Effect of various parameters on Homocysteinylation of Hemeproteins

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CERTIFICATE

This is to certify that the work titled **"Effect of various parameters on Homocysteinylation of Hemeprotein"** submitted by **Mr. Vasu Walia** in partial fulfilment for the award of degree of B. Tech Biotechnology of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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DECLARATION

I, **Vasu Walia**, student of B.Tech Biotechnology, Jaypee University of Information Technology, solan, Himachal Pradesh hereby declare that the project entitled **"Effects of various parameters on Homocysteinylation of Hemeprotein"** submitted is an original work done as during the period of seventh semester as a final year project, under the guidance of Dr. Saurabh Bansal to the best of my knowledge and has not been published anywhere.

Vasu Walia

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ACKNOWLEDGEMENT:

It gives me immense pleasure to express my gratitude towards the people who have helped me in innumerable ways to produce this piece of research work. Completing this work without their help would not have been possible.

All praise belongs to the almighty lord to whom I thank for the strength, courage and perseverance bestowed upon to me to undertake the course of the study.

I am indebted to my project guide **Dr. Saurabh Bansal** for his timely guidance and consistent support throughout the research. His great intellect, timely intervention and constant inputs have lead to complete my project with ease and grace.

I am grateful to thank **Dr. Laishram R. Singh** for giving me an opportunity to work in his lab and providing me with all the desired materials and equipments to carry forward my project and research. He guided me throughout my project period with his insightful thoughts.

I express my thanks to **Prof. Rajinder Singh Chauhan**, Dean, Department of Biotechnology and Bioinformatics, Jaypee University of Information and Technology for his constant support and guidance.

I would also like to extend my gratitude towards **Mr. Baleshwar**, **Mrs. Mamta Mishra**, **Mr. Kamlesh Kumar**, and other staff members for their constant help and motivation for successfully carrying my research work.

In the end I must say that I am privileged to be a part of **Department of Biotechnology, JUIT**, and **Dr. B.R. Ambedkar Centre for Biomedical Research, Delhi University** for providing me lab space, instruments and everything for conducting my research.

Signature of the student -

Name of Student - Vasu Walia

Date -

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ABBREVATIONS

Hcy	Homocysteine
HTL	Homocysteine thiolactone
Cyt c	cytochrome complex
Hb	Haemoglobin
H_2O_2	Hydrogen peroxide
CBS	Cystathionine-β-synthase
MTHFR reductase	Methylene tetrahydrofolate
μM	Micromolar
mM	Millimolar
UV	Ultraviolet
VIS	Visible
CD	Circular dichroism
ANS acid	8-anilinonaphthale-1-sulfonic
ThT	Thioflavin T

CHAPTER 1: INTRODUCTION

1.1 Summary/ Abstract

Elevated levels of homocysteine (Hcy) have been associated with various diseases and conditions. Homocysteine thiolactone (HCTL) is a metabolite of Hcy and reacts with amine groups in proteins to form stable amides, Homocysteinylated proteins. It has been proposed that proteins N-Homocysteinylation contributes to the cytotoxicity of elevated Hcy.

In vitro, addition of HCTL to purified proteins has a considerable effect on aggregation state, protein functions and protein structure. For example, N-homocysteinylation causes aggregation of many proteins, such as low-density lipoprotein, fibrinogen and RNase.

In the present study the structural properties and aggregation propensity of Hemeproteins were studied in the presence of increasing concentration of HCTL, using different spectroscopic techniques. As shown in this study, HCTL induces gross structural alterations and subsequently aggregation of Hemeprotein in a dose dependent manner. It was also observed that Protein loses its structure and function when it is in contact with homocysteine thiolactone.

1.2 Introduction

Homocysteinuria/ hyperhomocysteinemia is an inborn error of metabolism leading to increase in levels of sulphur containing amino acid, homocysteine in serum and urine of the affected person[1]. In a normal healthy individual, the serum homocysteine level range in 5-10 µM. However, mutations in the Hcy metabolizing enzymes, cystathionine β-synthase (CBS) or methylene tetrahydrofolate reductase (MTHFR), etc. cause an impaired ability to metabolize the toxic Hcy resulting in an increased levels of cellular and plasma Hcy. The total concentrations of plasma Hcy may range from 15 to 20 µM (mild forms) up to 500 µM (severe forms) [2, 3]. Increasing Hcy levels causes an increase in Homocysteine thiolactone (HTL), a cyclic thioester of Hcy, synthesised by certain aminoacyl tRNA synthetases (AARS) in proofreading reactions that prevent translational incorporation of Hcy into proteins [4, 5].

The mechanism of the conversion involves reaction of Hcy with ATP to form an AARS-bound homocysteinyl adenylate (Hcy~AMP). Subsequent rejection of the Hcy~AMP intermediate involves an intramolecular reaction in which the side-chain thiolate of Hcy displaces the AMP group from the carboxylate of the activated Hcy, forming HTL as a product.

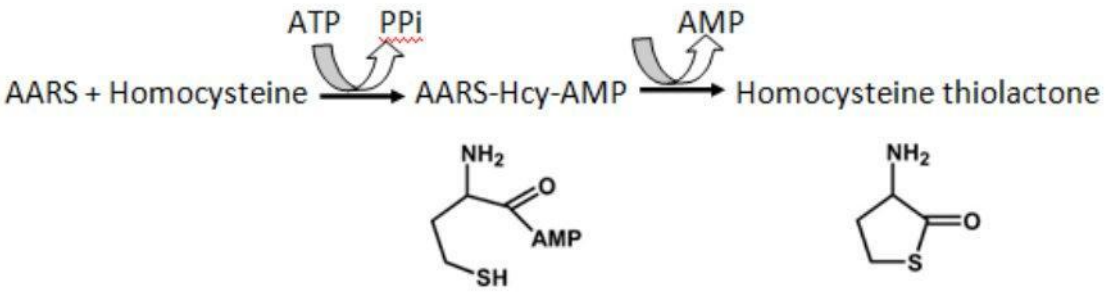


Figure 1: Formation of homocysteine thiolactone from homocysteine.

The basic cause of Hcy toxicity is known to be protein modification mediated by HTL. HTL forms an amide bond with the ϵ-amino group of protein lysine-residue a process known as protein N-homocysteinylation [6]. Protein N-homocysteinylation, has therefore, been considered to be one of the basic causes of HTL toxicity. Incorporation of HTL to the proteins are believed to result in loss of protein functions due to alterations in the protein structure and become susceptible to further damage by oxidation [7, 8].

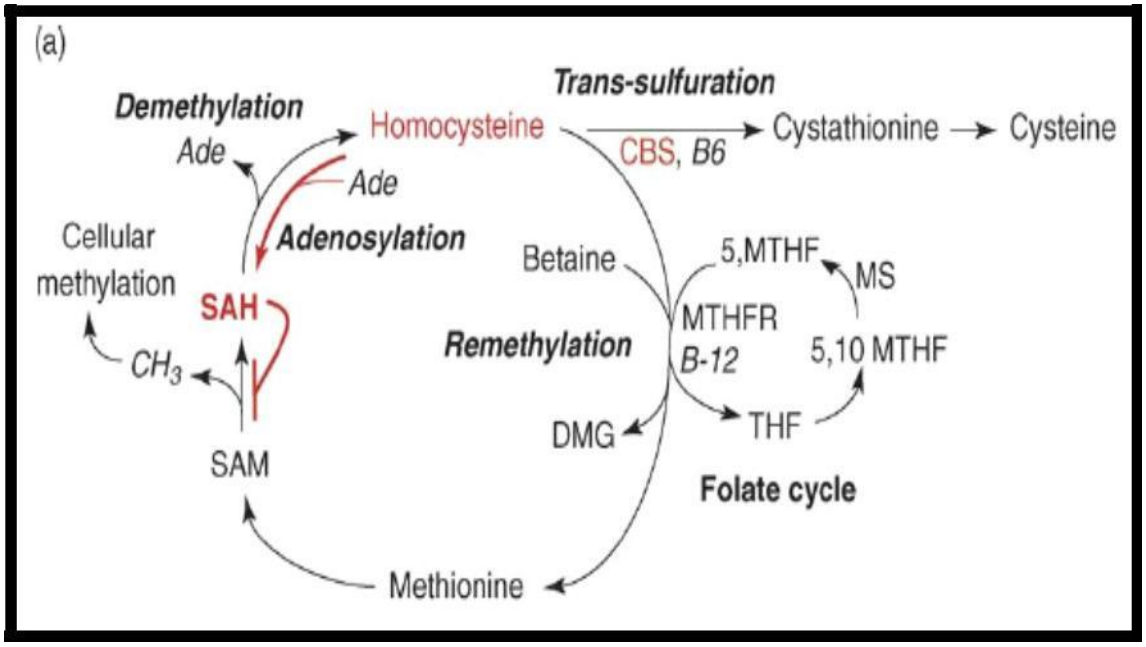


Figure 2: Hcy metabolism and biochemical mechanisms for Hcy pathologies.

In addition, it has also been observed in few proteins that N-homocysteinylation induces protein aggregation or amyloid formation and hence considered to be an independent risk factor for neurodegenerative diseases in human [1, 6, 9, 10].

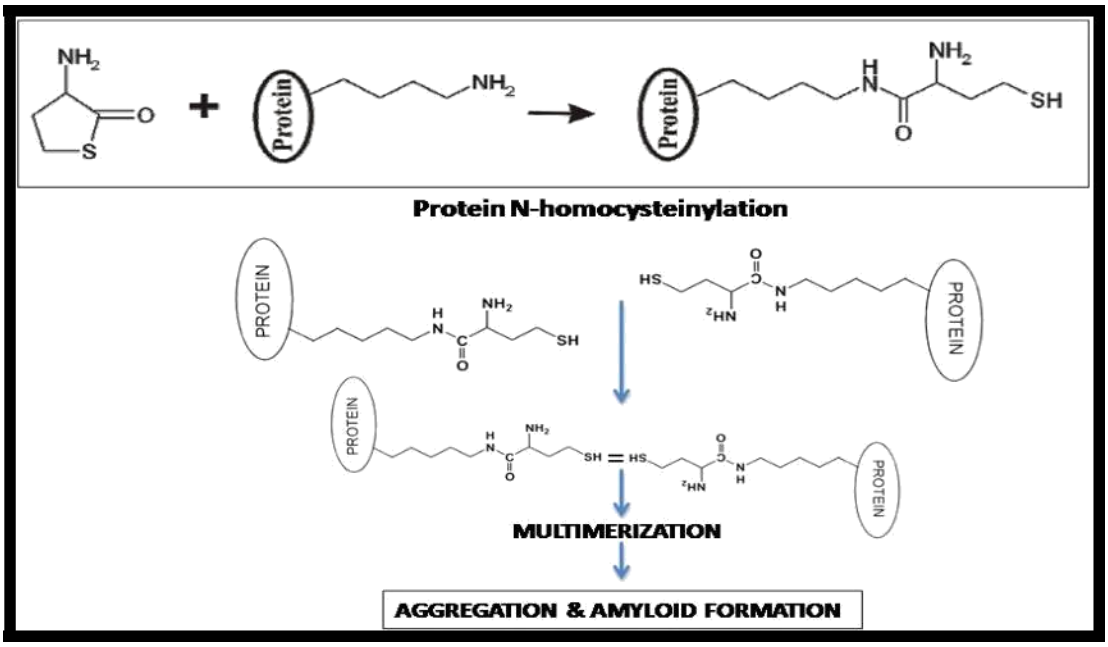


Figure 3: Homocysteinylation-induced multimerization and aggregation.

In the present study, we have analyzed the effects of N-homocysteinylation on the native state of Hemeprotein. We started with Myoglobin protein. Myoglobin is an oxygen-binding protein found in the muscle tissue of vertebrates in general and in almost all mammals. It is related to haemoglobin, which is the iron- and oxygen-binding protein in blood, specifically in the red blood cells. Structural analysis indicates that myoglobin undergoes structural alteration upon modification by HTL. The study provides what could be the cellular effects of HTL in muscle cells. Later work is on to study the effects in presence of different physical parameters on various Hemeproteins.

1.3 Review of Literature

Proper protein folding towards its functional native conformation is crucial for proper functioning of protein. It is not only the primary amino acids sequence of the polypeptide chain that dictates the final structure of the protein in question, but also the solvent condition and the environment in which the protein reside that also decide the functionality of the protein. A subtle change in the solvent condition and the environment can create havoc in the functionality of the protein and also may results in aggregation and amyloids formation as is case of various amyloids-associated diseases.

1.3.1 Protein Aggregation

Protein aggregation is a biological phenomenon in which mis-folded proteins aggregate (i.e., accumulate and clump together) either intra- or extracellularly. These protein aggregates are often toxic; protein aggregates have been implicated in a wide variety of disease known as amyloidosis, including Alzheimer's, Parkinson's and prion disease.

