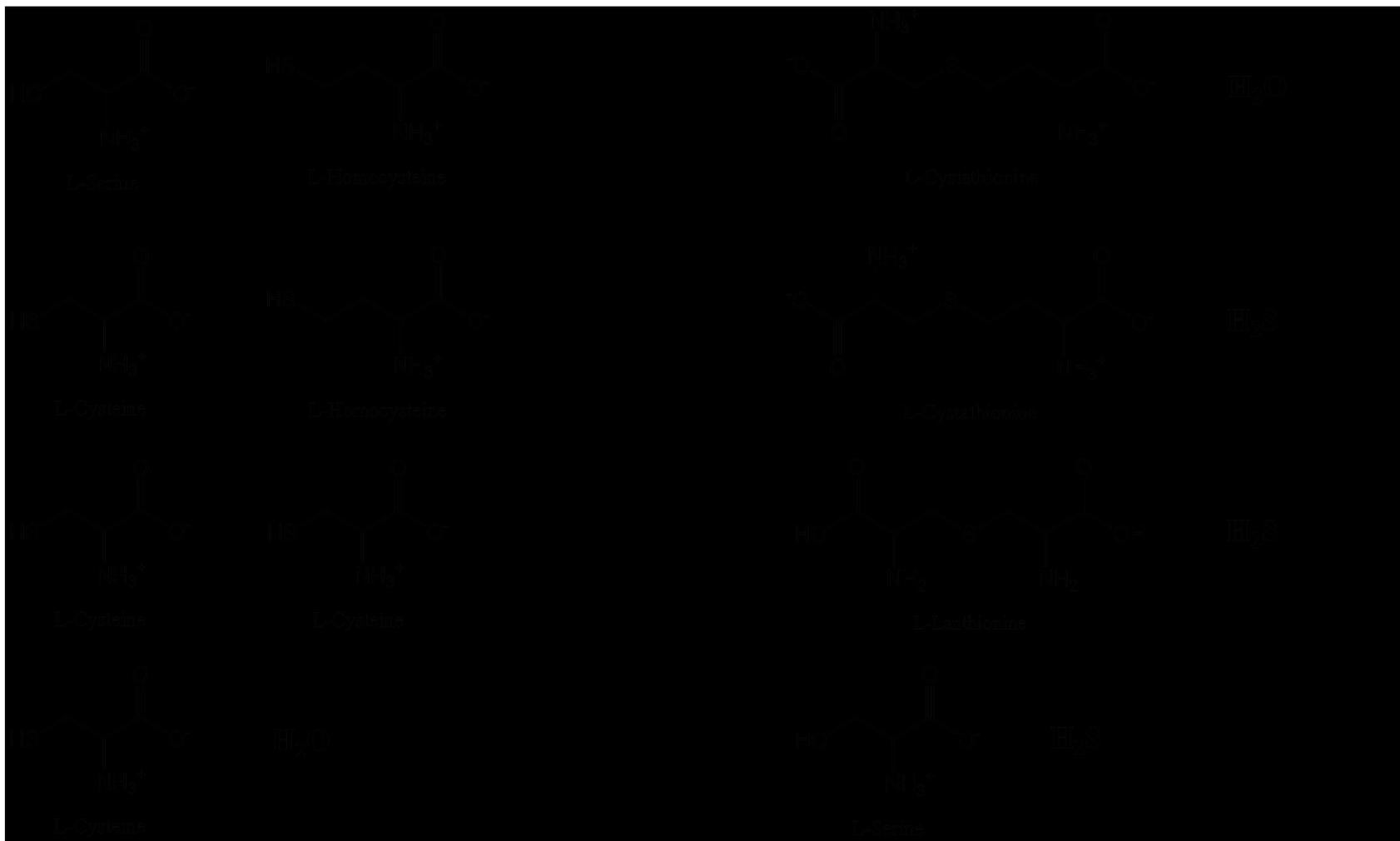


Figure 1.7: The four known β -replacement reactions catalyzed by CBS. Reaction 1 represents the canonical reaction while reactions 2-4 characterize the possible β -replacement reactions that yield H_2S (Yadav and Banerjee, 2012)

1.4.4. Proposed Roles of Heme in hCBS

The role of the heme cofactor has yet to be determined and several hypotheses have been proposed over the past two decades (Taoka *et al.*, 1998; Janosik *et al.*, 2001b; Majtan *et al.*, 2011). Majtan and colleagues (2008) demonstrated the production of stable and active hCBS in heme-deficient strains of *Saccharomyces cerevisiae* and *Escherichia coli* using chemical chaperones, porphyrin rings lacking a metallic core and exogenous heme. In a subsequent study, Majtan *et al.* (2011) described the purification of a full-length human enzyme with a cobalt-centred protoporphyrin IX ring substituted for heme. This approach yielded an enzyme with no significant change in specific activity, quaternary structure, and responsiveness to SAM and PLP saturation, compared to native hCBS (Majtan *et al.*, 2011). The authors concluded that heme cofactor serves a structural role within the enzyme (Majtan *et al.*, 2011). However, a consensus as to the role of heme has not been reached, as other researchers hypothesized the cofactor functions as a redox sensor (Taoka *et al.*, 1998).

In its ferric (Fe^{3+}) state, the hCBS heme is relatively inert to ligand exchange with exogenous compounds, while in its ferrous (Fe^{2+}) state, the iron atom is able to bind carbon monoxide (CO) and nitric oxide (NO) (Taoka and Banerjee, 2001; Banerjee and Zou, 2005). Binding of these molecules displaces C52 and concomitantly induces a tautomeric shift of PLP from its ketoenamine (active) form to its enolimine (inactive) state (Weeks *et al.*, 2009). The shift is believed to be induced by the removal of a salt bridge between C52 and R266, located at the C-terminal end of α -helix 8 (Figures 1.8 & 1.9). The resulting deprotonation of the nitrogen atom strengthens the imine bond and decreases the susceptibility of the adjacent C4' atom to a nucleophilic attack by L-Ser

(Figure 1.8). As a result of the tautomeric shift, the ketoenamine-stabilizing hydrogen bond between the O3' atom of PLP ring and active-site residue Asn149 is lost (Weeks *et al.*, 2009) (Figure 1.9).

Residue N84 of yCBS plays a role in reaction specificity as well as PLP orientation within the active site (Lodha *et al.*, 2010). Substitution of N84 in yCBS, the residue corresponding to hCBS-N149, is believed to cause a shift in the orientation of PLP within the active site, evident by the ~230-fold reduction in catalytic efficiency in addition to the development of marginal β -elimination activity (Lodha *et al.*, 2010). Residues located at the active-site end of α -helix 8 are involved in the stabilization of PLP *via* hydrogen bonding interactions with its phosphate moiety. Alteration of these residues resulted in the destabilization of PLP, while increasing the susceptibility to enzyme inactivation when bound to ferrous heme (Yadav *et al.*, 2012). This has led to the suggestion of bi-directional communication between PLP and heme through α -helix 8 (Yadav *et al.*, 2012). This brings credence to heme's proposed role as a redox regulator as the oxidation state of iron can be relayed to the PLP site *via* α -helix 8, with resulting modulation of enzymatic function.

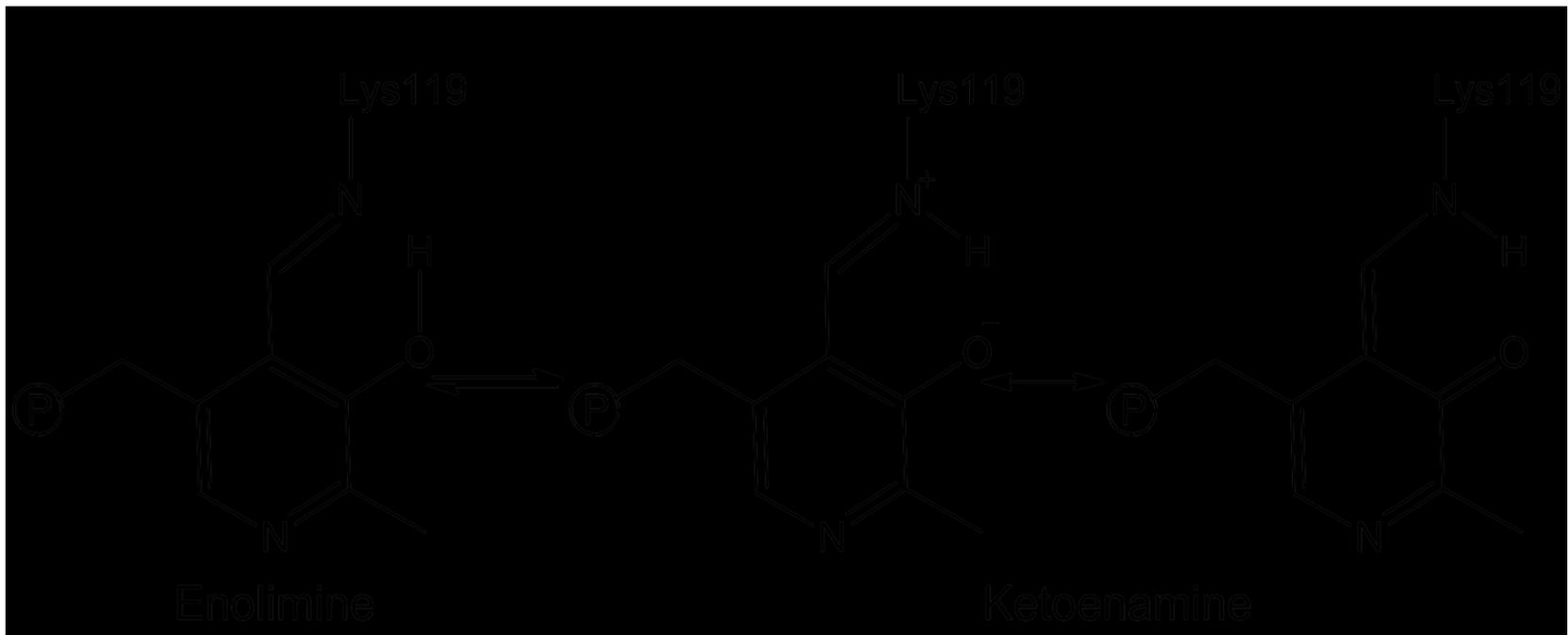


Figure 1.8: Enolimine and ketoenamine tautomers of the PLP-internal aldimine in human cystathionine β -synthase (Weeks *et al.*, 2009)

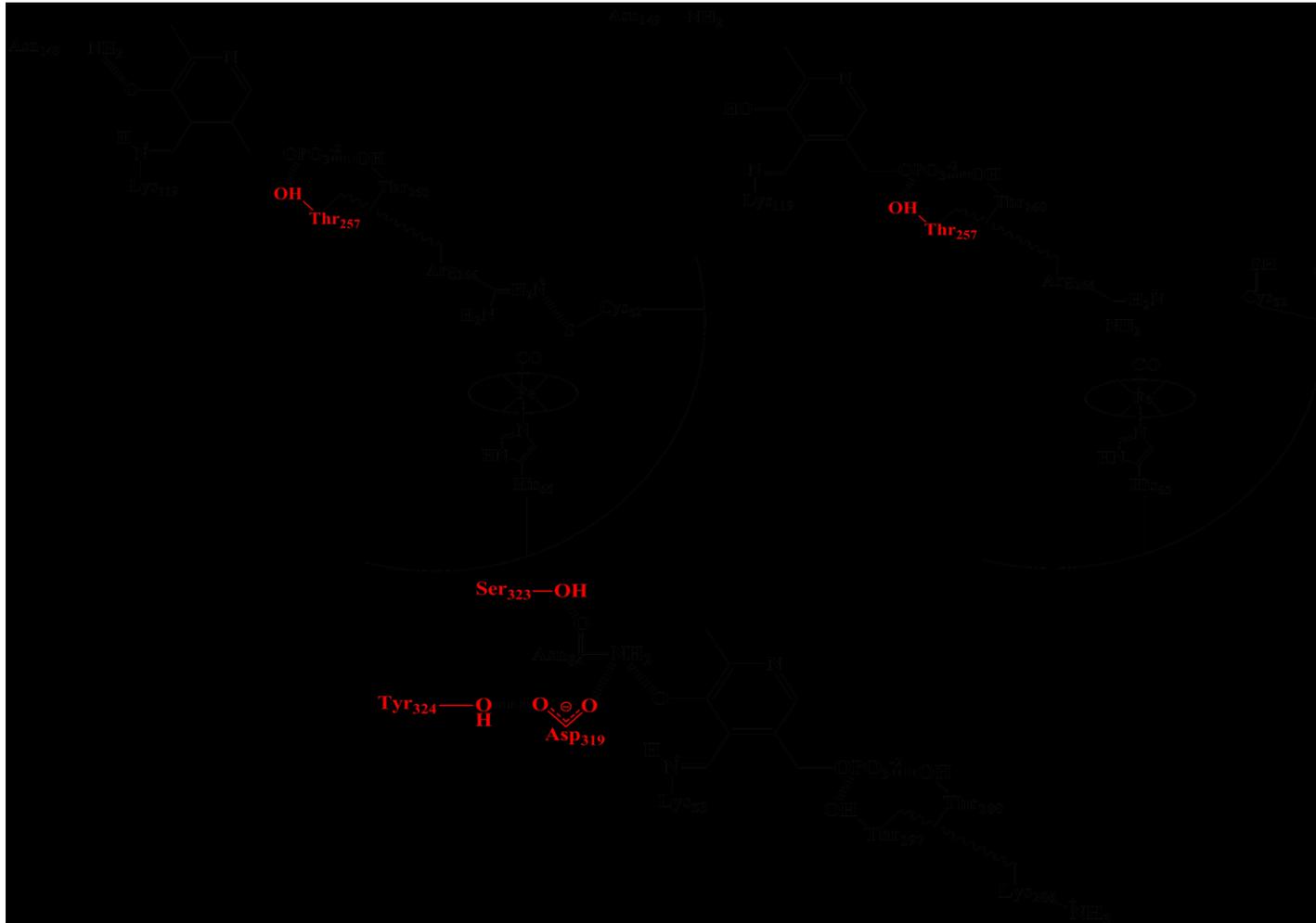


Figure 1.9: Proposed mechanism for the heme-CO linked inactivation of hCBS (Weeks *et al.*, 2009). Targeted residues of this study are highlighted in red. (A) Within the hCBS active site, CO replaces the thiol of C52 as a heme ligand resulting in the loss of the ketoenamine-stabilizing hydrogen bond between the O3' atom of PLP ring and N149. (B) Proposed contacts of corresponding residues in yCBS, which lacks the heme-binding region.

1.4.5. SAM Regulation in CBS

The C-terminal domain of hCBS contains a tandem repeat of a characteristic β - α - β - β - α fold, known as a "CBS" domain due to its initial discovery in hCBS (Bateman, 1997). These domains are found in other enzymes, including inosine monophosphate dehydrogenases, membrane channels and transporters, such as members of the ABC transporter family that transport glycine, betaine and L-proline (Bateman, 1997). Each hCBS monomer contains two such domains, designated CBS1 and CBS2, which are proposed to bind the allosteric regulator SAM, although the mechanism of activation remains to be elucidated (Finkelstein *et al.*, 1975; Ereno-Orbea *et al.*, 2013a). In the absence of SAM, hCBS activity is limited to a "basal" level by the autoinhibitory action of the C-terminal domain (Janosik *et al.*, 2001a; Scott *et al.*, 2004). Upon either SAM binding or removal of the C-terminal regulatory domain hCBS activity is increased ~2-4-fold (Kery *et al.*, 1998; Evande *et al.*, 2002). While not activated by SAM, removal of the C-terminal domain of γ CBS results in the same increase in activity observed for hCBS (Jhee *et al.*, 2000a). In contrast, the opposite effect occurs in dCBS when the C-terminal domain is removed (Koutmos *et al.*, 2010). This suggests that there is a difference in the arrangement of CBS domains such that it leads to a higher basal activity in dCBS while limiting basal activity in hCBS (Koutmos *et al.*, 2010; Ereno-Orbea *et al.*, 2013a).

Recent work has shown that there are two potential SAM-binding sites (S1 and S2) within the C-terminal domain of hCBS (Pey *et al.*, 2013) (Figure 1.10). Both sites are located in separate cavities between CBS1 and CBS2 and are structurally similar to sites reported to bind adenosyl ligands in other proteins with tandem CBS domains (Pey *et al.*, 2013; Ereno-Orbea *et al.*, 2013a). Both sites possess a hydrophobic pocket of residues

that can accommodate an adenine ring as well as conserved asparagine and threonine residues for interactions with the ribose moiety (Ereno-Orbea *et al.*, 2013a). Isothermal titration calorimetry (ITC) data has demonstrated that both proposed binding sites of hCBS are functional and that S2 functions as a high-affinity site, responsible for increasing the stability of the enzyme, while subsequent binding of SAM to the lower-affinity S1 site results in enzyme activation (Pey *et al.*, 2013). A mechanism of hCBS activation by SAM has recently been proposed in which SAM-binding to S2 causes both CBS motifs to rotate away from the catalytic core, thus exposing S1 for further SAM-binding (Pey *et al.*, 2013; Ereno-Orbea *et al.*, 2013a).

1.4.6. Homocystinuria-associated Mutations of CBS

Missense mutations constitute ~87% of all CBS mutations associated with homocystinuria and are not restricted to a particular region of the enzyme (Majtan *et al.*, 2010). This is exemplified by the location of the five most common missense mutations: I278T (subunit interface), T191M (catalytic core), G307S (catalytic core), R336C (subunit interface) and D444N (SAM-binding domain) (Meier *et al.*, 2003; Yamanishi *et al.*, 2006). Some CBS missense mutations associated with homocystinuria do not specifically target catalytic residues and result in the production of misfolded proteins, impairing function of protein levels (Majtan *et al.*, 2010). A thorough understanding of the structure-function relationships underlying the homocystinuria-associated mutations on the enzyme will provide insight for the enhancement of therapeutic treatments.

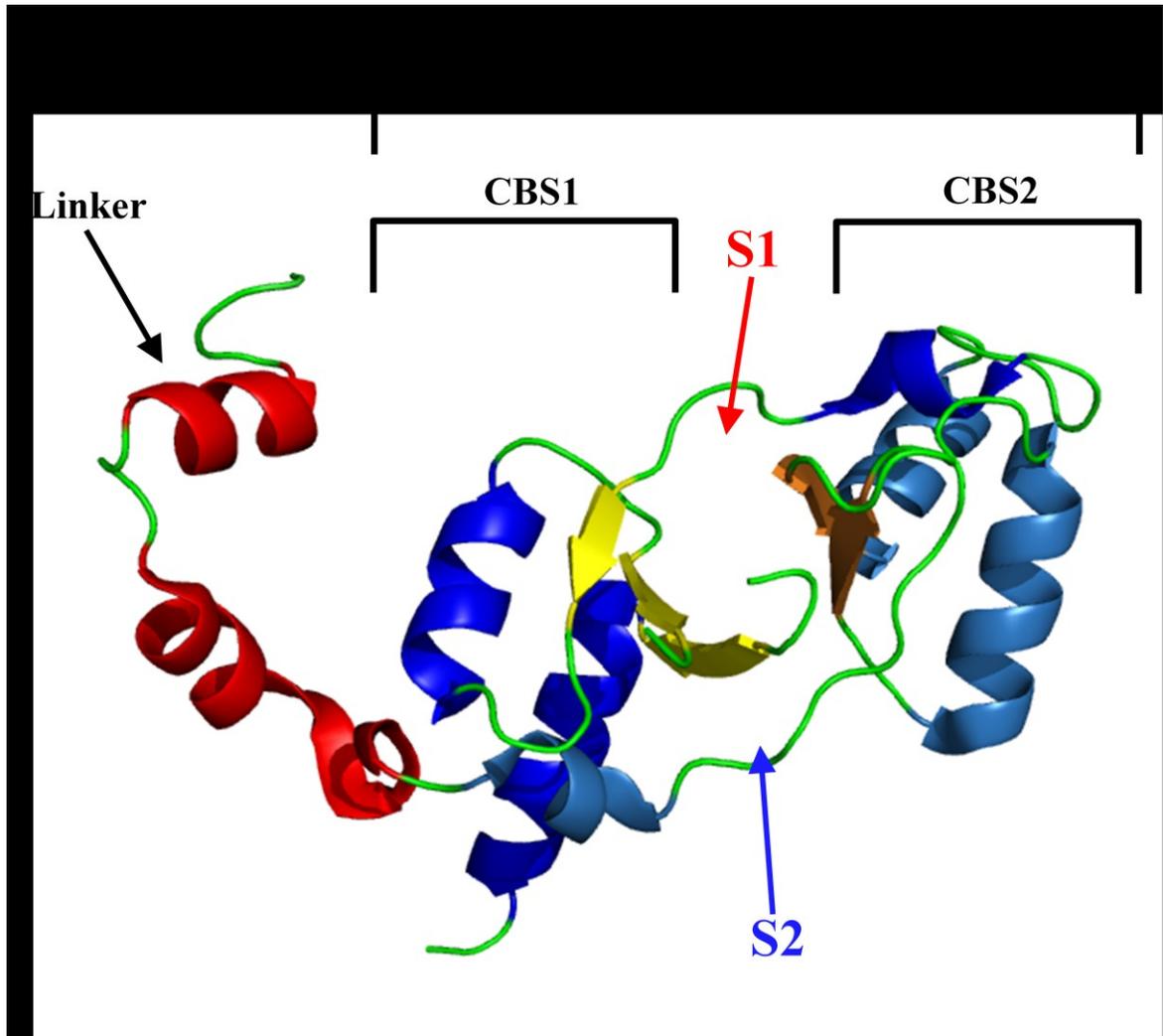


Figure 1.10: A 3D model of the hCBS $\Delta 516-525$ variant crystal structure showing both CBS domains (CBS1 and CBS2) and the location of the proposed binding sites of SAM (S1 and S2) (Ereno-Orbea *et al.*, 2013a).

1.5. Focus of Study and Objectives

Treatment of hereditary metabolic disorders, such as homocystinuria, is complicated by the need to consider the specific allele(s) an individual possesses when developing a treatment regime. For example, B₆ supplementation is only effective for approximately half of reported disease-associated alleles. This necessitates development of an understanding the structure-function relationships that underlie each distinct disease-associated allele and how these are manifested *in vivo*, as well as clinically. This study explores these structure-function relationships in the active site of the enzyme cystathionine β -synthase. The objectives of this study are:

1. Investigation of residues D319, S323 and Y324 in yCBS, located at the junction of the catalytic domain and the linker segment that connects the catalytic and regulatory domains. These residues are proposed to position active site residue yCBS-N84 (hCBS-N149), which regulates reaction specificity and PLP positioning (Lodha *et al.*, 2010). Site-directed mutagenesis was employed to remove only the polar group of each residue (D319A, S323A and Y324F) in order to probe its role within the active site hydrogen bonding network.
2. Exploration of homocystinuria-associated mutations in the hCBS active site. These included residues of the mobile loop (G148), bridging the active site cleft (E176, K384), interacting with the phosphate moiety (T257, G259) or pyridine ring (G305, S349) of the cofactor or with a proposed role in L-Hcys binding (G307). A series of 11 homocystinuria-associated variants (G148E/R, E176K, T257M, G259D/M, G305R, G307S, S349N and K384E/N) were created using a

hCBS construct lacking residues 398-551 (hCBS CAT_L397) allowing for direct comparison with the corresponding hCBS structure (Meier *et al.*, 2001).

2. METHODS

2.1. Materials

Isopropyl- β -D-thiogalactopyranoside (IPTG) was obtained from BioShop. L-cystathionine, β -NADH, L-Ser and D,L-homocysteine (D,L-Hcys) were purchased from Sigma. Dithiothreitol (DTT), ampicillin, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) and imidazole were acquired from Fischer Scientific. Nickel-nitriloacetic acid (Ni-NTA) resin and L-Lactate dehydrogenase (LDH) were purchased from Qiagen and Roche, respectively. The *Escherichia coli* cystathionine β -lyase (eCBL) coupling enzyme was expressed and purified as described by Aitken and Kirsch (2004).

2.2. Construction of Site-Directed Mutants

Oligonucleotide primers were designed for the introduction of the following site-directed mutations into the yCBS full-length sequence (yfCBS): D319A, S323A and Y324F and the truncated hCBS (htCBS) variant hCBS_CAT_L397 (residues 1-397): G148E/R, E176K, T257M, G259D/M, G305R, G307S, S349N and K384E/N (Table 2.1). The S323A and Y324F yfCBS constructs were prepared by Allison Jaworski. Overlap-extension polymerase chain reaction (OE-PCR), using the pSECseq0 and pSECseq7r flanking primers in addition to the mutagenic primers (Table 2.1), was utilized to construct the site-directed mutants (Higuchi *et al.*, 1988). The re-assembled amplicons were inserted between the *Nde*I and *Hind*III (hCBS) or *Nde*I and *Pst*I (yfCBS) restriction sites of the pTrc3 expression vector, which encodes a N-terminal 6-His affinity tag (Farsi *et al.*, 2009). The yfCBS and htCBS expression plasmids were transformed, *via* the heat shock method, with *E. coli* ER1821 cells (Froger and Hall, 2007). Nucleic acid sequencing was used to verify the presence of the site-directed mutations in each vector.

Table 2.1: Primers used in the construction of the yfCBS and htCBS site-directed mutants.

yfCBS Variant	Mutagenic Primer Sequence
D319A*	5' -ccatattcccaGCGtccatcaggtcg
S323A*	5' -gattccatcaggGCGtacctaacc
Y324F*	5' -gattccatcaggtcgTTCctaacc
htCBS Variant	Mutagenic Primer Sequence
G148E*	5' -cgagccgacatccGAAaacaccgggatcg
G148R*	5' -cgagccgacatccCGTaacaccgggatcg
E176K*	5' -ccagagaagatgagctccAAAaagggtggacgtgc
T257M*	5' -ggcttcagtgggcATGggcggcacc
G259D*	5' -ggcacgggcGACaccatcacg
G259M*	5' -ggcacgggcATGaccatcacg
G305R*	5' -cgaggtggaaCGTatcggctacg
G307S*	5' -ggaagggatcTCTtacgacttcaccc
S349N*	5' -gctgtgcggtggcAACgctggcagc
K384E*	5' -ggaactacatgaccGAAttcctgagcg
K384N*	5' -ggaactacatgaccAACttcctgagcg
pSECseq0*	5' -ggcgtcaggcagccatcggaagctg
pSECseq7r*	5' -gcccgccaccctccgggcccgttgcttcgc

*The second mutagenic primer is the reverse complement of the primers listed above

2.3. Expression and Purification of yfCBS Mutants

All yfCBS constructs was expressed and purified using the same procedure. Three 2.8-L Fernbach flasks, each containing 1L of CBS growth media (50 mM potassium phosphate, 50X V.B Salt, 7.2 g Tryptone, 14.4 g Yeast extract, glycerol) and 100 µg/mL ampicillin, were inoculated with an overnight culture (1:10 ratio) of the *E. coli* strain ER1821 containing the yfCBS construct. The cultures were grown at 30°C until the OD₆₀₀ reached 0.5 before inducing with 0.15 mM IPTG and further incubation for 16 hours. The cells were harvested *via* centrifugation at 5000 RPM at 4°C for 10 minutes. The resulting pellets were resuspended in 0.85% NaCl and centrifuged at 5000 RPM for 15 minutes before storage at -80°C.