After synthesis, proteins typically fold into a particular three-dimensional conformation: their native state. Only in their native state are they functional. This folding process is driven by the hydrophobic effect, a tendency for hydrophobic portions of the protein to shield itself from the hydrophilic interior of the cell by burying into the interior of the protein. Substantial data support the hypothesis that partially folded intermediates are key precursors to aggregates, that aggregation involves specific intermolecular interactions and that most aggregates involve beta sheets. [14]

Thus, the exterior of a protein is typically hydrophilic, whereas the interior is typically hydrophobic. However, newly synthesized proteins may not fold correctly, or properly folded proteins can spontaneously mis-fold. In these cases, if the cell does not assist the protein in re-folding, or degrade the unfolded protein, the unfolded protein may aggregate. In this process, exposed hydrophobic portions of the unfolded protein may interact with the exposed hydrophobic patches of other unfolded proteins, spontaneously leading to protein aggregation.

The aggregates usually consist of fibers containing mis-folded protein with a beta-sheet conformation, termed amyloid. There is partial but not perfect overlap among the cells in which abnormal proteins are deposited and the cells that degenerate. The most likely explanation is that inclusions and other visible protein aggregates represent an end stage of a molecular cascade of several steps, and that earlier steps in the cascade may be more directly tied to pathogenesis than the inclusions themselves. There is now increased understanding of the pathways involved in protein aggregation, and some recent clues have emerged as to the molecular mechanisms of cellular toxicity. These are leading to approaches toward rational therapeutics. [13]

Protein aggregation is a common characteristic of many neurodegenerative diseases and various cardiovascular diseases. The aggregates and/or oligomers appear to be toxic, causing injury or death to cells. In general, the greater the degree of aggregation, the greater is the severity of disease. Protein mis-folding and other errors in protein generation occur frequently within cells, and the cell has evolved a range of mechanisms to ensure proper folding and to eliminate aggregated or otherwise damaged proteins. A common characteristic of many neurodegenerative diseases and various cardiovascular diseases is protein aggregation due to a failure of clearance mechanism(s). [13,14]

1.3.2 Homocysteinuria/hyperhomocysteinemia

Homocysteinuria/hyperhomocysteinemia is an inborn error of metabolism leading to increase in levels of sulphur containing amino acid, homocysteine in serum and urine of the affected person [1-3]. It is an inherited disorder in which the body is unable to process certain building blocks of proteins (amino acids) properly. In a normal healthy individual, the serum homocysteine level range in 5-10 μM [8-9]. However, mutations in the Hcy metabolizing enzymes, cystathionine β-synthase (CBS) or methylene tetrahydrofolate reductase (MTHFR) cause an impaired ability to metabolize the toxic Hcy resulting in an increased levels of cellular and plasma Hcy. The total concentrations of plasma Hcy may range from 15 to 20 μM (mild forms) up to 500 μM (severe forms).

Homocystinuria caused by cystathionine β-synthase (CBS) deficiency is characterized by developmental delay/intellectual disability, ectopia lentis and/or severe myopia, skeletal abnormalities (excessive height and length of the limbs), and thromboembolism. Expressivity is variable for all of the clinical signs. Two phenotypic variants are recognized, B_6-responsive homocystinuria and B_6-non-responsive homocystinuria. B_6-responsive homocystinuria is typically, but not always, milder than the non-responsive variant. In the majority of untreated affected individuals, ectopia lentis occurs by age eight years. [16]

Some of the common and prominent symptoms include, knock knees, long limbs, mental retardation, arachnodactyly, intellectual deformities to name a few. Furthermore, deficiency in

folate (a key constituent of enzyme MTHFR) disrupts the remethylation pathway which effectively converts Hcy into less toxic Met. Disruption of this pathway eventually leads to build-up of Hcy levels in the blood too.

Complications of homocystinuria should be managed appropriately; e.g., by surgery for ectopia lentis. Treatment aims to correct the biochemical abnormalities, especially to control the plasma homocystine and homocysteine concentrations and prevent thrombosis. [17]

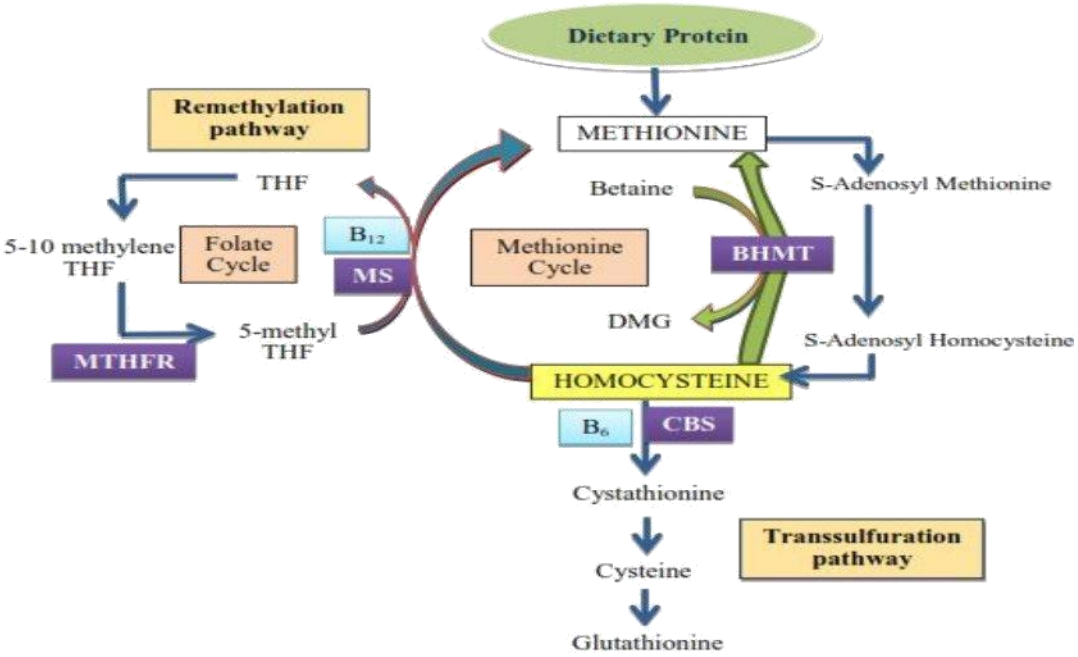


Figure 4: Hcy metabolism and biochemical mechanisms for Hcy pathologies.

Increasing Hcy levels causes an increase in Homocysteine thiolactone (HTL), a cyclic thioester of Hcy, synthesised by certain aminoacyl tRNA synthetases (AARS) in proofreading reactions that prevent translational incorporation of Hcy into proteins. The mechanism of the conversion involves reaction of Hcy with ATP to form an AARS-bound homocysteinyl adenylate (Hcy~AMP). Subsequent rejection of the Hcy~AMP intermediate involves an intramolecular reaction in which the side-chain thiolate of Hcy displaces the AMP group from the carboxylate of the activated Hcy, forming HTL as a product.

The basic cause of Hcy toxicity is known to be protein modification mediated by HTL. HTL forms an amide bond with the ϵ-amino group of protein lysine-residue [a process known as protein N-homocysteinylation].

1.3.3 Homocysteine and its Metabolism/ regulation

Homocysteine is sulphur containing non-protein [15] amino acid which is an intermediate [19] in methionine metabolism. It stands at the branch point of two amino acid metabolic pathways which are: irreversible degradation to cysteine via the Transsulphuration pathway and Remethylation back to methionine. [19]

The precise regulation of Homocysteine concentration is maintained by oxidation of approximately 99% of Homocysteine to disulfides and reduction of leftover 1% to free homocysteine. Homocysteine may be observed in either protein bound sate or non protein bound state out of which domination is of the protein bound state.

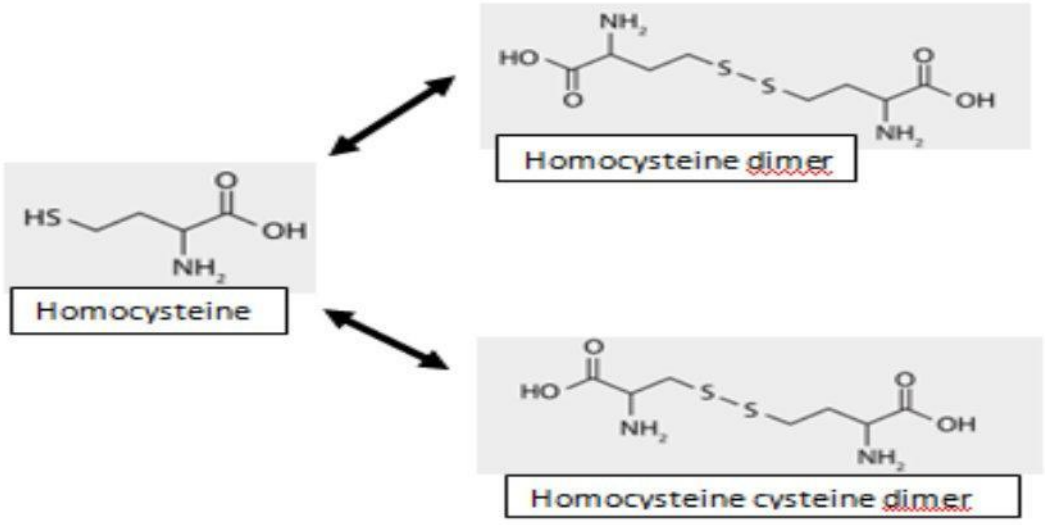


Figure 5: Different states in which homocysteine exists

Free Homocysteine

The free thiol (sulphydryl) molecule. Free unreduced homocysteine is rapidly oxidized at physiological pH and is present in plasma in only trace amounts (<0.3nmol/L). Homocysteine can be conjugated to another molecule of homocysteine by disulphide bonding to form homocystine. The oxidized fraction is largely composed of cysteine-homocystine which accounts for about 20-30% of total plasma homocysteine.

Bound to other proteins

The majority of homocysteine in plasma exists covalently linked via disulphide bonds to sterically unhindered peptide cysteine residues in various circulating proteins e.g. albumin and accounts for approximately 70-80% of total plasma homocysteine. The sum of free and bound homocysteine in plasma is denoted total Hcy and abbreviated 'tHcy'.

Healthy individual	5-15 µM
Mild forms	15-25 µM
Intermediate forms (impaired renal function)	25-50 µM
Severe forms (Homocysteinuria)	50-500 µM

As reported, Normal plasma level of Homocysteine vary from 5µmol/L to 15µmol/L. [16] Elevation in Homocysteine concentration is marked as an independent risk factor for CVD [18] and associated with increased incidences of atherosclerosis and thrombosis, pregnancy disorders,

Table 1: Homocysteine level

This medical condition in which blood plasma level of Homocysteine is elevated is referred to as Homocysteinemia. Elevation in Homocysteine level can be divided into three elevation ranges. Moderate elevation of Homocysteine concentration ranges from 15 to 30µmol/L, intermediate elevation ranges from 30-100µmol/L and severe elevation is above 100µmol/L [20]. These conditions are directly related to improper metabolism of homocysteine to methionine. This may be either due to genetic abnormality/ mutation in enzymes responsible or due to inadequate consumption of folate, vitamin B-12 and vitamin B-6. [21]

Homocysteine is involved in majorly three pathways in consideration to methionine and cysteine which includes Transmethylation, Remethylation and Transsulphuration.

Transmethylation is a biologically important chemical reaction for organic compounds in which a methyl group is transferred from one compound to another. Here, Transmethylation refer to the recovery of methionine from homocysteine. This requires presence of adequate level of vitamin B_{12} and folic acid which help in homocysteine mehtylation.