The stored cells were resuspended in buffer A (50 mM potassium phosphate, pH 7.8, 20 µM PLP and 10 mM imidazole) and lysozyme was added, to 1 mg/mL, with subsequent incubation for 20 minutes at room temperature. Following sonication (8x30 seconds at 50% duty cycle) the cellular debris was removed by centrifugation at 15000 RPM at 4°C for 60 minutes. The supernatant was loaded onto a 1.0 x 10cm column of Ni-NTA resin, equilibrated with 20 column volumes of buffer A. The affinity-tagged enzyme was eluted with a 200 mL linear gradient of 10-200 mM imidazole in buffer A. The fractions collected were assessed for protein purity *via* SDS-PAGE and fractions containing >95% pure target enzyme, as assessed by visual inspection, were pooled, concentrated and dialyzed against storage buffer (50 mM potassium phosphate, 1 mM EDTA, pH 7.8, 1mM DDT & 20 µM PLP). Glycerol was added to 20% (v/v) before storage at -80°C. The protein concentration of each yfCBS construct were determined *via* UV spectroscopy ($\epsilon_{280} = 55,900 \text{ M}^{-1} \text{ cm}^{-1}$) (Jhee *et al.* 2000a).

2.4. Expression and Purification of htCBS Mutants

All htCBS constructs were expressed and purified using the protocol reported by Belew *et al.* (2009). Three 2.8-L Fernbach flasks, each containing 1L of LB media, 75 mg/L δ -aminolevulinic acid (δ -ALA) and 100 μ g/mL ampicillin, were inoculated (1:50 ratio) with an overnight culture of the *E. coli* strain ER1821 containing the htCBS construct. These cultures were grown at 30°C to OD₆₀₀ = 0.5, induced with 0.2 mM IPTG and incubated for an additional 16 hours. The cells were harvested *via* centrifugation at 5000 RPM at 4°C for 10 minutes. The resulting pellets were resuspended with 0.85% NaCl, centrifuged at 5000 RPM for 15 minutes and the pellets were stored at -80°C.

The cells were resuspended and purified as described for yfCBS, with the exceptions that a 2mL Ni-NTA column and a 200 mL linear gradient of 10-300 mM imidazole were employed. The protein concentration of each htCBS construct were determined *via* UV spectroscopy ($\epsilon_{280} = 59,243 \text{ M}^{-1} \text{ cm}^{-1}$).

2.5. Spectroscopic Characterization of htCBS

The UV-Visible spectra of the CBS enzymes were recorded using an Agilent diode array model 8453 UV-visible spectrophotometer at 25°C. All htCBS variants were diluted in 50 mM Tris buffer (pH 8.6) to a final concentration of 4 μ M and their respective spectra were collected. A truncated yeast CBS enzyme (ytCBS), which was diluted to a final concentration of 4 μ M, was used to demonstrate the masking of PLP by the heme cofactor.

2.6. Enzyme Assays

Enzyme activity was measured in a total volume of 100 μL at 25°C with a Spectramax 390 spectrophotometer (Molecular Devices). The assay buffer consisted of 50 mM Tris (pH 8.6) and 20 μM PLP. A background rate, for all components except the CBS enzyme, was recorded for each sample before initiating the reaction *via* the addition of CBS. The data were fit using KaleidaGraph (Synergy Software).

2.6.1. Hydrolysis of L-Cystathionine by yfCBS

The reverse-physiological hydrolysis of L-Cth to L-Ser and L-Hcys was detected *via* the reaction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) with the thiol group of the product (Aitken and Kirsch, 2003). L-homocysteine reacts with DTNB to form a homocysteine/TNB⁻ (3-thio-6-nitrobenzoate) mixed disulfide, with concomitant formation of TNB⁻ ($\epsilon_{412} = 13,600 \text{ M}^{-1} \text{ cm}^{-1}$) (Figure 2.1). Reactions were carried out in assay buffer containing 2mM DTNB, 0.004-6.2 mM L-Cth and 0.675-2.175 μM yfCBS, depending on the activity of the variant. The data were fitted to the Michaelis-Menten equation (equation 1) to determine k_{cat} and K_m while k_{cat}/K_m was calculated independently using equation 2:

$$\frac{v}{[E]} = \frac{k_{cat}[S]}{K_m + [S]} \quad (1)$$

$$\frac{v}{[E]} = \frac{k_{cat}/K_m [S]}{1 + [S]/K_m} \quad (2)$$

2.6.2. Condensation of L-Serine and L-Homocysteine by htCBS

The CBL-LDH continuous assay was developed to monitor the condensation of L-Ser and L-Hcys, yielding L-Cth (Aitken and Kirsch, 2003). The L-Cth product is cleaved by eCBL, producing L-Hcys, NH_3^+ and pyruvate, which is reduced to lactate by LDH, with the concomitant oxidation of NADH ($\epsilon_{340} = 6,200 \text{ M}^{-1} \text{ cm}^{-1}$) to NAD^+ (Figure 2.2). Reactions were completed in assay buffer containing 1.5 mM NADH, 1 μM CBL, 0.5 μM LDH, 0.2-36 mM L-Ser and 2 mM Hcys as well as 1.23-4.11 μM htCBS. The data were fitted to the Michaelis-Menten equation while the k_{cat}/K_m values of the L-Ser substrate was calculated independently using equation 2.

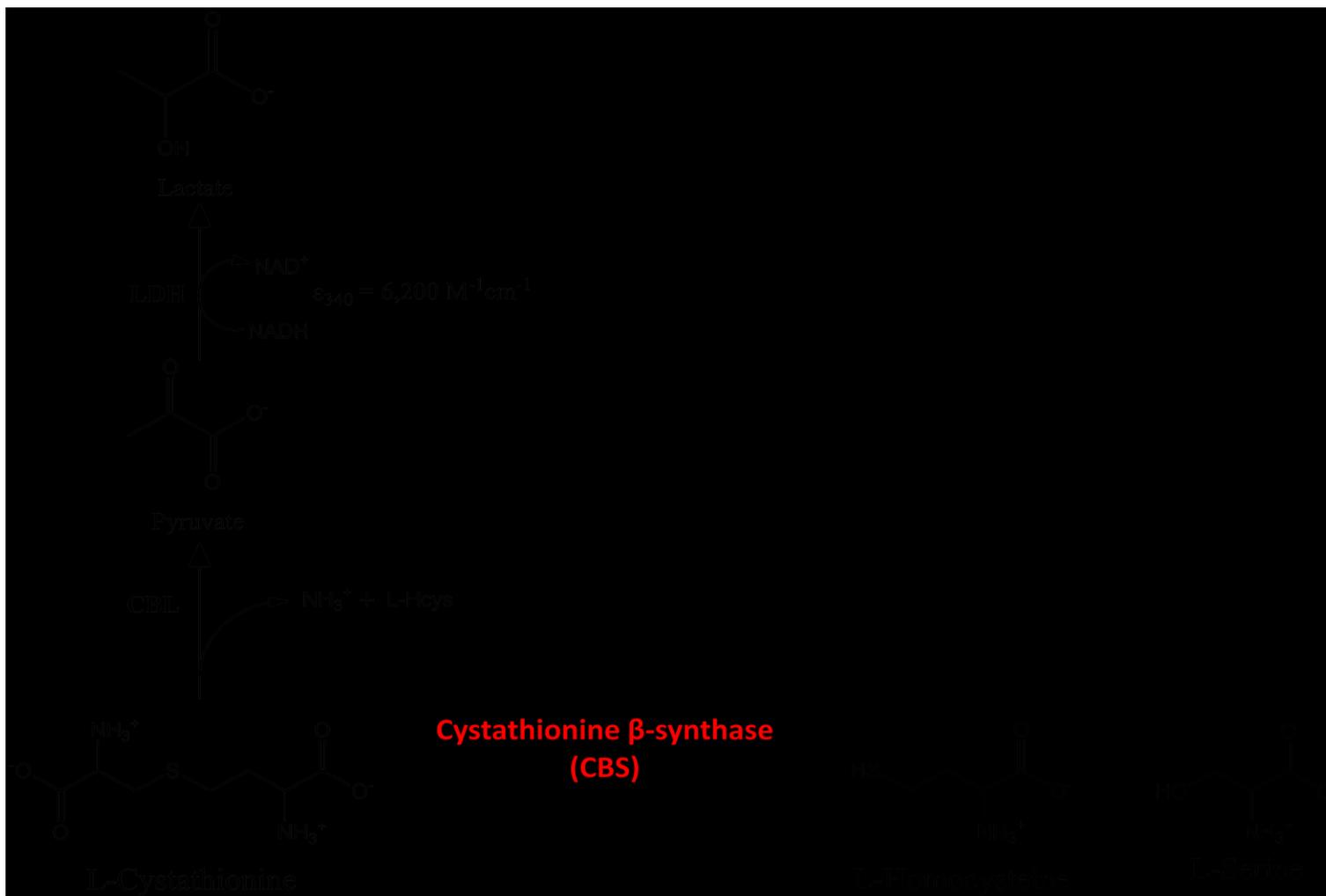


Figure 2.2: The assay employed to determine the β -replacement activity of CBS, *via* the use of CBL and LDH coupling enzymes (Aitken and Kirsch, 2003).

3. RESULTS

3.1. *The Construction and Purification of the Site-Directed Variants*

The yfCBS and htCBS variants were produced by overlap extension PCR (Figure 3.1). The amplicons introduced between the *NdeI* and *PstI* (hCBS) or *NdeI* and *HindIII* (yCBS) sites of the pTrec3 expressions vector and the resulting constructs were used to transform *E. coli* strain ER1821. The wild-type and site-directed variants were purified by Ni-NTA affinity chromatography and their purity was assessed *via* SDS-PAGE (Figures 3.2 & 3.3). The yields were between 0.33-1.98 mg and 0.41-4.44 mg per 1L culture for the yfCBS and htCBS variants, respectively (Table 3.1)

3.2. *Kinetic Characterization of the yfCBS Variants*

The kinetic parameters of the S323A and Y324F variants are similar to those of wild-type yfCBS (Table 3.2). The k_{cat}/K_{mR}^{L-Cth} of the S323A variant is decreased only ~6-fold, corresponding to an equivalent increase in K_{mR}^{L-Cth} . The Y324F substitution results in a reduction in k_{catR} and an increase in K_{mR}^{L-Cth} of a similar magnitude. No activity was detected for yfCBS-D319A.

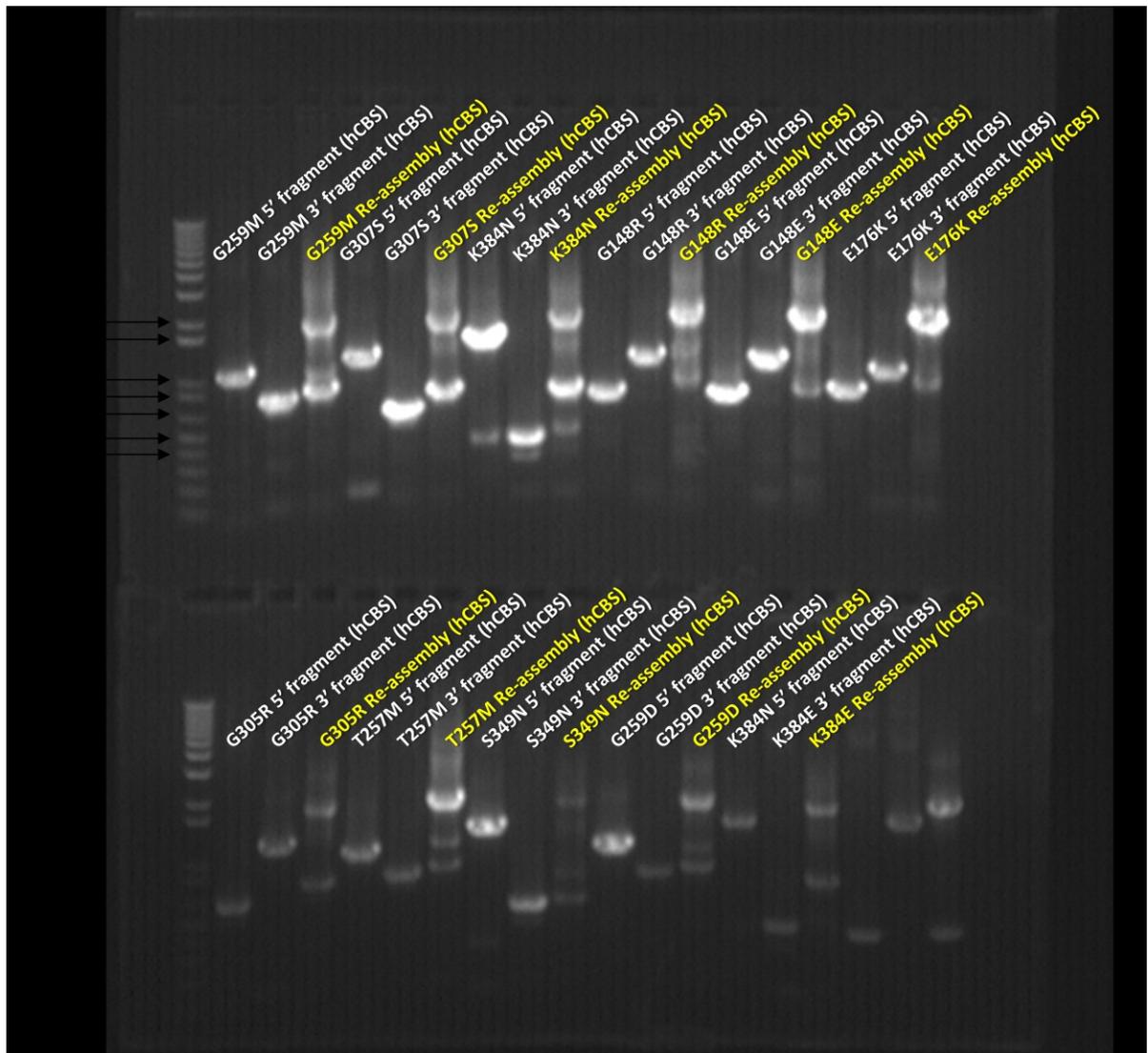


Figure 3.1: Agarose gel of PCR amplicons produced for the construction of the htCBS site-directed mutants *via* overlap-extension PCR. Each pair of 5' (Left Rxn; White) and 3' (Right Rxn; White) amplicons, which overlap by the mutagenic primer sequence, were combined and amplified with oligonucleotide primers flanking the start and stop codons to produce the reassembled htCBS sequence with the desired mutation (Yellow).

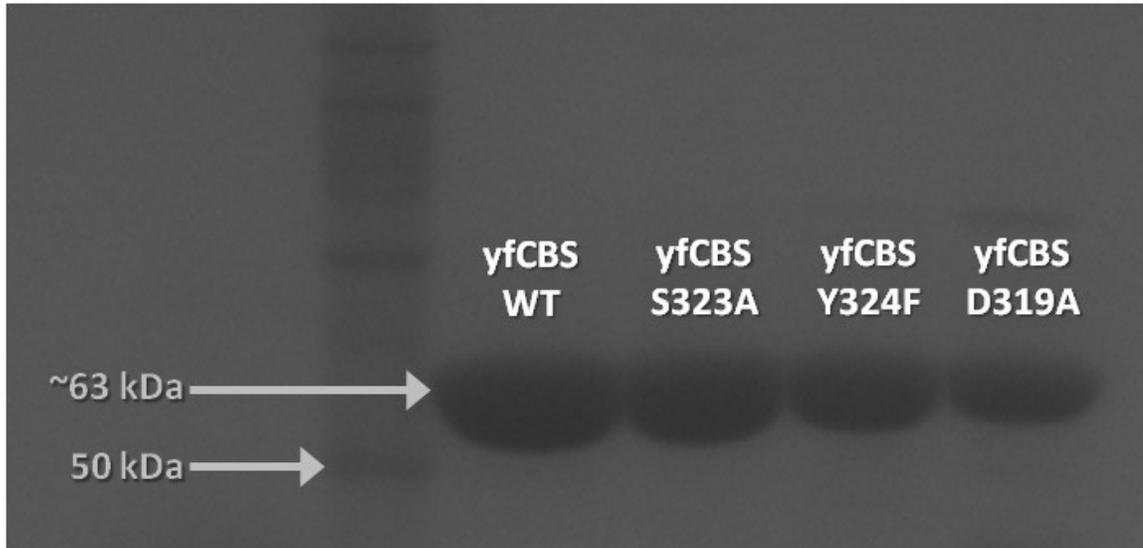


Figure 3.2: A SDS-PAGE gel, stained with Coomassie Blue, showing 4 μg each of the wild-type and S323A, Y324F and D319A site-directed variants of yfCBS. The approximate sizes of protein bands are indicated.

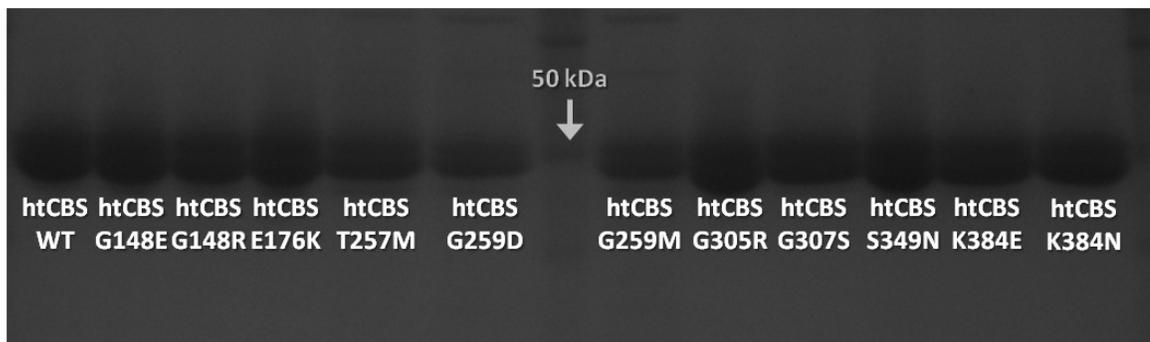


Figure 3.3: A SDS-PAGE gel, stained with Coomassie Blue reagent, showing 4 μ g of the wild-type and site-directed variants of htCBS. The 50 kDa protein marker band is indicated.

Table 3.1: Protein yield of yfCBS and htCBS variants.

yfCBS Variant	Yield (mg/L)
Wild-type	n.a.*
D319A	0.33
S323A	1.98
Y324F	1.03
htCBS Variant	Yield (mg/L)
Wild-type	4.44
G148E	2.46
G148R	1.64
E176K	2.18
T257M	0.68
G259D	0.41
G259M	0.67
G305R	2.20
G307S	2.79
S349N	3.63
K384E	3.39
K384N	2.25

*Wild-type yfCBS was purified by Sherwin Habibi and yield was not reported.

Table 3.2: Steady-state kinetics parameters of wild-type yfCBS and the D319A, S323A and Y324F variants for the reverse-physiological hydrolysis of L-cystathionine.

Enzyme	k_{catR} (s^{-1})	K_{mR}^{L-Cth} (mM)	k_{cat}/K_{mR}^{L-Cth} ($M^{-1}s^{-1}$)
wt yfCBS	0.147 ± 0.003	0.088 ± 0.008	$(1.7 \pm 0.1) \times 10^3$
S323A	0.145 ± 0.003	0.557 ± 0.005	$(0.26 \pm 0.02) \times 10^3$
Y324F	0.071 ± 0.002	0.20 ± 0.02	$(0.35 \pm 0.03) \times 10^3$
D319A	No Activity Detected		

*Kinetic measurements were determined at 25°C using a 50 mM Tris buffer (pH 8.6) containing 20 μ M PLP, 2 mM DTNB, 0.004-6.2 mM L-Cth and 0.675-2.175 μ M yfCBS. Data were fit to Eqs. (1) and (2).

3.3. Kinetic Characterization of the htCBS Variants

All of the homocystinuria-associated substitutions examined in this study impacted the catalytic efficiency of htCBS. A universal decrease in the turnover rate with respect to L-Ser (k_{cat}^{L-Ser}) was observed and ranges from 2.5-fold, for htCBS-K384N, to 19-fold for htCBS-G307S (Table 3.3). The kinetic parameter least affected by the various substitutions is the Michaelis constant for L-serine, as the K_m^{L-Ser} of 9 of the 11 variants is within 3-fold of the wild-type value, while the substitution of residue K384 to either the negatively charged glutamate (K384E) or the polar residue asparagines (K384N) led to a approximately 4- and 5- fold reduction in their respective K_m^{L-Ser} values (Table 3.3).

3.4. Spectral Characterization of the htCBS Wild-type Enzyme and Site-Directed Variants

The absorbance spectrum of the wild-type htCBS is characterized by a pair of absorbance peaks, of similar intensity, centred at 280 and 430 nm (Figure 3.4). The peak at 280 nm corresponds to the side chains of aromatic amino acids tryptophan, tyrosine and phenylalanine of the htCBS apoenzyme, while the 430 nm peak is attributable to the Soret absorbance of the heme cofactor. The PLP cofactor absorbs in the same region of the spectrum as the Soret band, but is masked by the 7-fold greater absorbance intensity of the latter (Figure 3.4).

The majority of the htCBS site-directed variants exhibit spectral profiles similar to the wild-type enzyme, with the exception of shifts in the intensity of the 280 and 430 nm peaks (Figure 3.5A). In contrast, the Soret absorbance of three variants (T257M,

G259D/M) is blue-shifted, compared to the wild-type enzyme (Figure 3.5B). The Soret peaks of both G259 variants are shifted ~25 nm, from 430 to 405 nm, while that of htCBS-T257M is only slightly shifted to 428 nm.

The ratio between the absorbances of the Soret and aromatic peaks ($A_{430/280}$) has previously been used as a qualitative measure of heme binding to hCBS (Majtan *et al.*, 2010). In this study, $A_{430/280}$ was used to examine the effect of each site-directed substitution on heme saturation. The wild-type truncated hCBS enzyme, lacking the C-terminal, regulatory domain, possesses an identical $A_{430/280}$ to that reported for the full-length enzyme (1.10; Majtan *et al.*, 2010). Eight variants exhibit $A_{430/280}$ ratios that range between 0.73 (htCBS-S349N) and 0.90 (htCBS-G148E) (Figure 3.5A). Substitution of residues involved in coordination of the phosphate group of PLP (T257M, G259D/M) show the largest reductions in the $A_{430/280}$ ratio (Figure 3.5B). The ratios of the T257M, G259D and G259M variants were reduced by 2-3.5-fold in addition to the observed blue-shift of the absorbance maximum.

Table 3.3: Steady-state kinetics parameters for the condensation of L-serine (L-Ser) and L-homocysteine (L-Hcys) by wild-type htCBS and homocystinuria-associated variants.



Kinetic Parameters	wt-htCBS	G148E	G148R	E176K	T257M	G259D
$^a k_{\text{cat}}^{\text{L-Ser}}$ (s^{-1})	1.69 \pm 0.03	0.195 \pm 0.004	0.17 \pm 0.01	0.30 \pm 0.02	0.25 \pm 0.01	0.36 \pm 0.01
$^a K_{\text{m}}^{\text{L-Ser}}$ (mM)	3.0 \pm 0.2	6.2 \pm 0.5	9 \pm 1	8.4 \pm 1.5	7 \pm 1	4.6 \pm 0.5
$^a k_{\text{cat}}/K_{\text{m}}^{\text{L-Ser}}$ ($\text{M}^{-1} \text{s}^{-1}$)	570 \pm 40	32 \pm 2	19 \pm 1	35 \pm 4	34 \pm 4	78 \pm 7

Kinetic Parameters	G259M	G305R	G307S	S349N	K384E	K384N
$^a k_{\text{cat}}^{\text{L-Ser}}$ (s^{-1})	0.33 \pm 0.02	0.15 \pm 0.01	0.09 \pm 0.01	0.117 \pm 0.004	0.103 \pm 0.003	0.69 \pm 0.03
$^a K_{\text{m}}^{\text{L-Ser}}$ (mM)	8 \pm 1	7 \pm 1	6.1 \pm 0.9	4.7 \pm 0.6	11 \pm 1	18 \pm 1
$^a k_{\text{cat}}/K_{\text{m}}^{\text{L-Ser}}$ ($\text{M}^{-1} \text{s}^{-1}$)	40 \pm 4	23 \pm 2	15 \pm 1	25 \pm 3	10 \pm 1	39 \pm 2

*Kinetic measurements were carried out in 50 mM Tris (pH 8.6) containing 20 μM PLP, 1.5 mM NADH, 1 μM CBL, 0.5 μM LDH, 0.2–36 mM L-Ser with 2 mM L-Hcys, and 1.23–4.11 μM htCBS at 25°C.