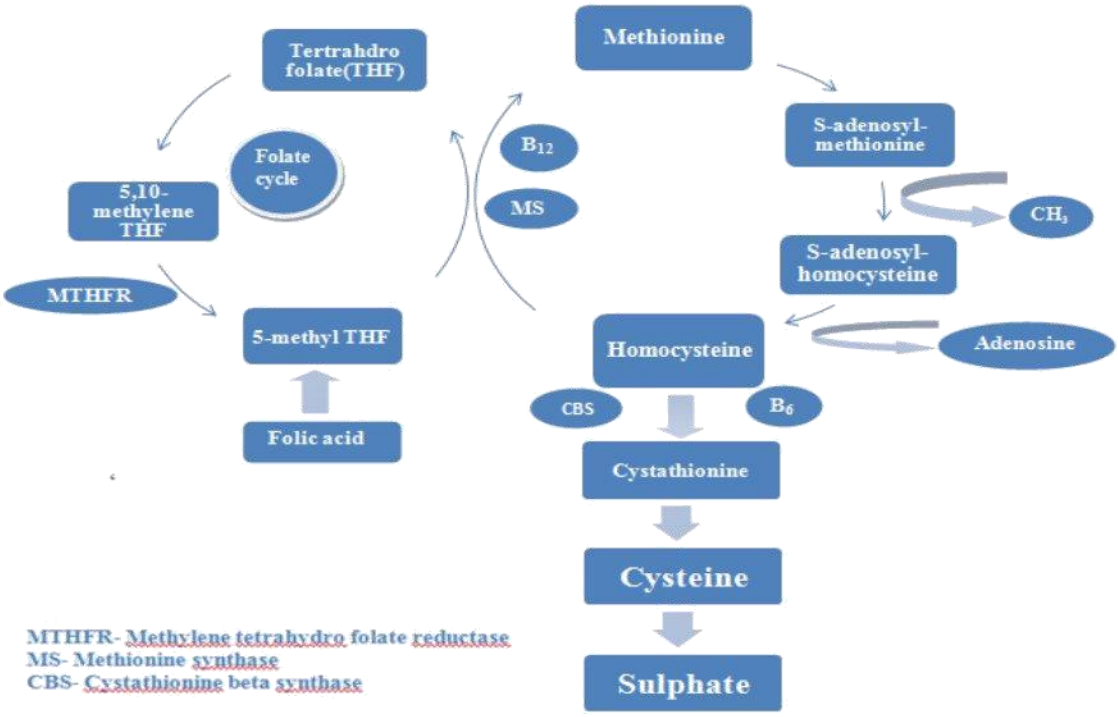


Figure 6: Homocysteine conversion and metabolism

Another pathway by which homocysteine metabolism takes place is Remethylation. It involves addition of methyl group on homocysteine which can either be due to conversion of 5-methyltetrahydrofolate to tetrahydrofolate or from the conversion of betaine to N, N-dimethylglycine[19]. This conversion happens in the presence of enzyme *methionine synthase* (MS) which uses biologically active form of vitamin B_{12} as its co-factor. In first case, which occurs in all tissues, methyl group is donated by 5-methyl-tetrahydrofolate (5-methyl-THF) which is produced by enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR) which uses active form of vitamin B_2 as co-factor. In Second case which is confined to liver and kidney, methyl group is donated by betaine with the help of enzyme betaine-homocysteine methyltransferase.[20]

Final fate of intracellular homocysteine metabolism is degradation of homocysteine to cysteine via irreversible Transsulphuration pathway, which is again mainly restricted to cells of the liver and kidneys. It is also found in muscle, brain and ovary and also during early embryogenesis in the neural and cardiac systems [19]. In this the condensation of homocysteine with serine occurs to form cystathionine, which is catalyzed by the enzyme *cystathionine-β-synthase* (CβS) which needs pyridoxal-5_-phosphate (vitamin B6) as a co-factor. Now the formed cystathionine gets converted to cysteine by *gamma-cystathionase*. Negative linkage of remethylation and positive linkage of CβS is observed with Transsulphuration when the methione concentration is found in excess in the cell [20]

As observed liver plays a major role in maintaining the adequate level of homocysteine concentration by degrading the excess methionine via unique set of enzymes as already discussed.

1.3.4 Homomcysteine Thiolactone

It is claimed that elevated levels of the homocysteine (Hcy) are associated with disease conditions in humans. But no way of its harming mechanism is reported. One consideration responsible for detrimental effects of elevated Hcy levels is the formation of homocysteine thiolactone. [15]

Homocysteine thiolactone is an intra molecular cyclic thioester of homocysteine, which is synthesized in all human cell types investigated by certain aminoacyl-tRNA synthetases specifically methionyl-tRNA synthetase (MetRS) in an error-editing or proofreading reaction that prevent translational incorporation of homocysteine into proteins [17]. As reported, course of action regarding Homocysteine thiolactone reactivity is not known, even though it is expected to acylate amino groups in proteins which can be considered and observed in various cases [18] .Chemically, homocysteine thiolactone was synthesized in 1930s(Baernstein 1934, Riegel and Du Vigneaud 1935) and almost 50 years later in 1980s is biological significance was reported with the discovery of enzymatic conversion of Hcy to Hcy thiolactone in error-editing reactions (as stated) both in vitro (Jakubowski and Fersht 1981) and in vivo (Jakubowski 1990). [17]

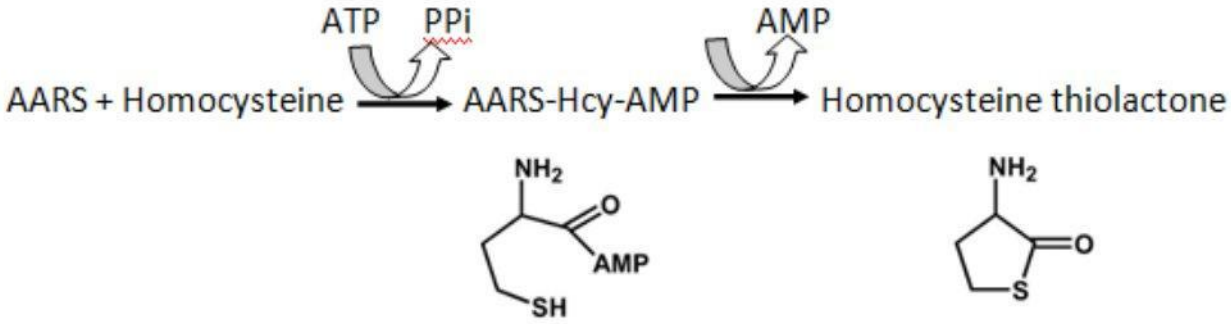


Figure 8: Formation of HTL

This conversion mechanism of Hcy thiolactone synthesis is a two step reaction, which involves reaction of homocysteine and ATP. In the first step involves Hcy carboxyl group activaton by ATP, forming a MetRS-bound homocysteinyl adenylate. This is followed by second step, in which displacement of AMP group (from the activated carboxyl group of Hcy) by the side chain thiolate of Hcy takes place [17] .This finally leads to the formation of Hcy thiolactone as a product.

In normal condition, as homocysteine concentration is relatively low, we observe lower concentration of Hcy thiolactone. But because of increased transmethylation and/or Transsulphuration of Homocysteine, the synthesis of Hcy thiolactone is enhanced. For example, in the absence of folate accumulation of Homocysteine takes place which directs the synthesis of Hcy Thiolactone in larger amount in the cell [17] .There are evidences that claim increased level of Hcy thiolactone is cytotoxic to the cardiovascular system and acutely toxic to the central nervous system [21].

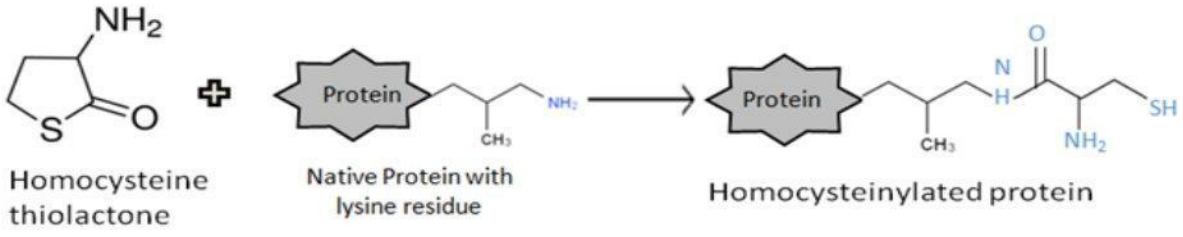


Figure 9: homocysteinylation of Protein

Homocysteine thiolactone is reported to damage the protein and lead to its structural imbalance and finally its functional loss (fig9) [17]. This is done under the process of Protein Homocysteinylation which is a post-translational modification in which e-amino group of a protein lysine residue is substituted with an Hcy residue containing a free thiol group which has detrimental effects on the protein structure and function. Protein Homocysteinylation leads to decrease in net positive charge of protein because of replacement by less basic a-amino group of N-linked Hcy[21] .This is finally linked to secondary and tertiary structural changes, along with protein aggregation which is a major highlighter of protein function loss.

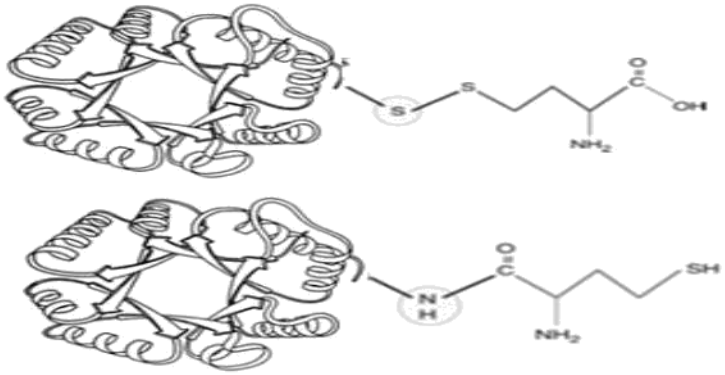


Figure 10: s-homocysteine and N-homocysteine

There are two kind of protein Homocysteinylation, one termed as protein-N-homocysteinylation in which acylation of free amino groups takes place, in particular, the binding of Hcy to the ***ε*** amino group of lysine residues and the terminal amino group of proteins as discussed above and second is the protein-S-Homocysteinylation in which oxidation of thiol groups, in particular directed towards cysteine (Cys) residues. Protein-N-Homocysteinylation is mediated by Hcy thiolactone, an Hcy derivative whereas protein-S-Homocysteinylation is mediated by Hcy in its free form. [23]

1.3.5 Determinants of plasma Hcy levels

Homocysteine levels are affected by a lot of factors that may be genetic or they may be related to lifestyle factors. Different factors affect plasma homocysteine level differently. Some of the factors are given below -

- Genetic factors - Alteration in the following genes
    - CβS deficiency
    - MS deficiency
    - MTHFR deficiency
    - TL-MTHFR (C677T)
- Physiological Age
- Smoking
- Coffee
- Sex
- Pregnancy
- Diet
- Alcohol
- Pathological Vitamin deficiencies
    - Renal disease
    - Renal/cardiac transplantation
    - Severe psoriasis
    - Leukaemia
- Medications Oral contraceptives/Hormone Replacement Therapy
- Steroids, drugs, hormones
- Pollution
- Physical activity

Genetics

To the matter of fact levels of homocysteine are considered to be dependent on both lifestyle and genetic factors. Studies have been conducted to identify the some of the common genetic variants that are involved in affected homocysteine concentration [45]. Due to lack or inefficiency of enzymes (the mediating molecules), making it difficult to metabolize homocysteine we reach a condition with elevated homocysteine level, called homocystinuria [46]. As already discussed, homocysteine is metabolized either by Remethylation or by Transsulphuration, where one of the most important enzyme is MTHFR. It plays a role in conversion of 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate which is folate's predominant circulating form [47]. Two major mutations (polymorphisms) observed are 677C>T and 1298A>C. Normal MTHFR helps in maintaining folate and methionine pool, and by preventing accumulation of homocysteine.

Both the polymorphism affects the level of homocysteine in one way or the other. The MTHFR 677C>T mutation (more common and more effective) leads to generation of *MTHFR* 677T allele which is found in relevance to the reduction in enzymatic activity. This leads to decreased concentration of folate and leading to mild homocysteine elevation [45, 47, and 6]. Another mutation is *MTHFR* polymorphism 1298A>C, which affects the activity but with no biological changes. In comparative study between double heterozygosity and single heterozygosity for *MTHFR* 677C>T and 1298A>C polymorphisms for revealed that double heterozygous polymorphisms result in a lower MTHFR activity [47]. So, to overcome and prevent the loss of enzyme activity by providing with optimal folate supply, enhancing the FAD binding, which was suppressed as a result of enzyme inactivation. [20]

The results of prevalence maybe conflicting at one point, but it mark the importance of genetic variation leading to elevated homocysteine level and acting as risk factor for various diseases [48, 49]. To combat this effect proper intake of folate is necessary ensuring proper metabolism of homocysteine.

Age and Sex

As the life progresses and the age increases, it is reported that it shows a positive co-relation to the increase in Plasma homocysteine levels. This may be related to decrease in efficiency of the enzyme CS as per increase in age or it may be due to Dietary changes. Both male sex and increasing age are related in higher homocysteine concentration in the body [23]. This difference in homocysteine concentration may be supported with the larger muscle mass in men which is associated to creatinine synthesis.

Another adding factor is difference in vitamin status between sexes and also due to influence of sex hormones which was confirmed in a study with transsexual males and females (Giltay et al., 1998). Observed results suggest Men have 25% higher concentration of homocysteine in comparison to premenopausal women. Although this difference was decreased when the comparison was made with post menopause women. This justifies that Homocysteine level is higher in post menopausal women in comparison to pre menopausal women. Also, levels are decreased by intake of oral contraceptive pill.