^aData were fitted to Eqs. (1) and (2)

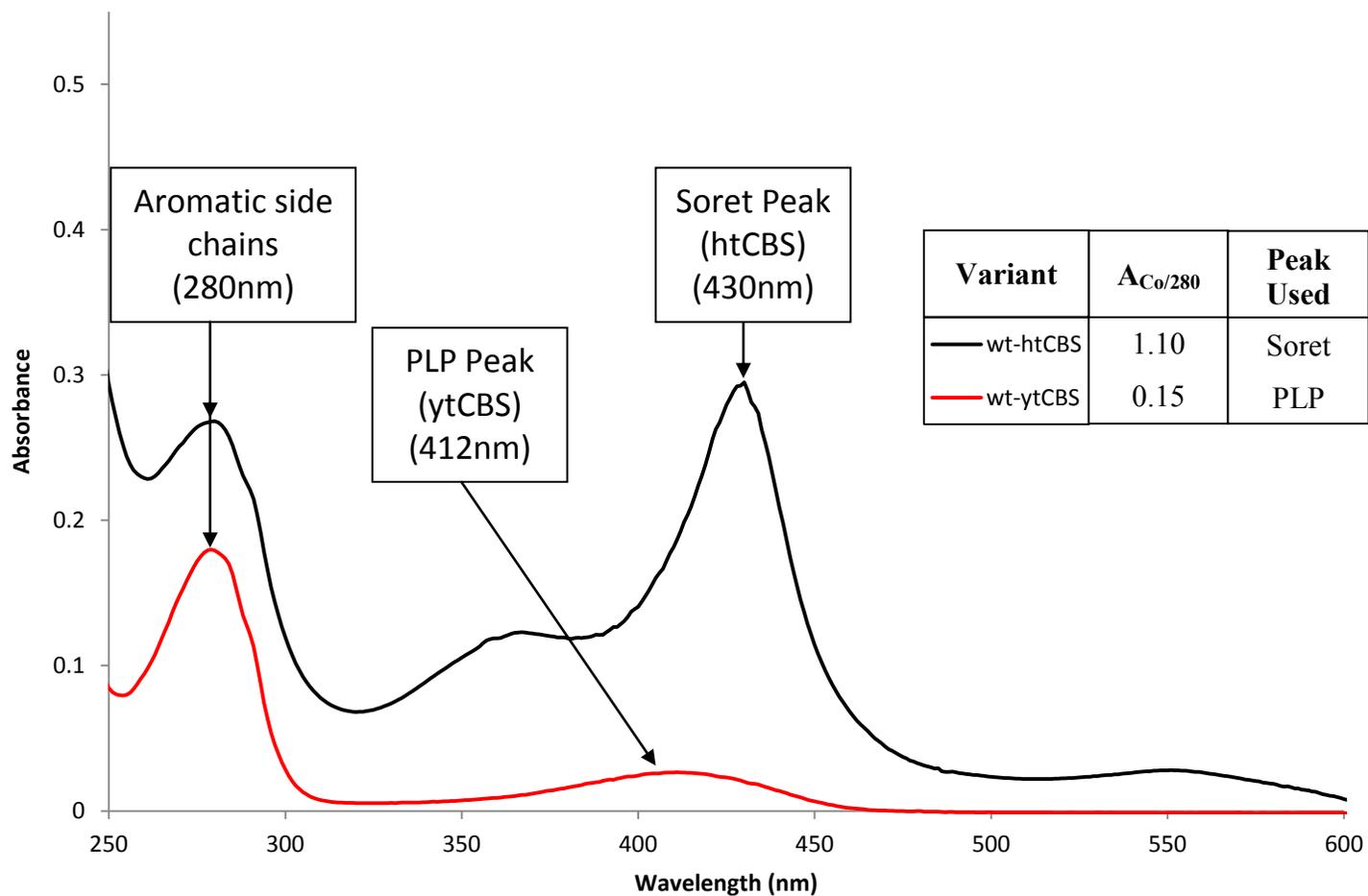


Figure 3.4: UV-Visible spectra of the wild-type htCBS (black) and ytCBS (red) enzymes. Comparison of the spectra of the two enzymes demonstrates that the Soret absorbance of the heme cofactor masks that of the PLP in the 400-460 nm region.

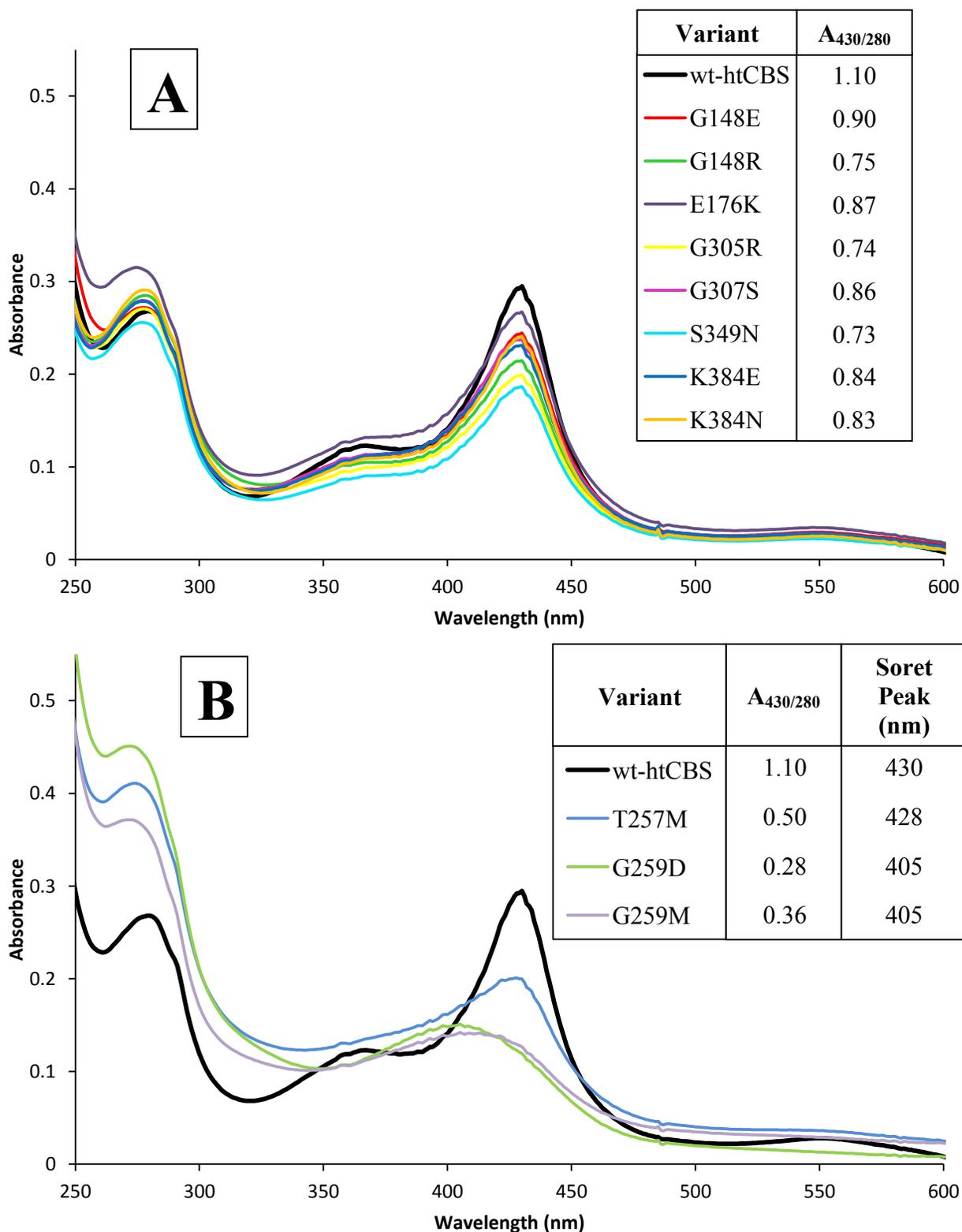


Figure 3.5: UV-Visible spectra of the purified htCBS variants examined in this study. Comparison between spectra of the wild-type wt-htCBS enzyme and (A) the 8 variants (G148E/R, E176K, G305R, G307S, S349N, K384E/N) similar to the wild-type enzyme, and (B) the T257M and G259D/M variants. The ratio of the absorbance at 430 and 280 nm peaks provides an indication of heme saturation.

4. DISCUSSION

As the first enzyme in the reverse transsulfuration pathway, CBS catalyzes the condensation of the branch-point metabolite L-Hcys with L-Ser to produce L-Cth. The flow of metabolites from the L-Hcys branch point has been well-studied as it is associated with homocystinuria (Mudd *et al.*, 2001). However, relatively few studies have probed the structure-function relationships that underlie the many (>100) unique disease-associated alleles of CBS. In this study, the effect of a series of homocystinuria-associated substitutions, located in proximity to the active site, on the kinetic parameters of the enzyme was investigated. The results enhance our understanding of the molecular basis of homocystinuria and contribute to a body of knowledge that can form the basis for the development of novel therapeutics.

The common theme of the two studies that comprise this thesis is exploration of the hydrogen bonding networks of the active site. The role of three residues (D319, S323 and Y324) that interact with N84, which tethers the O3' atom of the PLP cofactor, in the active-site of the model yeast CBS enzyme was probed. A series of 11 homocystinuria-associated mutations (G148E/R, E176K, T257M, G259D/M, G305R, G307S, S349N, K384E/N), situated in proximity to the active-site of human CBS were also investigated.

4.1. *The yfCBS Variants (D319A, S323A & Y324F)*

Residues D319, S323 and Y324 are located at the junction of the catalytic domain and the linker segment that connects the catalytic core and the regulatory domain of yCBS. These residues are within hydrogen bonding distance of N84 (N149 in hCBS numbering), which forms a ketoenamine-stabilizing hydrogen bond with the O3' position of the PLP cofactor (Figure 4.1) (Lodha *et al.*, 2010; Weeks *et al.*, 2009; Koutmos *et al.*,

2010). Substitution of N84 with asparatate in ytCBS causes a reduction in k_{cat}/K_{mF}^{L-Ser} of four orders of magnitude and the development of a marginal β -elimination activity (Lodha *et al.*, 2010). A set of conservative substitutions that remove the hydrogen bonding capacity of the side chains of the targeted residues (D319A, S323A and Y324F) was constructed to examine their effect on the activity of the model yeast enzyme. A schematic representation of the yCBS active site is presented in Figure 4.1 in which the targeted S323, Y324 and D319 are shown, with respect to key residues involved in substrate binding and catalysis. The full-length yfCBS enzyme was employed for this study because of the location of the 3 residues in proximity to the linker and regulatory domain.

Analysis of the structures of hCBS and dCBS suggests that the side-chain carboxylate moiety of D319 forms a hydrogen bond with the amide nitrogen of the N84 side chain (Figure 4.1). The lack of detectable activity observed for the yfCBS-D319A variant, which differs by only the missing side-chain carboxylate group of D319, may be due to improper positioning of the PLP cofactor. The negatively charged carboxylate group may also play an important role in modulating the strength of the hydrogen bond between N84 and O3' of the cofactor, thereby influencing electron distribution in the ring of the cofactor during catalysis. Loss of this influence and repositioning of N84, resulting from removal of the D319 carboxylate group, may thereby disturb the subtle active-site dynamics required for catalysis. This residue is conserved across all known CBS enzymes including hCBS (D376) and dCBS (D345), providing indirect evidence of the requirement for a negatively charged and/or hydrogen bonding side chain at position 319 (Chang and Vining, 2002, Kabil *et al.*, 2011; Oyenarte *et al.*, 2012).

The side-chains of residues D319 and Y324 are connected by a hydrogen bond, raising the possibility that Y324 may play an indirect role (D319-N84-mediated) in positioning the cofactor and determining active-site architecture (Figure 4.1). The removal of the Y324 side-chain hydroxyl moiety, through a tyrosine-to-phenylalanine substitution, results in a negligible effect on the function of the enzyme as both K_m^{L-Cth} and k_{cat} are within ~2-fold of the wild-type enzyme. This demonstrates that the hydrogen bonding capacity of the Y324 side chain is not a key determinant of active-site architecture or cofactor positioning.

Residue S323, like D319, is situated within hydrogen bonding distance of the side-chain amide moiety of N84 (Figure 4.1). The K_m^{L-Cth} of yfCBS-S323A is increased ~6-fold, while k_{cat} is unchanged (Table 3.2). This suggests that while the cofactor positioning within the active site is not changed by removal of the hydrogen bond between S323 and N84, the binding site of the L-Ser substrate may be subtly altered. Interestingly, S323 is a yeast-specific residue as its equivalent in all other known CBS enzymes is an asparagine residue.

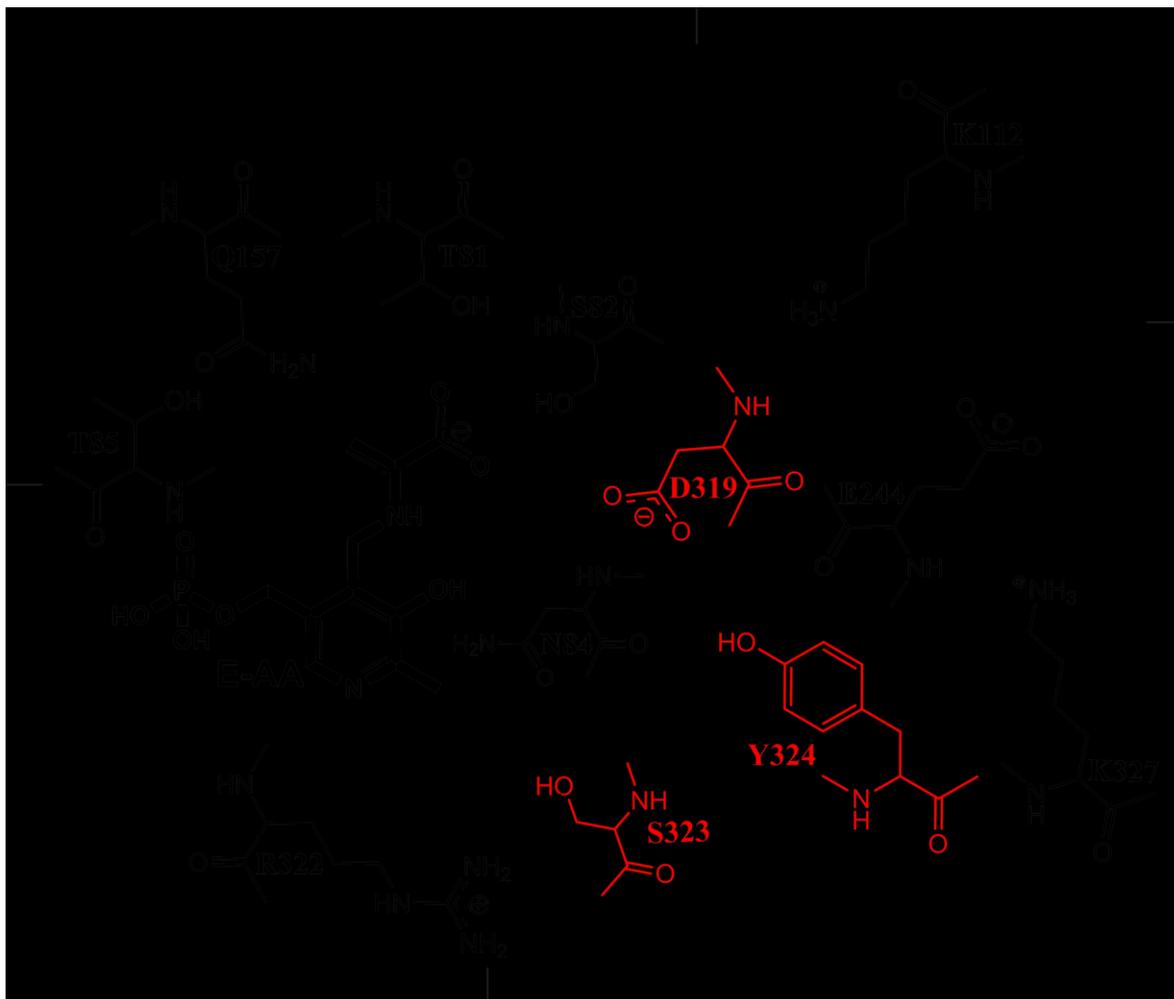


Figure 4.1: Proposed active site contacts in the yCBS-aminoacrylate complex. The putative hydrogen bond distances (represented by dotted lines; $<3.2\text{\AA}$ between heteroatoms) are based on interactions observed in the structure of dCBS (Koutmos *et al.*, 2010; Lodha *et al.*, 2010). Residues numbering corresponds to yCBS.