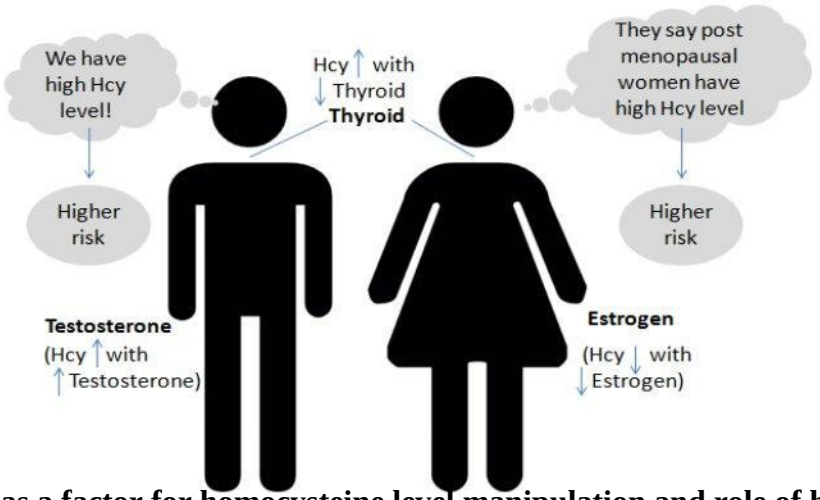


Figure 11: Sex as a factor for homocysteine level manipulation and role of hormones

Hormone replacement therapy has also been reported as a reducing factor of Homocysteine level. Approximately 60% fall in homocysteine concentration is observed in case of pregnancy due to reduction in albumin which bounds significant amounts of homocysteine which is prevented by an increase in the activity of the enzyme betaine homocysteine methyltransferase. [24]

Smoking

The act of inhalation of smoke of burning tobacco which is enclosed in cigarettes, pipes and cigars is referred to as smoking. Addiction of tobacco products is responsible for several diseases, such as cancer, respiratory diseases and cardio vascular diseases. Directly or indirectly tobacco smoking leads to 6 million deaths each year [39].

Tobacco abuse is marked to be independent risk factor for various diseases one of which is cardiovascular diseases and same goes for homocysteine level [40]. Is there any relation between the two? Reports suggest a positive relationship between homocysteine concentration and smoking. Smoking affects the body in various ways out of which a one may be linked to homocysteine concentration. Smoking is observed to have an effect of vitamin B as a whole. It forces the release of stress hormones in the body which increases the need of all vitamin B including folic acid. Also, low level of vitamin B12 is observed in smoker's blood [40]. We have already studied the need and probable effects of vitamin B deficiency in case of homocysteine concentration. Diet is something which is not proper in case of smokers, which points to the fact that they will have less of vitamin B intake or even exclusion of vitamins in some cases (Dallongeville et al., 1998). This is because comparative demand is more than the normal individual and to the matter of fact smokers don't understand this fact.

Deduction of exact mechanism by which smoking affects homocysteine level is not known, but it is reported to have an influential effect on changing the thiol redox status which is linked to plasma homocysteine concentration. Another report suggests that action of smoking via inhibiting the enzymes like methionine synthase is another way of positively contributing to homocysteine levels. According to a study, per day 20 cigarettes consumption is marked as high risk factor is associated with 2.01 μmol/l increase in homocysteine level (Hordaland Homocysteine Study). So, we have witnessed effects of smoking on homocysteine levels which can be linked to various diseases, hence, there is a serious need to combat smoking for better living of individuals.

Coffee

Most versatile and one of the most popular beverage in the world, consumed by one third world population. Coffee was always in picture of discussion as it was linked to cardiovascular diseases and other health conditions, but recently health beneficial association with coffee consumption, including decreased risk of diabetes, Parkinson's disease, and liver disease have been reported. With more than 1000 compounds coffee is reported to b positively associated with homocysteine level in both male and female in almost all the cases. Intervention studies have shown that high levels of coffee consumption (6 to 10 cups of coffee per day) increase homocysteine and homocysteine decrease if regular coffee consumers stop drinking coffee [31, 36]. Caffeine is one of the major components in coffee linked with homocysteine concentration, but reports suggest that it is only partly related.

Caffeine by acting as vitamin B6 antagonist inhibits the conversion of Homocysteine to cysteine and hence contributing as a factor in the above level increase. Other compounds in coffee like chlorogenic acid also contribute to the effect of homocysteine. Chlorogenic acid required methyl group from methionine for its metabolism which leads to increase homocysteine level [32, 37]. Significant reports suggests that caffeine lead to only 25-50% homocysteine raise in comparison to coffee with same amount of caffeine content. Also the effects of coffee were observable within a few hours of coffee uptake which was not the case in caffeine consumption. Thus others compounds might be responsible for homocysteine raise [34].

Additionally, recent evidence showed that chlorogenic acid, a polyphenol that is present in coffee in the same amount as caffeine, may also partly be responsible for the increase in the tHcy concentration. When polyphenols are metabolized, methyl groups from methionine are necessary, which results in a higher production of homocysteine Both caffeine and chlorogenic acid are also present in tea, although in smaller doses, which explains the absence of a clear association between tHcy and tea consumption.

Supplemental and Dietary B vitamin Intake

As mentioned Folic acid and vitamin B are essential cofactors in Homocysteine metabolism, hence its supplementation in diet plays a role in maintaining homocysteine concentration, which is supported by several intervention studies. An inverse relationship exists between folate and B12 intake and homocysteine levels. This suggests that homocysteine levels are lower in those individuals who go for vitamins supplementation [24]. A Meta analysis study suggests that, In adults, homocysteine concentration was observed to decrease by about 25% when dietary folic acid was added to routinely diet at dose of 500 to 5000 g per day. Also, levels of homocysteine supplementation was given at a dose of 500 g lowered by 7% when additional oral vitamin B12 ? factors per day. The observation above is independent of other dietary factors and life style ? (Rasmussen et al., 2000; de Bree et al., 2001c; Jacques et al., 2001).

Also the above result is found to complement the result found when dietary folate intake studies were done in middle-aged(Shimakawa et al., 1997; Ubbink et al., 1998; Saw et al., 2001) and elderly sample population space (Selhub et al., 1993; Bates et al., 1997; Koehler et al., 2001; Saw et al., 2001. This may be supported with mal absorption of vitamin B12 from the diet, commonly due to atrophic gastritis as age progresses. As discussed earlier in the metabolism of homocysteine, where folate is required for the conversion of homocysteine to methionine by acting a substrate which donates the methyl group whereas vitamin B2, B6, and B12 act as cofactors for various enzymes in homocysteine metabolism, so we can conclude that folate is the most important dietary determinant, along with vitamin B because of their metabolic role.

Plasma homocysteine is directly related to methionine intake and levels are therefore lower in individuals who consume a diet low in protein of animal origin. Homocysteine levels are inversely correlated with vitamin intake and tend to be lower in diets rich in fresh fruit and vegetables. There is an inverse relationship between folate and B12 intake and homocysteine levels. Homocysteine levels are lower in individuals whose diet is supplemented with vitamins. Elevated homocysteine levels may occur in individuals with B12 or folate deficiency [26].

Alcohol

Today consumption of Alcohol is a status symbol which proudly marks it presence as the most common drink around the world. It happens to be the most deadly drug when it comes to war of drugs, even cocaine and heroin stands next to it. Alcohol is leading death taking drug in the society where the number reaches 100,000 deaths per year which is a huge number in comparison to 20,000 deaths per year which is combined contribution of other drugs [29]. This cruel drug is slow, so effects in starting are minimal which marks its more consumption, maybe to celebrate, socialize or relax. [28]

Alcohol consumption and homocysteine level show a correlation, but the relation is highly complex [30]. According to a report, chronic alcoholism is found to be associated with a derangement in the sulfur amino acid metabolism, and homocysteine is found to be one of them. The association between Alcohol consumption and Homocysteine level follows a J-shaped curve (Halsted, 2001). The explanation of this curve is like lower homocysteine concentration is observed in moderate alcohol consumers in comparison to non drinkers irrespective of their sex. On the other hand, homocysteine level were found elevated in case of chronic alcoholics. This is very similar to alcohol consumption and cardiovascular mortality curve, which is again in J-shaped fashion, following the similar pattern [32]. The purpose of calling this relation to be complex is that, there is another report which reports that there is no relation between the two (Lussier Cacan et al., 1996; Gudnason et al., 1998). Here comes the point, if it follows a J-shaped pattern, so it deduce that moderate amount of alcohol consumption is beneficial for the individual. Also, different types of alcoholic beverage consumed have varied effects. A 3 week study was conducted which suggest that beer had no effect on homocysteine level whereas wine and spirits have positive effect of increasing homocysteine levels. In fact the results were astonishing when results of that study showed that high amount of beer have inverse effect on homocysteine concentration which is not in line with any other study. Reports say this may be due to folate, vitamin B presence in beer useful for metabolism of homocysteine [33]. So this is the relation between alcohol consumption, vitamin B, folate and homocysteine level which is a risk factor for cardiovascular diseases. [34]

It has been reported that elevated level of homocysteine are found to induce neuronal cell damage by free radical formation and by stimulating NMDA receptors which plays important role in controlling synaptic plasticity and memory function [30] . This may lead to neurotoxicity as overstimulation of these receptors is taking place and hence linking chronic alcoholism to elevated homocysteine level and linking this elevation to neurodegenerative diseases.

Drugs

Following other factors, drugs were the next target to be studied for exploring its effect on homocysteine levels. Drugs given for prevention or treatment of various diseases like cardiovascular diseases, etc. Several drugs are found to modulate homocysteine levels [52, 54]. Drugs given to type 2 (non-insulin-dependent) diabetes mellitus and to those who are at high risk of cardiovascular diseases are observed to raise homocysteine level. Dyslipidaemias and metformin (fibric acid derivatives) belong to the above category. This raises a question of decreasing the effectiveness of drug, negating its functionality and counteracting the desired cardio protective effect [54]. Homocysteine increase is about 20-50%, which cannot be totally avoided, but can be reduced. Another drug, HMG-CoA reductase inhibitors (statins), ACE-inhibitors are found to have no effect on homocysteine elevation [53]. Some drugs like ß-blockers were observed with lowering of homocysteine. Effects of some drugs like nicotinic acid and n3-fatty acids are not clear, more research required.

The mechanism of drug affecting homocysteine concentration varies greatly. A few drugs interfere with vitamin absorption in gut, for example cholestyramine and metformin. Another set is of those who interfere with folate and homocysteine metabolism directly, including drugs like methotrexate, nicotinic acid (niacin) and fibric acid derivatives. Drugs like glucophage and cholestyramine act by blocking absorption of folic acid from the intestines [54, 53]. This is how different drugs target homocysteine levels directly or indirectly. Also, effects of drugs decreasing homocysteine levels are found to be associated with improvement in cardiovascular diseases [52]. So, this brings us to the conclusion that while prescribing drugs to cardiovascular patients, doctors should always suggest folic acid, pyridoxine uptake for regulation of homocysteine

[52, 53]. concentration

Hormones

Several reports have appeared in the literature proving facts that hormones play a role in modulating homocysteine metabolism and play a role in maintaining their concentration. Different hormones show different results, but with unclear mechanisms.

Talking about Thyroid hormone on the first place, its deficiency (hypothyroidism) is found to be associated with increased risk of cardio vascular diseases. New research direction recognizes homocysteine level as one of the risk factor for atherosclerosis in patients with hypothyroidism. Study suggests elevation in homocysteine level in case of thyroid deficiency and is observed to decrease when patient is subjected to thyroid replacement therapy. When studied further it was found that folate metabolism and enzymes like MTHFR are affected (decreased) by hypothyroidism. Another hormone under consideration is Insulin where results are very conflicting; varying from patients to apparently healthy population sets. An unexpected result shown by one of the study is the negative correlation between insulin resistance and serum homocysteine.

Another study highlighted the correlation of homocysteine and insulin in obese children and adolescents, pointing in direction that suggests the contribution of fat mass–associated hyperinsulinism in impairment of homocysteine metabolism.

Now talking about effect of sex hormones found to affect homocysteine levels and is indicated by gender differences [20]. Higher plasma levels of total homocysteine are observed in man than in women, without much detail of mechanism involved. Male hormone testosterone is observed to have a negative effect on CBS expression, which is involved in catalysis s of Transsulphuration pathway. This is done via posttranscriptional mechanism which increases the susceptibility to oxidative stress conditions. All reports suggest that postmenopausal women are at higher risk of coronary artery disease and atherosclerosis in comparison to Premenopausal women. This is because of estrogen (female sex hormone) effect, which diminishes homocysteine level and its effect. A lot is not known about its working and mechanism. The effects can be minimized by using hormone replacement therapy (HRT) which is observed in lowering of homocysteine levels. [36, 23]

Pollution

We live because of two basic things air and water which our mother earth has given us. Without them considering earth will be just like other planets- with no man, no life. Today the intervention and spoiling of nature has gone up so high that pollution of air and soil is reaching the mark which is high above danger level. Water and air pollution is contamination or addition of unwanted contaminant, which is a risk factor for various diseases and is also supposed to be linked with cardiovascular diseases. Its linkage to homocysteine is also reported.

Water pollution is caused by addition of a lot of unwanted contaminants, one of them is Arsenic. Water contaminated with arsenic has been reported to increase the risk of atherosclerosis. Homocysteine is shown to have modifying effect on biomethylation of ingested arsenic, which of its involvement in S-adenosylmethionine. A positive correlation exists between the plasma homocysteine level and arsenic exposure. Elevated homocysteine levels may worsen the formation of atherosclerosis, which is guided by arsenic exposure. This is how homocysteine levels are affected by the interference of water pollutants.

Air pollution, another major pollution is linked with induction of short term inflammatory changes that may be the determinant of hyperhomocystenemia. The mechanism and involved participants are not well understood, but to some extent it is associated with activation of systemic inflammation, hypercoagulity and increased peripheral blood pressure [28, 29]. Also, cigarette smokers are found with increased risk for developing hyperhomocystenimia. They have a preexisting pro-inflammatory status, where particles between the two may interact. Subject (smokers) with higher concentration of ambient PM_{10} is found to have increased plasma homocysteine level [17]. Another study suggests the relation of all the genes, which are involved in interaction with air pollution, had observable effects on total plasma homocysteine. Variations of oxidative stress-related genes were found to modify the effects of black carbon/$PM_{2.5}$ on homocysteine level, showing the mediation through oxidative stress pathway [49, 50]. This is how air pollution is linked with higher risk of increasing homocysteine concentration.

Physical Activity

Physical activity, daily workout, etc all comes under one category, category promoting fitness and health. A lot of studies have been conducted to determine the relation between homocysteine level and physical activity. In one line if I have to sum up, physical activity is very weakly associated with homocysteine concentration in an inverse order.

Homocysteine level elevation is constantly marked as risk factor for cardiovascular diseases. Reports suggest that physical activity, exercises and training program (aerobics, etc) are acting as controllers of atherosclerosis. Now the question is whether physical activity targets the above via affecting homocysteine route or some other pathway. It is observed that it induces protein and amino-acid metabolism changes [38]. The results of acute exercises and physical training are very different, in fact opposite. Reports confirm that acute exercises are linked with elevated homocysteine level in plasma, whereas aerobic physical training is reported to decrease homocysteine level [40]. Other reports (Wright et al., 1998) showed that there is no effect of exercises on homocysteine concentration [20]. This makes it highly difficult to decode what is the exact scenario. Folate dependency was established in case of exercise- induced homocysteine increase, but no link to vitamin B12. In a study athletes with highest training volume, were found with lower homocysteine level and high folate level.

Training types differ in form of intensity, duration, repetition and volume. All are found to have comparatively different effects of homocysteine level [38]. High intensity exercises are reported to stimulate metabolic pathways that produce energy, which rely on vitamin B6, B12 and folate. So, in order to maintain the optimal functionality, consumption of vitamin B and folate should be high, leaving fewer vitamins for homocysteine metabolism and finally leading to elevated level of homocysteine [41]. In case of elders, correlation between homocysteine and physical activity is even more complex and controversial in case on elderly people. A study revealed the importance of vitamins required for the regulation of homocysteine concentration. The affect observed in homocysteine levels were found, and conclusion was made that they are independent of vitamin B in elderly people [42]. This makes clear the importance of maintaining good health and doing physical activities daily for ensuring better life.

1.3.6 Hemeprotein

A **hemeprotein** (or **haemprotein**; also **hemoprotein** or **haemoprotein**), or heme protein, is a metalloprotein containing a heme prosthetic group- an organic compound that allows a protein to carry out several functions that it cannot do alone. Heme remains bound to the protein permanently, either covalently or noncovalently bound or both.

The heme contains a reduced iron atom, Fe^{2+} in the center of a highly hydrophobic, planar, porphyrin ring. The iron has six possible coordination bonds. The porphyrin ring has 4 nitrogen atoms that bind to the iron, leaving two other coordination positions of the iron available for bonding to the histidine of the protein and a divalent atom.

Hemeproteins probably evolved to incorporate the iron (Fe) atom contained within the protoporphyrin IX ring of heme into proteins. As it makes hemeproteins responsive to molecules that can bind divalent iron (Fe), this strategy has been maintained throughout evolution as it plays crucial physiological functions. Oxygen (O_2) nitric oxide (NO), carbon monoxide (CO) and hydrogen sulfide (H_2S) bind to the iron atom in heme proteins. Once bound to the prosthetic heme groups, these molecules can modulate the activity/function of those hemeproteins, affording signal transduction.

Hemeproteins have diverse biological functions including oxygen transport, which is completed via Hemeproteins including hemoglobin, myoglobin, neuroglobin, cytoglobin and leghemoglobin. Catalysis occurs with hemeproteins cytochrom P450s, cytochrome c oxidase, ligninases and peroxidases.

Myoglobin

Myoglobin is an iron- and oxygen-binding protein found in the muscle tissue of vertebrates in general and in almost all mammals. It is related to hemoglobin, which is the iron- and oxygen-binding protein in blood, specifically in the red blood cells. In humans, myoglobin is only found in the bloodstream after muscle injury. It is an abnormal finding, and can be diagnostically relevant when found in blood.

Myoglobin is the primary oxygen-carrying pigment of muscle tissues. High concentrations of myoglobin in muscle cells allow organisms to hold their breath for a longer period of time. Diving mammals such as whales and seals have muscles with particularly high abundance of myoglobin. Myoglobin is found in Type I muscle, Type II A and Type II B, but most texts consider myoglobin not to be found in smooth muscle.

Myoglobin belongs to the globin superfamily of proteins, and as with other globins, consists of eight alpha helices connected by loops. Human globin contains 154 amino acids. Myoglobin contains a porphyrin ring with an iron at its center.

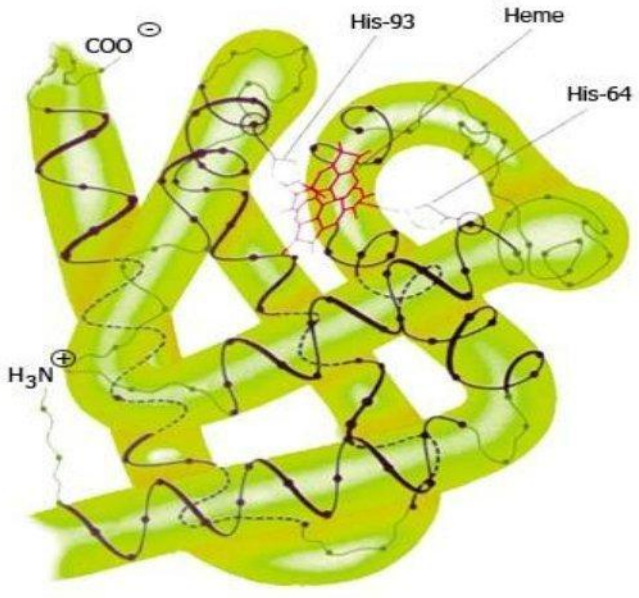


Figure 12: Structure of Myoglobin.

Hemoglobin

Hemoglobin abbreviated Hb or Hgb, is the iron-containing oxygen-transport metallo-protein in the red blood cells of all vertebrates as well as the tissues of some invertebrates. Hemoglobin in the blood carries oxygen from the respiratory organs (lungs or gills) to the rest of the body (i.e. the tissues). There it releases the oxygen to permit aerobic respiration to provide energy to power the functions of the organism in the process called metabolism.

Hemoglobin is involved in the transport of other gases: It carries some of the body's respiratory carbon dioxide (about 10% of the total) as carbaminohemoglobin, in which CO_2 is bound to the globin protein. The molecule also carries the important regulatory molecule nitric oxide bound to a globin protein thiol group, releasing it at the same time as oxygen. Hemoglobin is also found outside red blood cells and their progenitor lines. Other cells that contain hemoglobin include the A9 dopaminergic neurons in the substantia nigra, macrophages, alveolar cells, and mesangial cells in the kidney. In these tissues, hemoglobin has a non-oxygen-carrying function as an antioxidant and a regulator of iron metabolism.

Hemoglobin and hemoglobin-like molecules are also found in many invertebrates, fungi, and plants. [8] In these organisms, hemoglobins may carry oxygen, or they may act to transport and regulate other things such as carbon dioxide, nitric oxide, hydrogen sulfide and sulfide. A variant of the molecule, called leghemoglobin, is used to scavenge oxygen away from anaerobicsystems, such as the nitrogen-fixing nodules of leguminous plants, before the oxygen can poison the system.

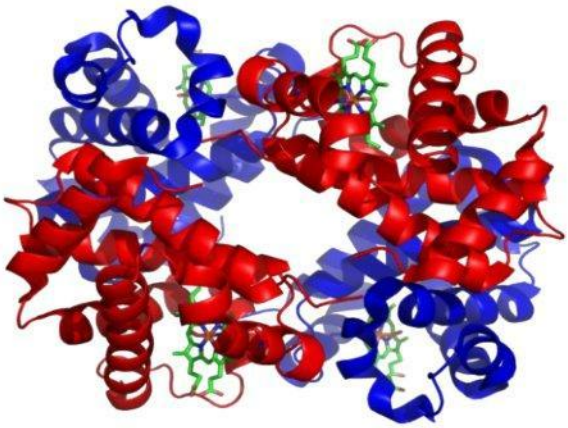


Figure 13: Structure of haemoglobin.

Cytochrome C

Cytochrome complex or cyt *c* is a small hemeprotein found loosely associated with the inner membrane of themitochondrion. It belongs to the cytochrome c family of proteins. Cytochrome c is a highly water soluble protein, unlike other cytochromes, with a solubility of about 100 g/L and is an essential component of the electron transport chain, where it carries one electron. It is capable of undergoing oxidation and reduction, but does not bind oxygen. It transfers electrons between Complexes III (Coenzyme Q – Cyt C reductase) and IV (Cyt C oxidase). In humans, cytochrome c is encoded by the *CYCS* gene.

Cytochrome c is a component of the electron transport chain in mitochondria. The heme group of cytochrome c accepts electrons from the bc_1 complex and transfers electrons to the complex IV. Cytochrome c is also involved in initiation of apoptosis. Upon release of Cytochrome c to the cytoplasm, the protein binds apoptotic protease activating factor-1 (Apaf-1).

Cytochrome *c* can catalyze several reactions such as hydroxylation and aromatic oxidation, and shows peroxidase activity by oxidation of various electron donors such as 2,2-azino-*bis*(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2-keto-4-thiomethyl butyric acid and 4-aminoantipyrine. Cytochrome *c* is involved in one form of nitrite reductase

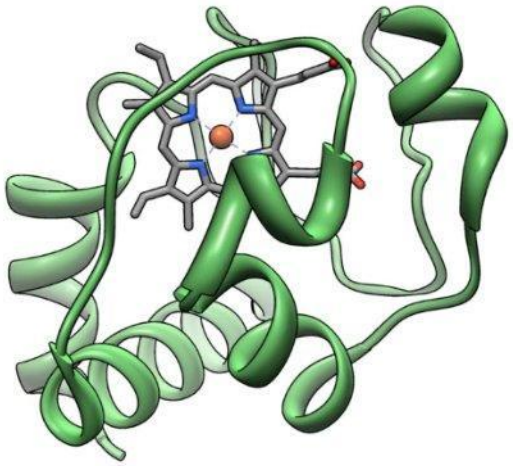


Figure 14: Structure of Cytochrome C

1.3.7 Physical parameters

When we talk about any experiment, any reaction we define certain parameters that define the whole system. To some extent those parameters have a major role in the reaction. These parameters have a major effect on the reaction and its mechanism.

By changing these parameters we observe changes in the results as it hampers with the structure and function of certain reaction substrates, intermediates and products which finally may lead to certain disruptions or some disease condition.

Parameters under our consideration will be –

- **Salt concentration-** change in salt concentrations. Salts like calcium chloride and potassium chloride. This lead to change in ion level which leads to various changes.
- **pH –** change is pH has always been observed as a key parameter in the reaction as different reaction results are observed at different pH. So range for pH we will consider is 6 to 8.
- **Temperature –** change in temperature always reports with change in structure of proteins. So we will consider a moderate change in temperature as body temperature is more or less constant.
- **Alcohol –** To measure or observe what the effect of homocysteinlyation on people is who drink alcohol more frequently.
- **Nicotine –** To measure or observe what the effect of homocysteinlyation on people is who smoke more frequently.

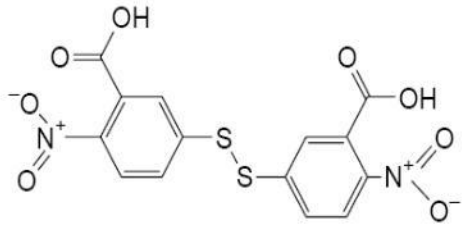
Time based study for all parameters as time itself is one of the most important parameter.