4.2 The Truncated Human CBS Enzyme

Belew *et al.* (2009) determined the kinetic parameters of the 6-His tagged, full-length hCBS (hfCBS) and the affinity-tagged truncated human enzyme constructed for this study was compared to this version of the full-length wild-type enzyme. In keeping with reports that removal of the C-terminal domain, which blocks substrate access to the active-site, increases enzyme activity (Evande *et al.*, 2002; Ereno-Orbea *et al.*, 2013a), the k_{cat}^{L-Ser} of htCBS are 1.4-fold higher than the full-length enzyme ($k_{cat}^{L-Ser} = 1.69 \pm 0.06 \text{ s}^{-1}$). The observed minor changes in K_m^{L-Ser} may be due to slight differences in the architecture of the active site resulting from loss interactions between residues of the regulatory and catalytic domains, in the context of the truncated enzyme. The effect of 11 homocystinuria-associated mutations, of residues located in, or close to, the active-site of htCBS, on activity was investigated: G148E/R, E176K, T257M, G259D/M, G305R, G307S, S349N and K384E/N.

4.2.1 Residue G148

G148 is situated in the middle of the mobile loop (T146-S147-G148-N149-T150) which undergoes a conformational shift upon binding of the L-Ser substrate to the active site. The hydrogen bond between the backbone amide of G148 and the hydroxyl group of T146 may aid in positioning of the latter, enabling formation of a hydrogen bond with the L-Ser substrate (Figure 4.2). Substitution of G148 with either a negatively-charged (G148E) or a positively-charged residue (G148R) is detrimental to the function of the enzyme. Both variants result in a decrease of approximately 9-fold in k_{cat}^{L-Ser} and a 2-3-fold increase in K_m^{L-Ser} (Table 3.3). The hydrogen side chain of G148 likely provides

flexibility required by the mobile loop as it moves between the open and closed conformations of the active site. Substitution of this residue with one possessing a bulky side chain, such as glutamate or arginine, would be expected to limit the flexibility of the loop and limit its range of motion compared to the wild-type enzyme.

The G148R variant has been classified as nonresponsive to vitamin B₆ supplementation and the clinical responsiveness of the G148E variant has not been assessed (Kraus *et al.*, 1999). Based on comparable effects to the kinetic parameters of both variants, it may be inferred that a patient with the G148E phenotype is also likely to be B₆-nonresponsive. The presence of the bulky side chain at position 148 may interfere with proper substrate positioning rather than cofactor binding, such that the effect of the substitution is not alleviated by B₆ supplementation. The observed changes in the turnover of the htCBS-G148E/R variants would be expected to reduce L-Hcys flux through the reverse transsulfuration pathway, thereby leading to its observed accumulation in homocystinuric patients.

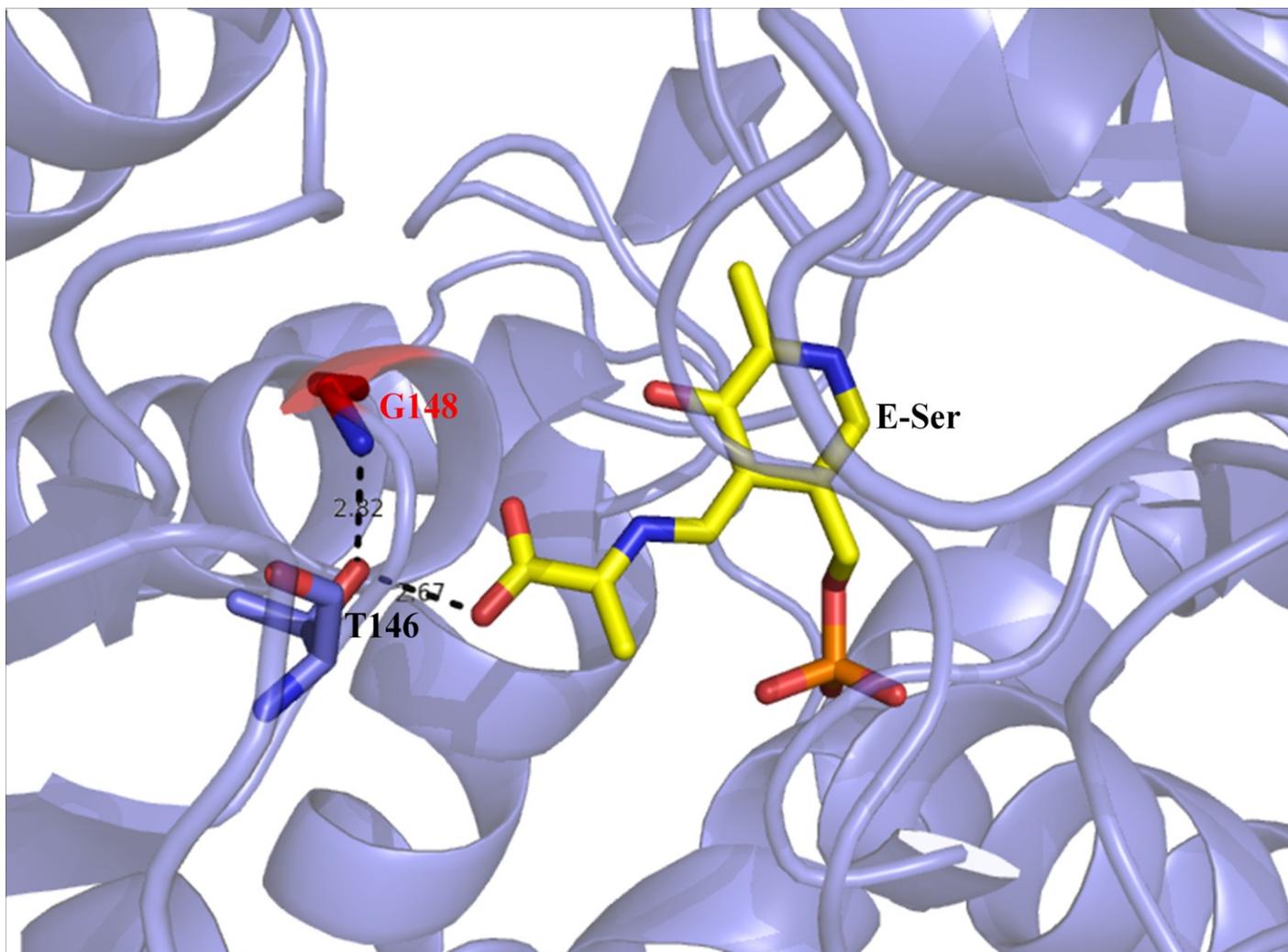


Figure 4.2: The placement of residue G148 (red) within the CBS active site and its direct and indirect interaction with T146 (pale blue) and the external aldimine (E-Ser; Yellow), respectively. Proposed hydrogen bond distances are indicated and shown in yellow (Koutmos *et al.*, 2010).

4.2.2 Residues E176 and K384

Residues E176 and K384 are members of the K177 hydrogen bonding network, which also includes E304, T383 and S147 (Figure 4.3). The network is responsible for bridging the mobile loop of the active site with the C-terminal portion of the catalytic core. The segments connected by this hydrogen bonding network (the mobile loop as well as loops 296-316 and 374-388) constitute a large portion of the surface of the active site. Lodha and colleagues showed that conservative substitution of the residue corresponding to K177 in ytCBS (K112) with arginine produces a 471-fold reduction in catalytic efficiency of L-Ser, thereby illustrating the importance of this network in maintaining proper active site architecture (Lodha *et al.*, 2009).

Charge-reversal substitution of position 176 (E176K) produces a 3-fold increase in K_m^{L-Ser} and a 6-fold decrease of the turnover rate. Substituting the negatively charged carboxylate group of E176 with a positively charged lysine would not necessarily eliminate the E176-T326 hydrogen bond, but may cause subtle changes in active-site architecture or communication with the regulatory domain, not present in htCBS (Figure 4.3). Hnizda *et al.* (2012) showed that the E176K variant in the full-length sequence was more susceptible to protein cleavage than the other variants examined in their study, suggesting that the effect of this B₆-unresponsive substitution may prevent the enzyme in adopting a stably folded structure (Kraus *et al.*, 1999; Hnizda *et al.*, 2012).

The effect of substituting the lysine at position 384 with glutamate (K384E) was more detrimental to htCBS turnover than a replacement with asparagine (K384N) evident by the 7-fold increase in the k_{cat}^{L-Ser} of K384N compared to K384E. Although the asparagine substitution removes the positive charge of the native lysine side chain and the

glutamate substitution results in a charge reversal, both have a similar effect of reducing the size of the residue at position 384, thereby repositioning the hydrogen bonding component of the side chain. Surprisingly, individuals with the K384E phenotype responded to vitamin B₆ treatment while those with the K384N variant were only partially responsive, as total homocysteine concentration remained >50μM following to vitamin B₆ treatment (Kraus *et al.*, 1999). This may indicate that the presence of excess PLP shifts the equilibrium of active-site conformations of the K384E, and to a lesser extent K384N variant, in favor on one that is catalytically productive, thereby alleviating the L-Hcys buildup in individuals expressing either genotype.

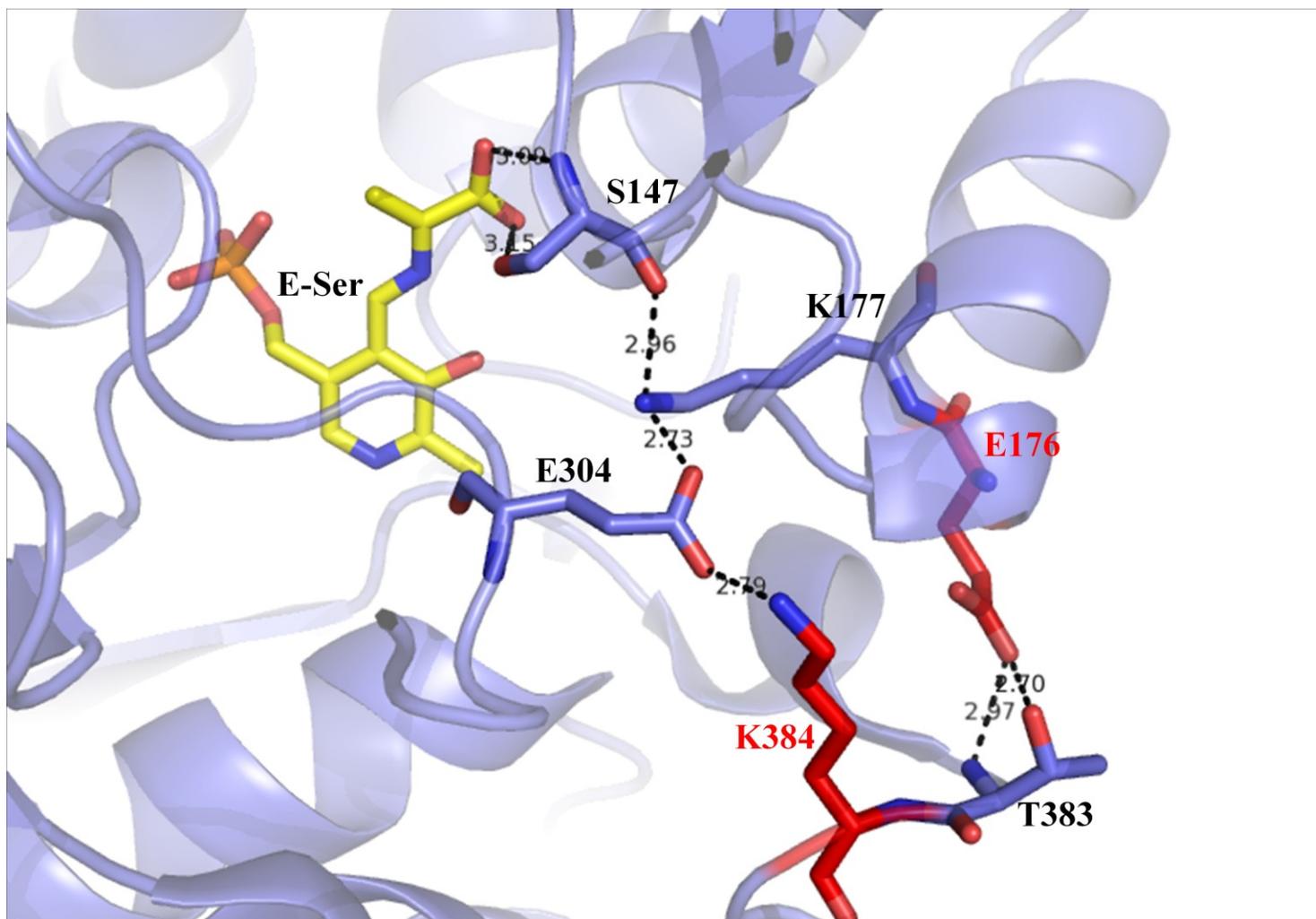


Figure 4.3: The location of the K177 hydrogen bonding network to which K384 and E176 are members of said group. The putative roles of K384 and E176 (red) within the network as well as the network's interaction with S147 in the closed conformation of CBS are shown (E-Ser; Yellow). Proposed hydrogen bond distances are indicated and shown in yellow (Koutmos *et al.*, 2010).