1.3.8 Techniques

Ellman's Test:-

In 1959 Ellman1 introduced 5,5'-dithio-*bis*-(2-nitrobenzoic acid), also known as DTNB, as a versatile water-soluble compound for quantitating free sulfhydryl groups in solution (Figure 1, see Additional Information section). A solution of this compound produces a measurable yellow-colored product when it reacts with sulfhydryls. Consequently, Ellman's Reagent is very useful as a sulfhydryl assay reagent because of its specificity for -SH groups at neutral pH, high molar extinction coefficient and short reaction time. DTNB reacts with a free sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid.

The target of DTNB in this reaction is the conjugate base (R—S-) of a free sulfhydryl group. Therefore, the rate of this reaction is dependent on several factors:2 1) the reaction pH, 2) the pKa' of the sulfhydryl and 3) steric and electrostatic effects. TNB is the "colored" species produced in this reaction and has a high molar extinction coefficient in the visible range.



Ellman's Reagent;
5,5'-Dithio-*bis*-(2-nitrobenzoic acid)
$C_{14}H_8N_2O_8S2$
Mol. Wt.: 396.35

Figure 1. Structure of Ellman's Reagent

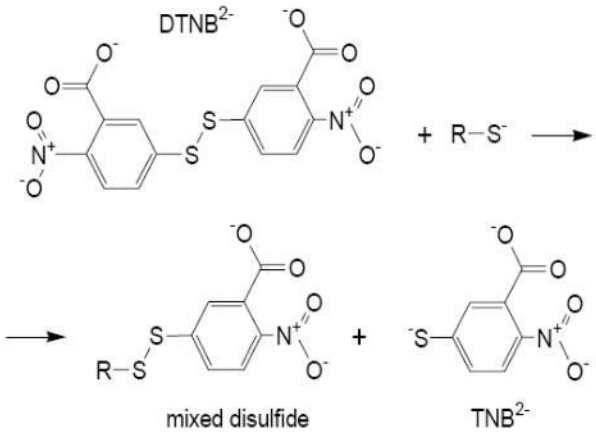


Figure 2. Reduction of Ellman's Reagent

Figure 15: Structure and reaction of Ellmans's reagent

Thioflavin T

Thioflavin T is a benzothiazole dye that exhibits enhanced fluorescence upon binding to amyloid fibrils, both *ex vivo* and *in vitro*. The superiority of ThT for detection of amyloid was further confirmed by comparing congo red, crystal violet, van gieson and ThT dyes for their binding to several positive and negative control tissues.

Due to the specificity of binding of thioflavin T to amyloid fibrils, it has found many applications such as diagnosis of amyloid in tissue sections using fluorescence microscopy, monitoring extracted amyloid and in vitro amyloid fibril formation using fluorescence spectroscopy.

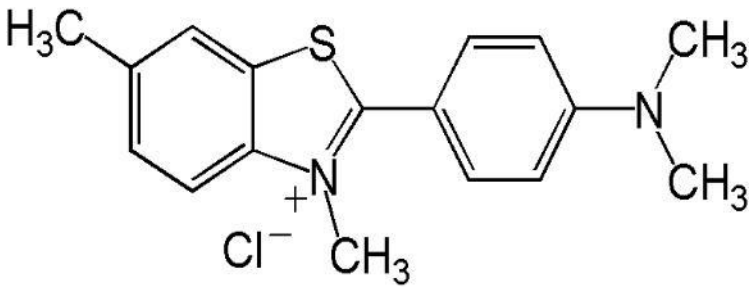


Figure 16: Structure of ThT

The fluorescence property of ThT shows appearance of a new excitation peak at 450nm, upon binding to amyloid fibrils that is responsible for enhanced fluorescence emission at 482nm.

The structure of thioflavin T has a hydrophobic end with a dimethylamino group attached to a phenyl group, linked to a more polar benzothiazole group containing the polar N and S.

This combination of polar and hydrophobic regions creates the possibility for thioflavin T molecules to form micelles in aqueous solution, with hydrophobic interiors and the positively charged N pointing toward the solvent. The thiazole nitrogen of the dye and hydroxyl groups of tissue structures form hydrogen bonds to give rise to specific binding of these dye molecules to amyloid and other tissue structures.

Thioflavin T is also known to bind to many structures other than amyloid, including nucleic acids, keratin, and elastin fibrils. The importance of electrostatic interactions in ThT binding is

evident in the binding to the negatively charged nucleic acids. Presumably, the positive charges on the surface of the ThT micelles interacts with the negative charges on the nucleic acids to give strong binding, leading to enhanced fluorescence.

ANS (8- Anilino-1-napthalene sulphonic acid*)*

The fluorescence dye 8-anilino-1-naphthalene sulfonate (ANS) is a charged hydrophobic fluorescent molecule of molecular mass 673 Da. It is a valuable probe for the detection and analysis of conformational changes in proteins and in the studies of biological membranes. The "preferable interaction" of this dye with equilibrium and kinetic compact intermediates in relation to native and completely unfolded proteins is well established. It has a low fluorescence yield in polar environments, which is greatly enhanced on interaction with many proteins. ANS binds non covalently to proteins and its fluorescence varies with changes in the probe environment.

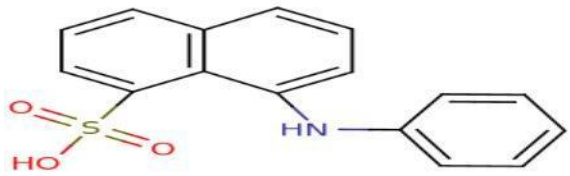


Figure 17: Structure of ANS

Fluorescence enhancement with a hypsochromic shift is resulted from the interaction of the charged group of lysine and arginine with the sulfonate group of ANS. Ion pairing between Arg (or Lys) and the sulfonate group of ANS reduce the intermolecular charge transfer (CT) rate constant that leads to enhancement of fluorescence. A positive charge near the -NH group of ANS changes the intramolecular CT process producing a blue shift of fluorescence. The Arg side chain compared to that of Lys more effectively interacts with both the -NH and sulfonate groups of ANS.

CHAPTER 2: MATERIAL AND METHODS

Material and Methods

2.1 Materials:

Commercially lyophilized preparations of Hemeprotein, DL-Homocysteine thiolactone hydrochloride (HTL), 8-anilino-1-naphthalene sulfonic acid(ANS), Guaiacol, Hydrogen peroxide (H_2O_2), Phosphate Buffer (pH 7.4) were purchased from Sigma Chemical Co. Potassium chloride (KCl), Potassium phosphate were purchased from Merck.

2.2 Methods:

2.2.1 Analytical Procedures:

All protein solutions were dialyzed extensively against 0.1 M KCl at pH 7.0 in cold (~ 4°C). Protein stock solution was filtered using 0.22 μm Millipore syringe filter. Concentration of the protein solution was determined experimentally using ε, the molar extinction coefficient values of 171000 $M^{-1}cm^{-1}$ at 409 nm. All experiments were carried out in 0.05 M potassium phosphate buffer (pH 7.4) containing 0.1 M KCl at 37° C.

2.2.2 Protein Modification:

Protein was incubated in presence of varying concentrations of HTL (0-5mM) in 0.05M potassium phosphate buffer, pH 7.4 overnight at 37°C. The HTL treated/untreated protein samples were further used for subsequent studies.

2.2.3 Ellman's Assay:

Protein sulfhydryl (SH) group estimation was carried out as described by Ellman [11]with some minor modifications. The levels of thiol groups in control and homocysteinylated protein samples were essayed using 5, 5'- Dithiobis (2-nitrobenzoic acid), the Ellman's reagent. The absorbance of the samples was measured at 412 nm, using a 1cm path-length cuvette. The amount of 5'-nitrothiobenzoate released was estimated from the molar extinction coefficient of 13,700 $M^{-1}cm^{-1}$.

2.2.4 Circular Dichroism (CD) Measurements:

CD measurements were done Jasco J-810 spectropolarimeter equipped with a Peltier-type temperature controller with three accumulations. Protein concentration used for the CD measurements was 0.5 mg/ml. Cells of 0.1 and 1.0 cm path lengths were used for the measurements of the far and near-UV spectra, respectively. The protein samples were pre-incubated overnight with HTL. Necessary blanks were subtracted for each measurement. All readings were recorded at 37° C.

2.2.5 Peroxidase Activity Measurements:

In order to measure peroxidise activity, we used guaiacol as the substrate, hydrogen peroxide as the oxidising agent as reported earlier [12]. The reaction was followed in Jasco V-660 UV/Visible Spectrophotometer. The protein concentration used was 2 μM. For peroxidase activity estimation, 5 mM H_2O_2 and 10 mM guaiacol were used. The formation of tetraguaiacol was followed spectrophotometrically at 470 nm.

2.2.6 Fluorescence Measurements:

Fluorescence spectra of the protein samples were measured in a Perkin Elmer LS 55 Spectrofluorimeter in a 3 mm quartz cell, with both excitation and emission slits set at 10nm. Protein concentration was taken as 2 μM. For intrinsic fluorescence measurement, cyt c was excited at 295 nm and the emission spectra were recorded in the wavelength region 300–450 nm.

For ANS-protein binding experiments, the excitation wavelength was 360 nm, and emission spectra was recorded from 400–600 nm. ANS concentration was kept 16 fold that of protein concentration. For ThT binding experiments, the excitation wavelength was 450 nm, and emission spectra were recorded from 470–600 nm. ThT concentration was kept 25 μM.

2.2.7 Calcium Chloride Intervention:

Calcium is an important part of cell system, so it is always present in the cytoplasm and other related cell parts. So, effect of calcium on homocysteinylation is to be studied. For this we used CD spectroscopy as discussed above. We took 4 varied concentration of calcium chloride for 5 different concentration of homocysteine thiolactone. This was done in order to study the effect of varied level of homocysteine thiolactone and calcium chloride concentration on each other. Protein concentration used for the CD measurements was 0.5 mg/ml. Cells of 0.1 and 1.0 cm path lengths were used for the measurements of the far and near-UV spectra, respectively. The protein samples were pre- incubated overnight with HTL. Necessary blanks were subtracted for each measurement. All readings were recorded at 37° C.

CHAPTER 3: RESULTS AND DISCUSSION

Results and Discussion

3.1 Results And Discussion

To investigate the effects of N-homocysteinylation on the structural and functional consequences of proteins, we have chosen myoglobin as our model protein. The choice of protein was made keeping in that myoglobin plays a critical role as oxygen carrying pigment of muscle tissues. We have extensive characterised the protein for the effects of HTL on the native structure of myoglobin and aggregation.

3.1.1 Sulfhydral estimation through Ellman's assay suggest adduct formation between myoglobin and HTL:

To modify proteins by HTL, each of the protein samples (0.06 mg/ml) were treated with different concentrations of HTL ranging from 0-1000 uM (incubated overnight at pH 7.4). Modification of protein lysine residue by HTL results in availability of one free SH group for each HTL incorporated to the protein molecule, and the resulting SH group can be easily determined via Ellman's Essay. The increase in SH group in the modified protein is regarded to be a good signature of protein covalent adduct formation by HTL. The results for the Ellman's assay are presented in Table 1. It can be seen in the table that the protein has been incorporated with HTL as suggested by large increase in the free SH contents of the modified protein. Thus, it is clear that the protein has been homocysteinylated upon overnight incubation with HTL.

Table 2: Protein free sulfhydral contents on modification by HTL

HTL (µM)	Free SH (µM/mg)
0	2.05
10	2.55
50	35.65
100	56.08
200	123.35
500	838.55
1000	2694.03

3.1.2 Intrinsic fluorescence spectroscopic study of myoglobin modified by HTL:

Our initial analysis through Ellman's assay confirmed covalent modification of myoglobin by HTL. To see if the modification brought about any structural perturbations in myoglobin, we have performed intrinsic fluorescence by measuring the Trp fluorescence of myoglobin. Figure 3 shows the Trp fluorescence spectra of native and modified myoglobin. In native state, when the Trp is buried in hydrophobic environment, fluorescence is mainly quenched by disulfide bonds in the protein and the fluorescence emission yield is low. However, any structural perturbation in the folded state of the protein which causes disruption of the tertiary contacts and the exposure of the fluorophore into more polar environment ultimately leads to an increased fluorescent behaviour of the protein with a prominent red shift. It can be seen clearly in Figure 3 that the native myoglobin has a very low fluorescence. However, modification of the protein by HTL induces global conformational changes resulting in an enhanced fluorescence as well as a very prominent red shift in the peak maxima confirming the unfolding of the protein upon modification by HTL. The result suggests that modification via N-homocysteinylation of lysine residues in myoglobin results in structural destabilization.

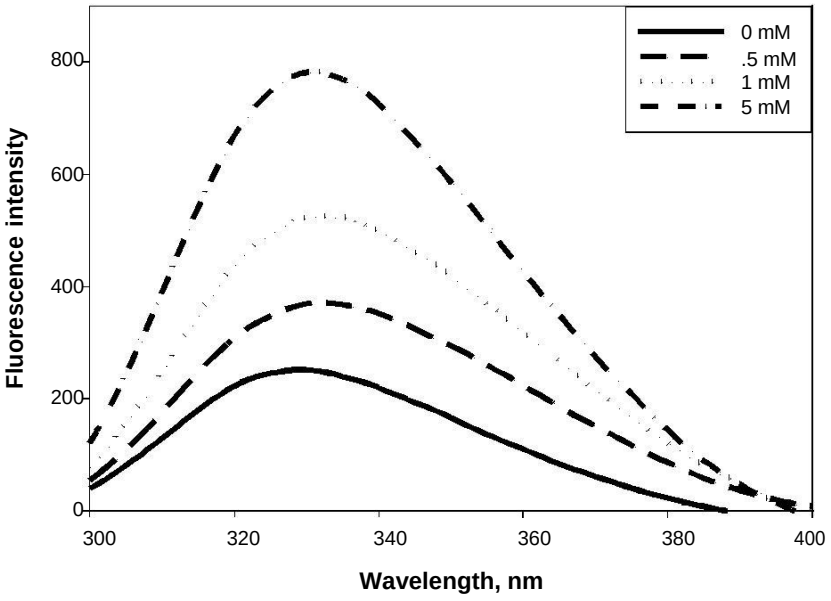


Figure 18: Intrinsic fluorescence of myoglobin modified with HTL treatment

3.1.2 Tertiary and secondary structure analyses through CD spectroscopy:

Circular dichroism is a useful technique to explore conformational changes in protein structure. We were further interested in assessing the disruption of the tertiary and the secondary structural elements caused as a result of HTL-induced protein modifications. For this purpose, we carried out circular dichroism spectropolarimetry in the near- and far- UV region. Respective CD signals were recorded and plotted against wavelength. Figure 4 shows the near- and far-UV CD spectra of native and homocysteinylated proteins. The near-UV CD spectra tell us about the gross tertiary structure of the protein. It was observed that, on being treated with HTL, the native state tertiary structural components gets destabilized as evident from the decrease in CD signal. The far-UV CD spectrum of control myoglobin shows a minimum at 208 nm and 222 nm, which is indicative of α-helical structure. We noticed that in the presence of HTL, the CD signals did not change significantly. The observation from the Near UV-CD leads us to a conclusion that on being treated with HTL, the protein's tertiary structure gets lost whereas the Far UV- CD data, hints at no loss in secondary structure which implies that while the secondary structure remains intact, it is the tertiary structure of myoglobin that is targeted by HTL.

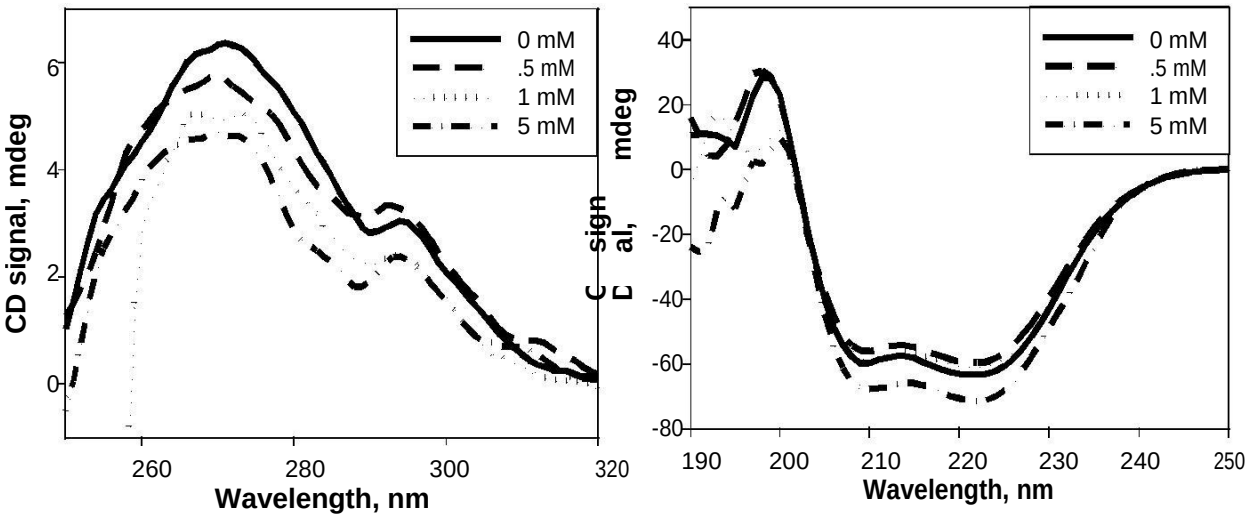


Figure 19: Near and far UV-CD spectra of myoglobin: Near (left panel) and far (right panel) UV-CD spectra of cyt c modified with HTL

3.1.3 N-homocysteinylation induces the activation of peroxidase-like activity in myoglobin:

To further confirm the destabilization of myoglobin induced by N-homocysteinylation, we performed peroxidase activity assay. Peroxidase activity assay serves as a very sensitive analysis for studying structural perturbations and unfoding in several heme binding protein, including myoglobin. Indeed the folded native state of myoglobin does not show any peroxidase activity. However, unfolding and exposure of the heme to solvent environment is known to induce the peroxidase activity. In an attempt to check whether N-homocysteinylation could induce such peroxidase function in myoglobin, we have performed peroxidase activity assay for both unmodified and modified myoglobin. Figure 5 shows the peroxidase activity of myoglobin induced upon modification via N-homocysteinylation. In the figure we can clearly see that the native folded myoglobin does not show any peroxidase activity. However, modification of myoglobin by HTL induced peroxidase function indicating structural destabization and exposition of the heme moiety to solvent environment. The heme iron of myoglobin is always hexacoordinate except for the reduced, deoxygenated derivative, and it is the pentacoordinate form which can behave as peroxidases. Hence the induction of peroxidase function upon modification by HTL can be attributed to the fact that the protein has undergone structural destabilization leading to disruption of certain ligation in the heme moiety, thus converting the protein into a pentacoordinate form.

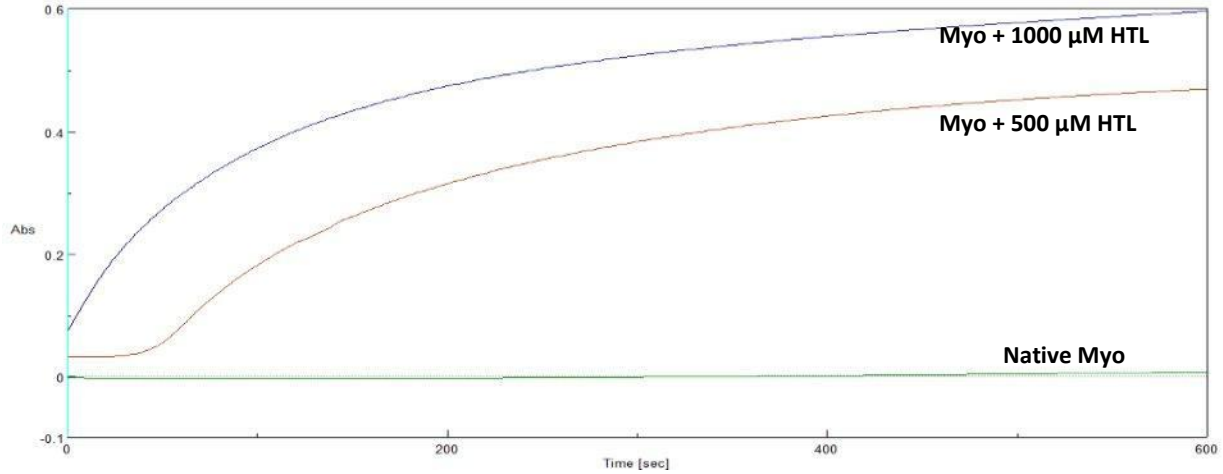


Figure 20: Induction of peroxidase-like activity of myoglobin upon modification by HTL

3.1.4 ANS binding study:

To confirm whether the tertiary structural perturbations induced by HTL lead to exposure of hydrophobic patches of the protein, ANS binding essay was performed. ANS is an extrinsic fluorophore which binds to exposed hydrophobic clusters. However, the dye does not bind to native globular proteins. Results for ANS binding experiments are presented in Figure 6. Unbound ANS gives a peak maximum at 520 nm upon excitation at 360 nm. On binding to exposed hydrophobic clusters, there is a significant blue shift with a peak maximum at 475 nm and enhanced fluorescence. Thus, we can be seen in the figure that after 24 hours of incubation with HTL, there is an increase in surface hydrophobicity of myoglobin as suggested by the blue shift and enhanced fluorescence. Also as HTL concentration increases in the environment, the efficiency of ANS binding to myoglobin increases which is accompanied by a concomitant blue shift in maximum emission is significantly increased. The enhanced fluorescence and the blue shift in HTL treated samples results from the increase in solvent exposed hydrobhobic regions of the protein. Such non-native species with exposed hydrobhobic regions are known to have a high tendency of self association and aggregation.

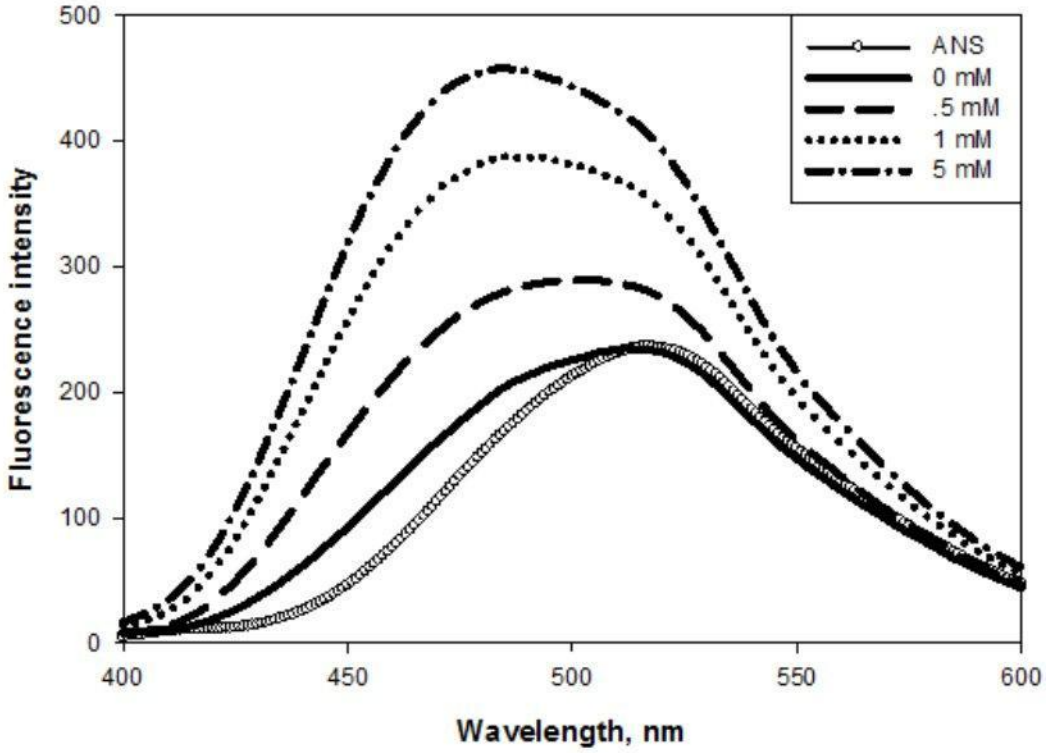


Figure 21: ANS fluorescence spectra of native and N-homocysteinylated myoglobin.

3.1.5 ThT binding study:

ThT is the dye which specifically binds to the beta-sheet (amyloid). Hence ThT binding assay is a confirmatory test for formation of aggregates/amyloids. It was observed that there was no binding of ThT with native, thus showed negligible fluorescence behavior, overlapping with that of ThT alone. In case HTL treated protein there was enhanced fluorescence intensity with a peak at 485 nm suggesting binding of ThT to the HTL-modified myoglobin. However, the fluorescence was not much pronound (only two fold-increases at 5 mM HTL concentration). Thus it might be possible that the aggregate species generated might not be amyloidogenic in nature, but some disordered amorphous aggregates.