4.2.3 Residues T257 & G259

Residues T257 and G259 are a part of a conserved glycine rich loop (G256-T257-G258-G259-T260) that forms multiple hydrogen bonds with the phosphate moiety of PLP and plays an important role in anchoring the cofactor within the active site (Figure 4.4). While G259 does not directly interact with the cofactor, its backbone amide moiety is within hydrogen bonding distance of the hydroxyl side-chain group of T257. Therefore, G259 may play an indirect role by positioning T257 to optimally align the latter to hydrogen bond with the phosphate group of the cofactor (Meier *et al.*, 2001; Koutmos *et al.*, 2010).

Replacement of T257 with methionine removes the hydrogen bonding capacity of this residue and increases the bulk of the side chain. Therefore, the observed 7-fold decrease in the k_{cat}^{L-Ser} of T257M is likely due to a shift in the position of the cofactor within the active site, offering an explanation for this variant's unresponsiveness to vitamin B6 (Kraus *et al.*, 1999). A 2 nm blue-shift, compared to the 430 nm maximum of the Soret peak of wild-type wt-htCBS, and a 2-fold decrease in $A_{430/280}$ are also observed for this variant. Ojha *et al.* (2002) observed a slight blue-shift of the Soret band of a H65R full-length variant to 424 nm, similar to the T257M variant examined in this study. As the overall shape of the Soret peak remained intact, indicating that the six-coordinate state of heme is retained, Ojha *et al.* (2002) hypothesized that the slight shift in the Soret peak may be due to a replacement of H65 as the axial iron ligand of the heme cofactor by the proximal H67 (Figure 4.5). The similar characteristics of the Soret peaks of the H65R and T257M variants, suggest that H65-H67 heme ligand switching may be the source of the altered spectral profile of htCBS-T257M.

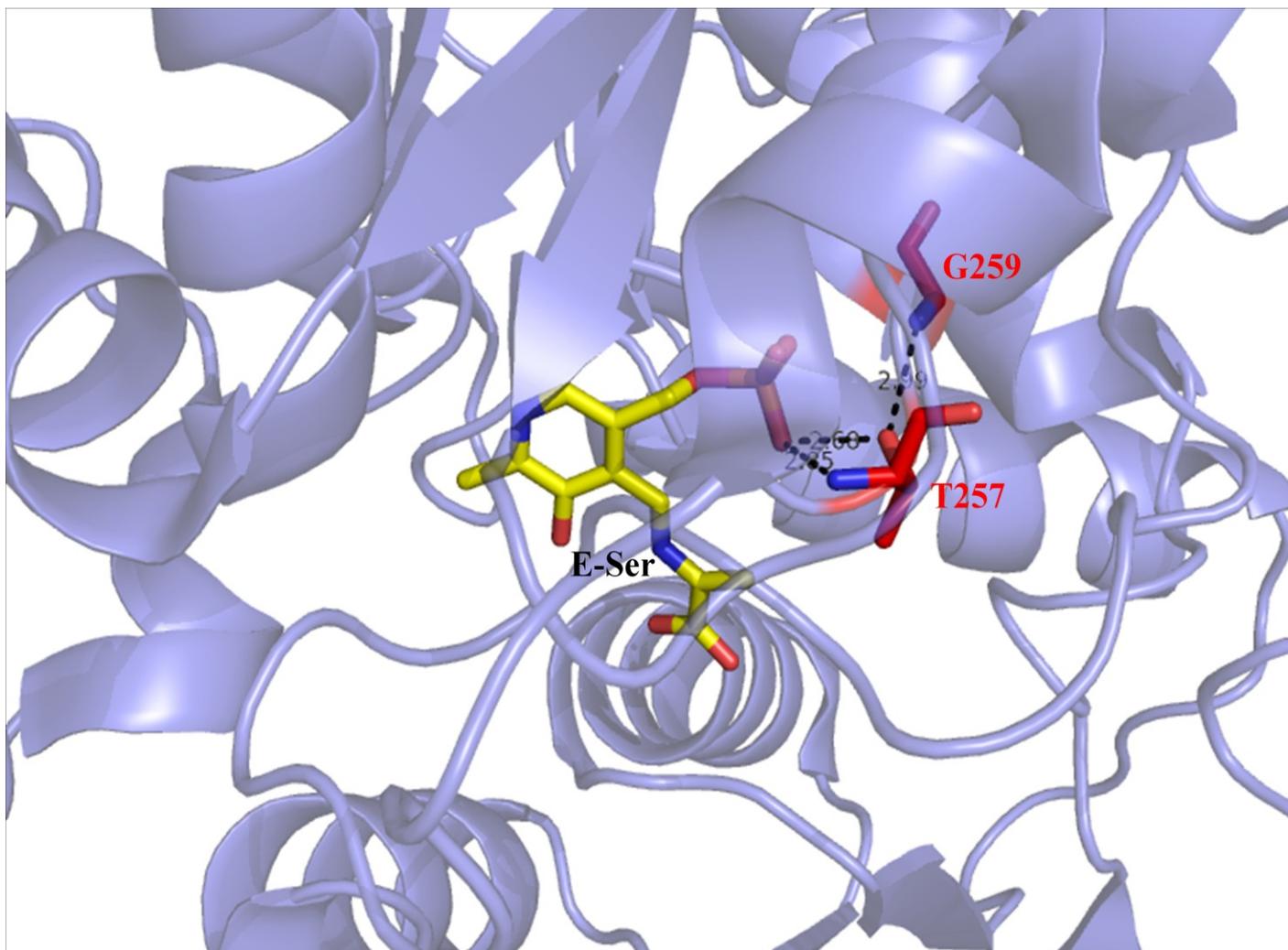


Figure 4.4: A view of the glycine-rich loop that positions the PLP cofactor *via* interactions with the phosphate moiety. Interactions between residues T257 and G259 (red) and the phosphate moiety of the aminoacrylate complex of the cofactor are shown (Yellow). Proposed hydrogen bond distances are indicated and shown in yellow (Koutmos *et al.*, 2010).

Yadav *et al.* (2012) observed that while the substitution of either threonine residue (257 or 260) of the Gly-rich loop resulted in impaired heme binding, neither interact directly with this cofactor. The authors proposed that α -helix 8, which extends from the heme cofactor to the PLP cofactor may be involved in bi-directional communication through the interactions of the Gly-rich loop at the N-terminal end of the helix and residue R266, which interacts with the thiolate atom of C52, one of the axial ligands of the heme iron (Figure 4.5) (Weeks *et al.*, 2009; Yadav *et al.*, 2012). The results of this study suggest that residue T257 may participate in this α -helix-8-mediated communication as its proximity to the phosphate moiety of PLP allows the residue to function as a signal transducer between the cofactor sites.

Previous work has focused on the Thr residues of the Gly-rich loop while the Gly residues have not been investigated (Yadav *et al.*, 2012). The replacement of G259 with aspartate or methionine results in a similar reduction in catalytic efficiency as the T257M variant despite their more pronounced effect on the electronic environment of heme than htCBS-T257M. The Soret peak of both G259-substitution variants has blue-shifted 25 nm to 405 nm (Figure 3.5B). This is accompanied by a broadening of the Soret peak and a 3-4-fold reduction in the $A_{430/280}$ ratio. Ojha and colleagues (2000) demonstrated the use of mercury chloride, a thiol chelator, in broadening the Soret peak of hfCBS as well as a peak shift to \sim 395nm. X-Ray absorption data indicates a loss of C52 without a replacement (Ojha *et al.*, 2000). The broadening of the G259D/M Soret peaks and their reported blue-shift may indicate the occurrence of a similar effect. The insertion of bulkier side chains at position 259 may shift the positioning of PLP and α -helix 8, resulting in loss of the thiolate heme ligand as a result of the C52-R266 interaction.

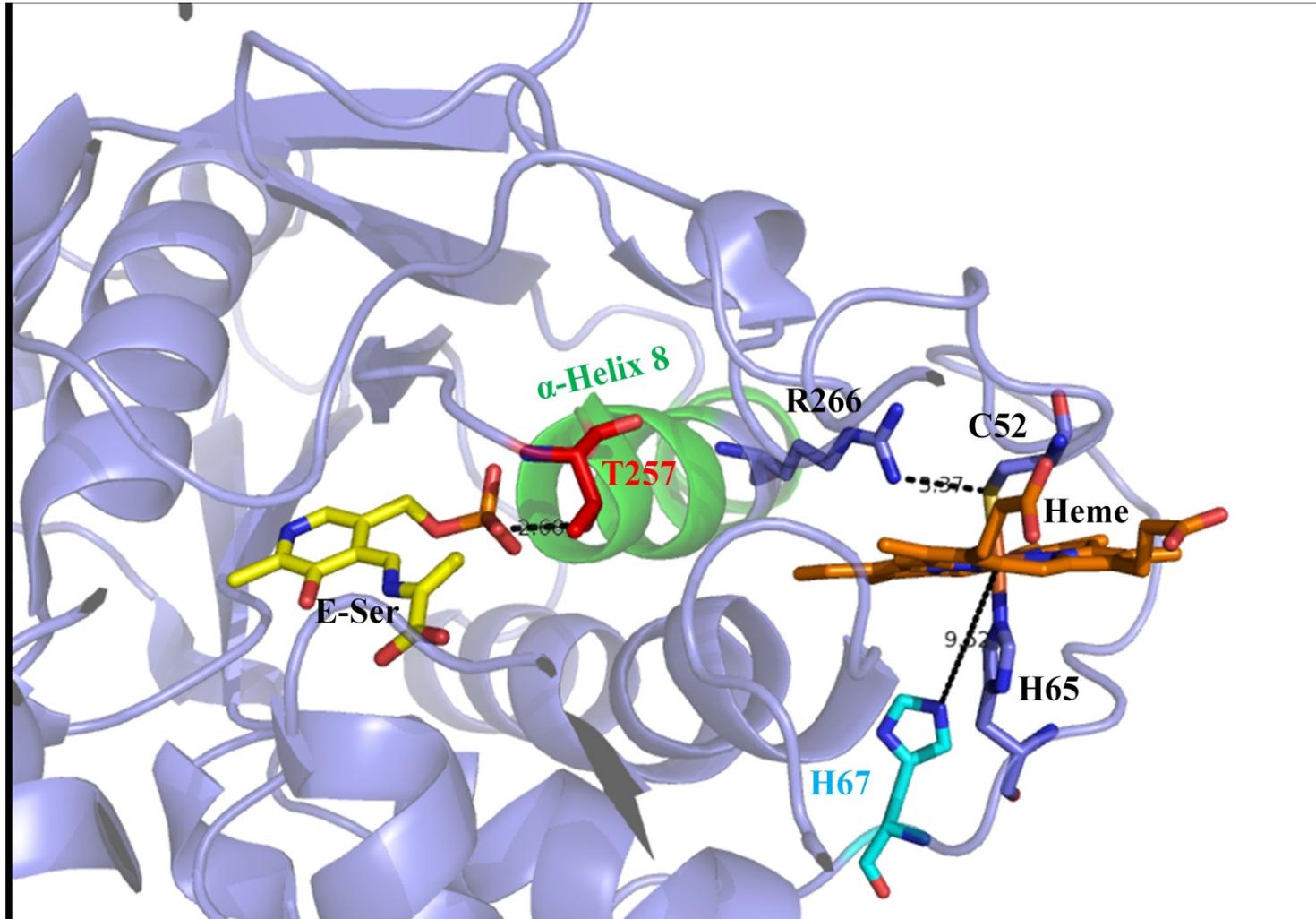


Figure 4.5: Location of key residues and α -helix 8, which extends from the heme cavity to the PLP-binding site, proposed to be involved in bi-directional communication between the two cofactors. H67 (grey) is proposed to replace H65 (teal) as a heme ligand in the T257M variant. (Yadav *et al.*, 2012; Ojha *et al.*, 2002; Koutmos *et al.*, 2010)

4.2.4 Residue S349

Residue S349 is situated in the C-terminal segment of the catalytic core of CBS. The hydroxyl group of the S349 side chain participates in positioning and modulating the electronic properties of the PLP cofactor *via* a hydrogen bond with the N1 nitrogen of the pyridine ring (Figure 4.6) (Meier *et al.*, 2001; Koutmos *et al.*, 2010). Unlike the other residues examined in this study, which are conserved in all known CBS enzymes, S349 is conserved more broadly, across the members of the fold-type II group of PLP-dependent enzymes. This is suggestive of its importance to the family's ability to catalyze β -replacement reactions.

The introduction of an asparagine residue at this position interferes with the enzyme's ability to catalyze its physiological reaction, as demonstrated by the 14-fold reduction in k_{cat}^{L-Ser} . Replacement of the corresponding S289 of the model ytCBS enzyme with alanine results in a 20-fold decline in k_{cat} , while substitution with aspartate, present in this position in many fold-type I PLP-dependent enzymes, leads to a loss of β -replacement activity (Quazi and Aitken, 2009). While there was no change in the overall spectral profile of htCBS-S349N, the $A_{430/280}$ ratio is decreased 1.5-fold and is the lowest among the htCBS variants that maintained a similar absorbance spectrum to the wild-type enzyme. The altered interaction between the substituted asparagine residue and the pyridine ring may have been communicated to heme, *via* α -helix 8, thereby reducing heme binding or altering the intensity of the Soret peak. Although the S349N variant is not B₆-responsive, it is interesting to note that substitution of S349 with asparagine, which is of similar size to aspartate, is accommodated in the context of htCBS, in contrast with the inactive ytCBS-S289D variant (Urreizti *et al.*, 2003; Quazi and Aitken, 2009).

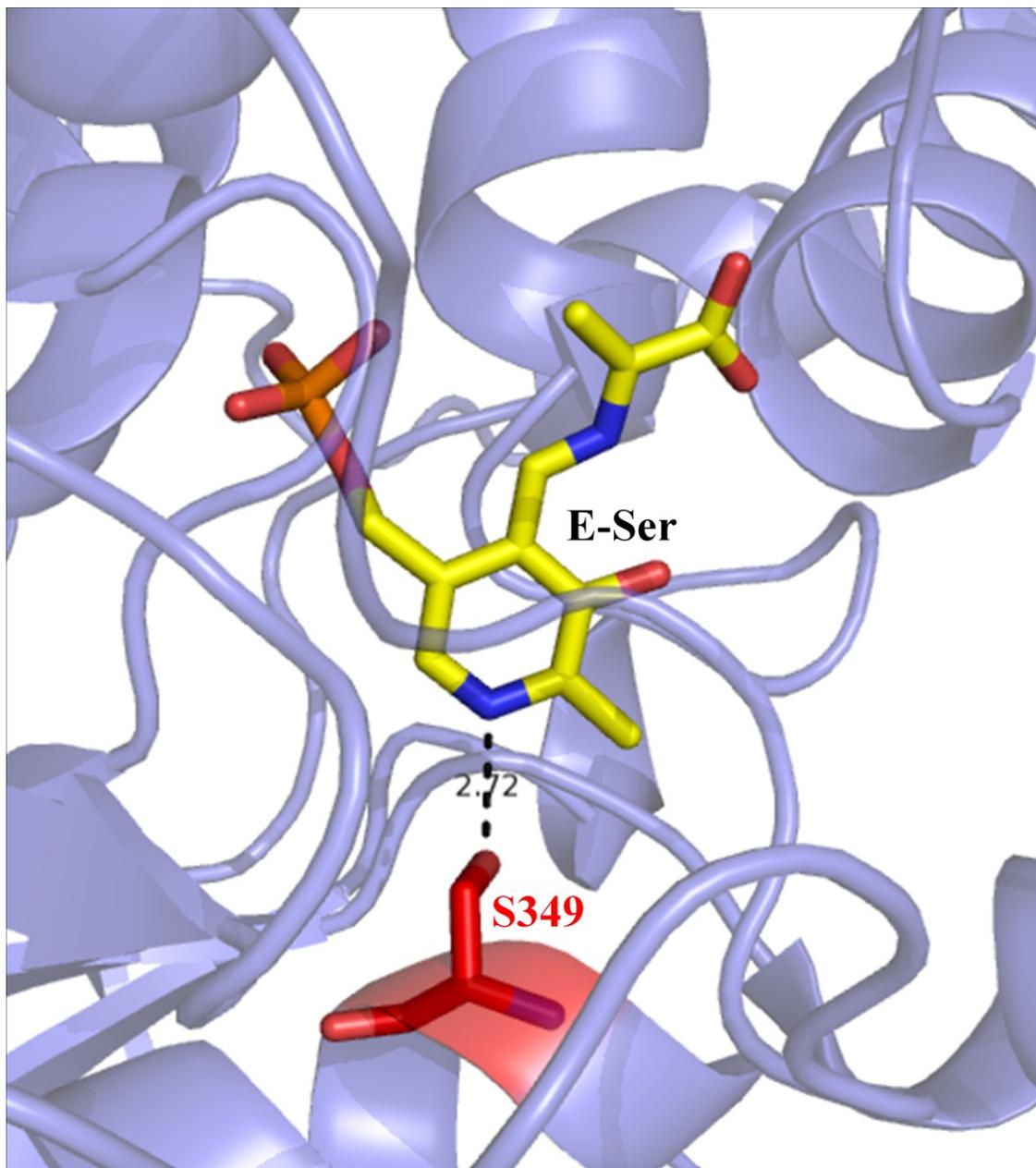


Figure 4.6: The interaction between residue S349 (red) of hCBS and the N1 position of the PLP cofactor (E-Ser; yellow). The proposed hydrogen bond and hydrogen bond distance is indicated in yellow (Lodha *et al.*, 2010; Meier *et al.*, 2001; Koutmos *et al.*, 2010).

4.2.5 Residues G305 & G307

Residues G305 and G307 are located at the mouth of the active site (Figure 4.7). G305 aids in the orientation of PLP through Van der Waals interactions with the *re* face of pyridine ring. Located at the bottom of the active site opening, G307 is situated in a tightly packed pocket with P282, S285 and T301 (Meier *et al.*, 2003). Meier *et al.* (2003) hypothesized that the G305R substitution likely would affect cofactor binding because of the need to accommodate the large, positively charged side chain of arginine. The 11-fold reduction in k_{cat}^{L-Ser} for htCBS-G305R imply that the cofactor remains in a catalytically-productive position within the active site, suggesting that the loop of residues including G305 can adopt an alternate conformation to accommodate the bulky arginine side chain (Kraus *et al.*, 1999). Remarkably, a patient heterozygous for both the I278T and G305R variants was found to be B₆-responsive suggesting that the treatment may be sufficient for G305R homozygous patients (Sperandeo *et al.*, 1996).

The insertion of a serine residue at position 307 is believed to cause a protrusion within the tightly packed group of residues located at the bottom of the active site entrance. The small size of G307 allows space for other bulky residues within its vicinity such as P282 and T301 and to accommodate L-Hcys binding. Modeling of the G307S substitution in hCBS suggested the formation of a new hydrogen bond between the hydroxyl groups of the side chains of S307 and T301, causes a conformational change, resulting in the loss of hydrogen bonds between the phosphate moiety of PLP and residues G256 and T257. This may explain the reduced activity and unresponsiveness to vitamin B₆ observed for the common G307S allele (Lodha *et al.*, 2009; Kraus *et al.*, 1999). While the corresponding substitution (ytCBS-G247S) resulted in a lack of β -replacement activity in the model yeast enzyme (Lodha *et al.*, 2009), the truncated human

counterpart exhibits detectable activity. Comparison of the htCBS-S349N and G307S substitutions with the corresponding substitutions in the model yeast enzyme illustrate the subtle structure-function relationships that underlie enzyme function, as differences are observed despite the 47% amino acid sequence identity shared by the catalytic cores of the human and yeast CBS enzymes (Jhee *et al.*, 2000a; Jhee *et al.*, 2000b).

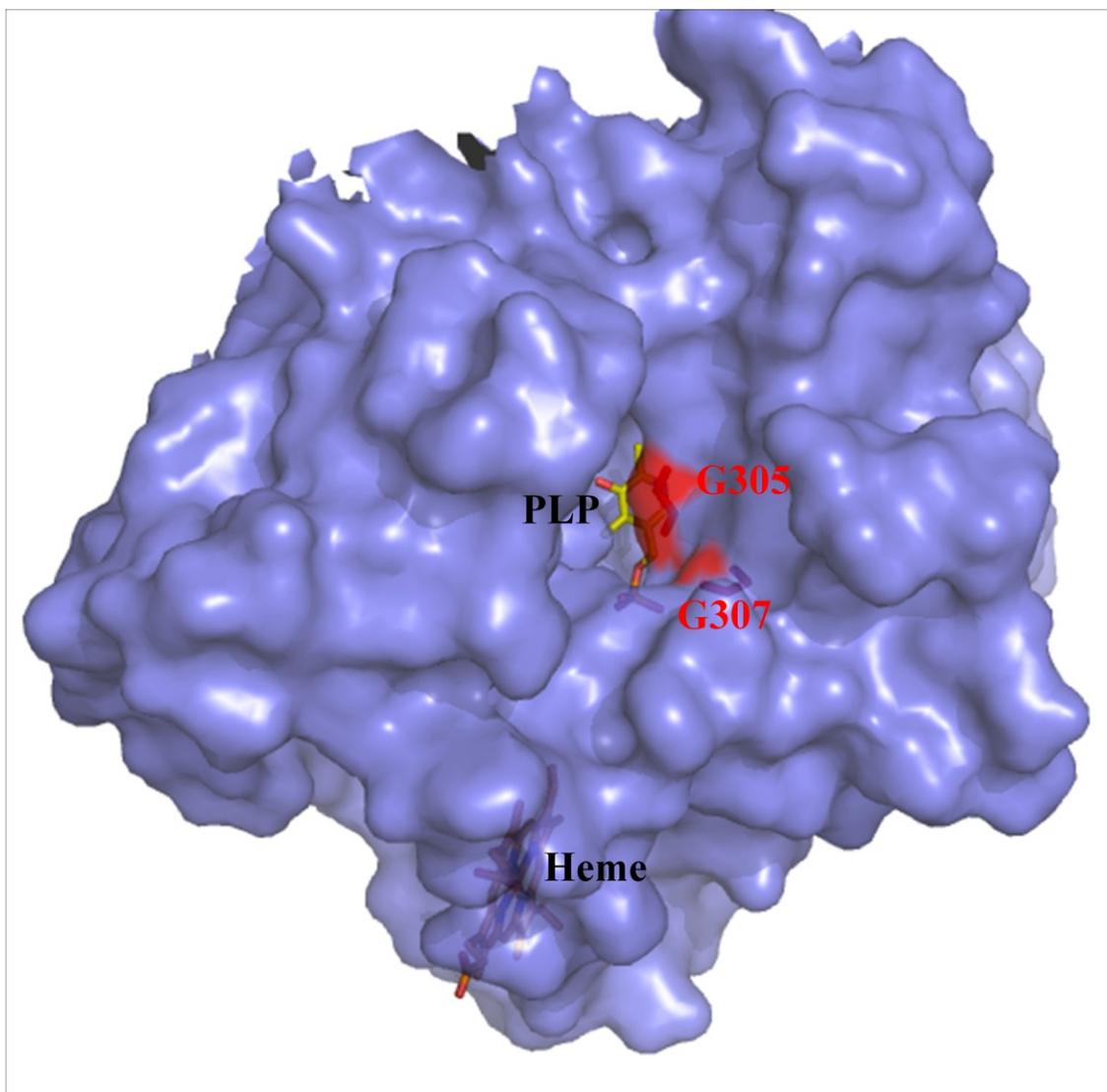


Figure 4.7: Position of G305 and G307 (red) at the mouth of the hCBS active site. The PLP (yellow) and heme (orange) cofactors are highlighted (Meier *et al.*, 2001).

5. CONCLUSION

This study focused on the exploration of the structure-function relationships that underlie the interaction networks and the effects of a selection of homocystinuria-associated mutations in the active site of CBS. The role of active-site residues D319, S323 and Y324 in positioning of the pyridoxal 5'-phosphate cofactor was investigated in the model yeast CBS enzyme. In the context of yfCBS, the separate removal of the side-chain hydrogen bonding capacity of residues S323 and Y324 resulted in minor changes to enzymatic function suggesting that neither is a key determinant of cofactor orientation or active-site architecture. The inactivity of the D319A variant may be due to a shift in the orientation of PLP. Removal of the carboxylate moiety of D319 from the active-site hydrogen bonding network may lead to a repositioning of N84 or a change in the strength of the N84-PLP (O3') interaction.

The effect of a set of 11 homocystinuria-associated substitutions, located within the active-site, on the kinetic parameters of human CBS was also investigated. Residue G148 allows for the flexibility of the mobile loop, essential for proper transitions between the open and closed active site conformations. Residues E176, G307 and K384 are responsible for maintaining proper active site architecture. Residues in proximity to the phosphate moiety of PLP (T257, G259) are vital to the effective binding of PLP along with G305 and S349 in addition to the bi-directional communication between the heme and PLP cofactors. This is the first step to a better understanding of the molecular mechanism of homocystinuria. As there are >160 reported mutations associated with the disorder that span all three major domains of hCBS, gaining insight into the structure-function relationship can help postulate novel therapeutics for patients with homocystinuria.

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