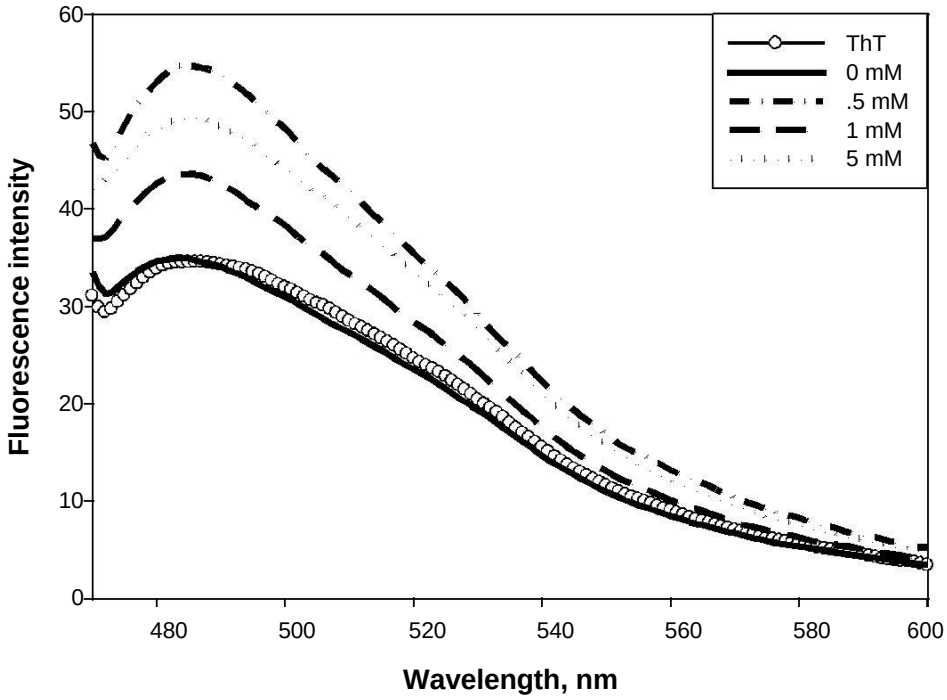
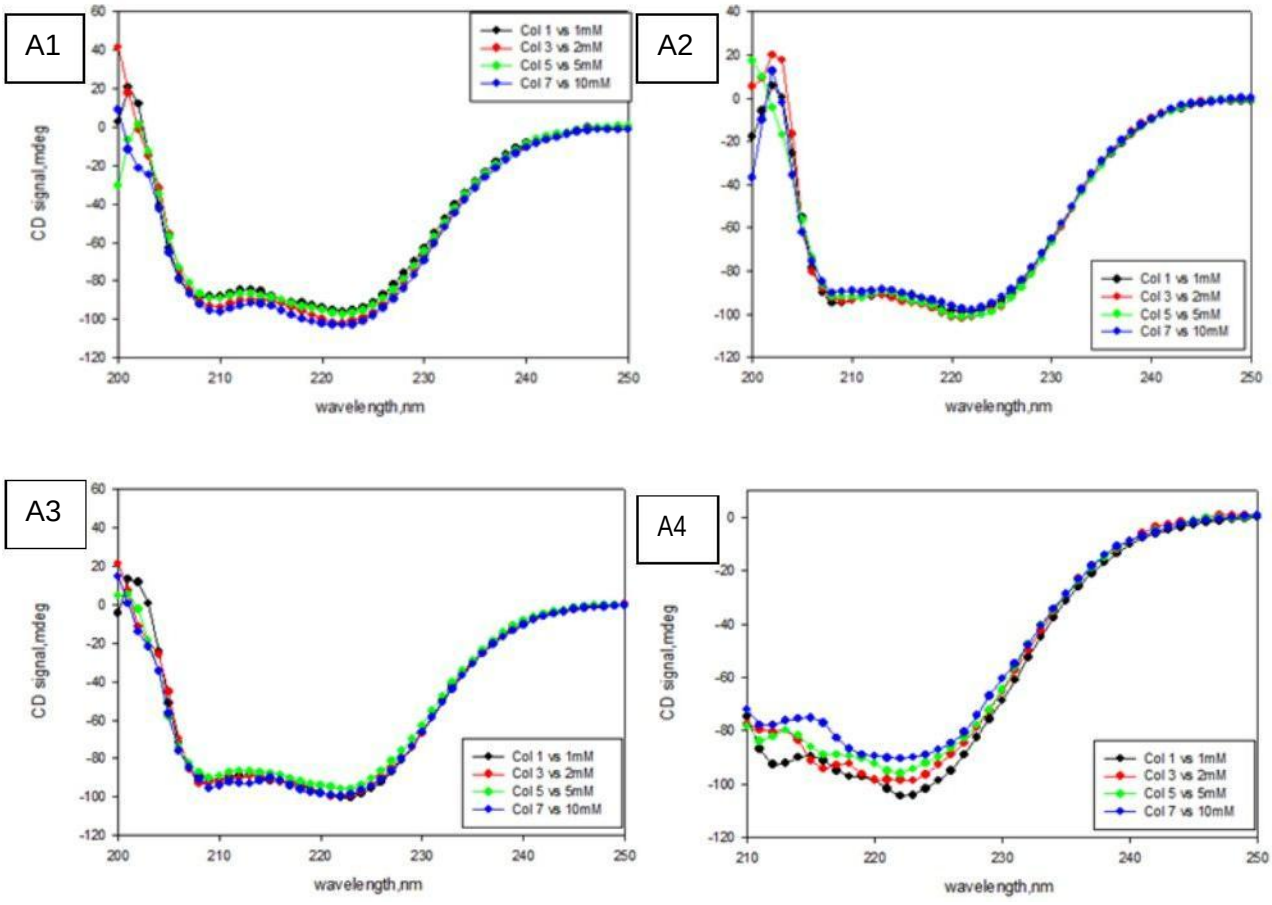


Figure 22: ThT fluorescence spectra of native and N-homocysteinylated myoglobin.

3.1.7 Tertiary and secondary structure analyses of Calcium intervention through CD spectroscopy-

Initially we performed CD for assessing the disruption of the tertiary and the secondary protein structure that was supposed to be caused as a result of HTL-induced protein modifications. But the previous study was independent of any factor or determinant that is present in natural system. So, further studies were done to assess the role or intervention of calcium ion in close proximity to homocysteine, as it is present in the cell. For this we went for a CD spectroscopy of various samples with varied concentration of both homocysteine thiolactone and calcium chloride. We obtained very convincing graphs for both far and near UV- CD. Respective CD signals were recorded and plotted against wavelength. Figure shows the near- and far-UV CD spectra of native and homocysteinylated proteins in presence of calcium chloride. The near-UV CD spectra tell us about the gross tertiary structure of the protein.



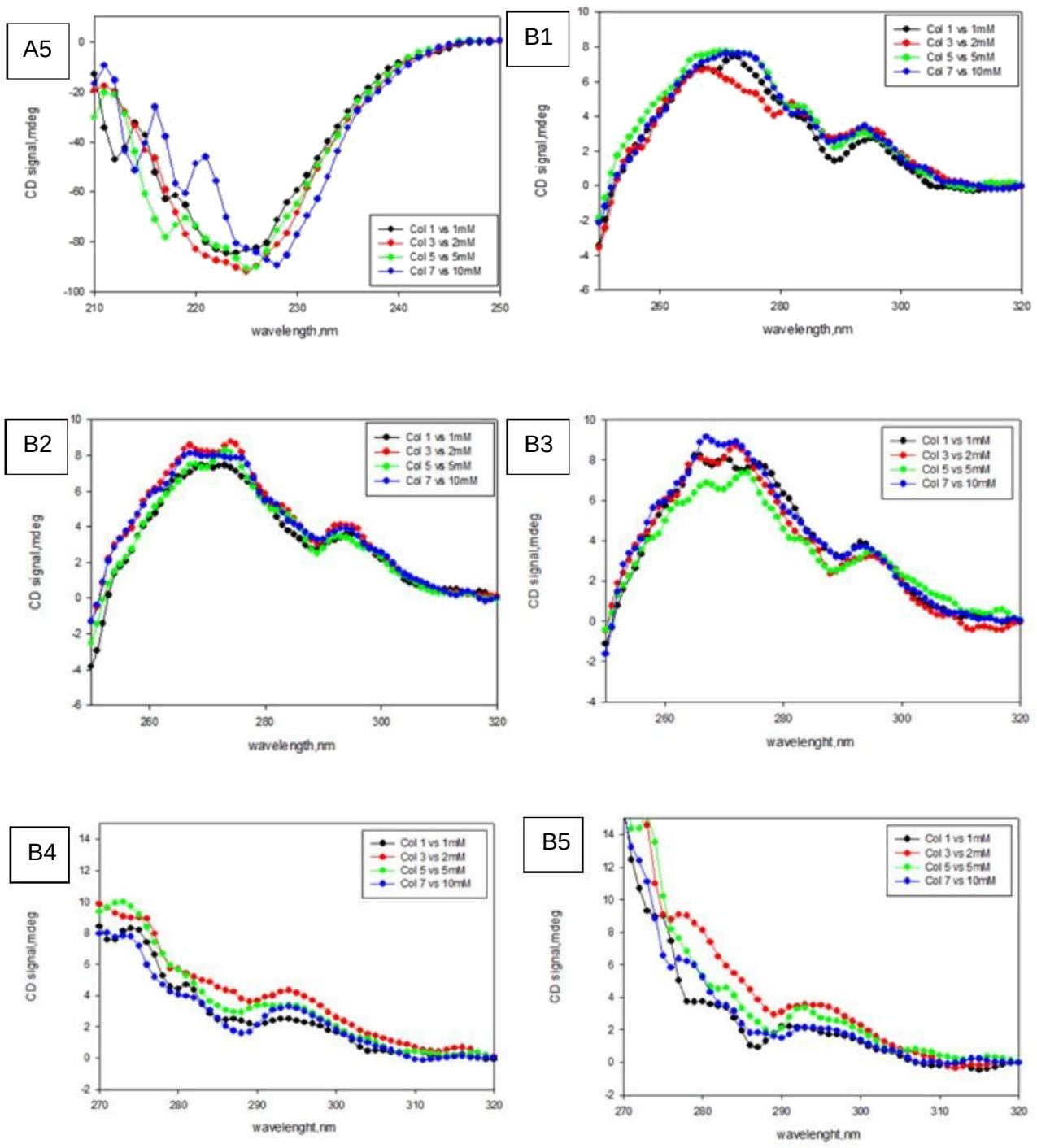
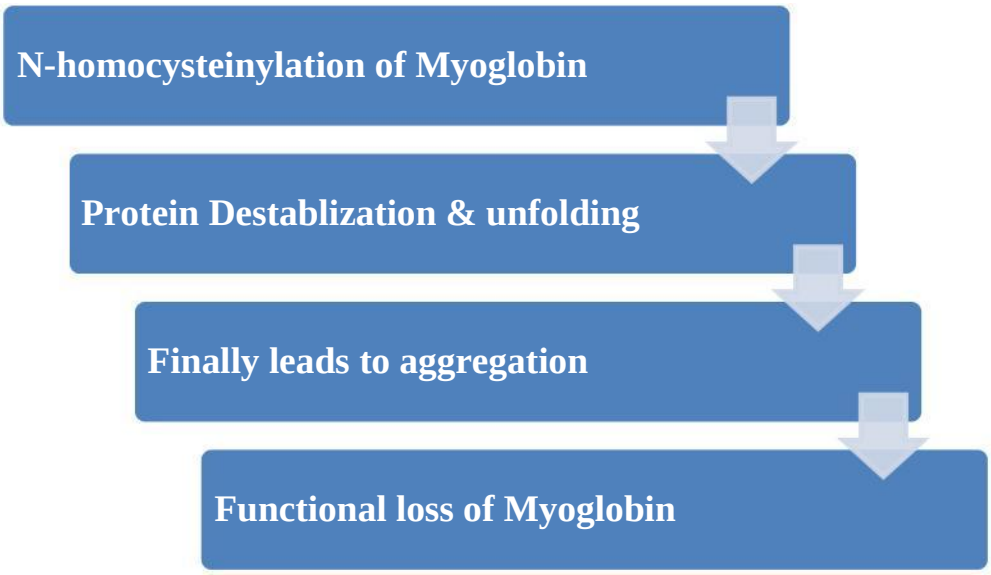


Figure23: Near and far UV-CD spectra of myoglobin in presence of calcium chloride: A1-A5 shows Far-UV CD and B1- B5 shows Near-UV CD with increasing concentration of homocysteine thiolactone for varied concentration of calcium chloride.

Destabilization in the native tertiary and secondary state structural components were observed with the increasing concentration of calcium chloride and homocysteine thiolactone. This may be because of the increase in overall charge, which may have lead protein to disrupt more quickly and lose its functionality. We observed peak loss and peak shift at higher concentration in both Far-UV and Near- UV. This gives clear indication about the role played by calcium chloride in homocysteinylation process. This proves that calcium chloride presence is surely linked to homocysteine action, may be not directly. Targeted protein may lose its function in even comparatively less concentration of homocysteine thiolactone in presence of Calcium chloride as it adds on to the charge which finally leads to loss of protein structure and its function.

3.2 CONCLUSION

As we have taken Myoglobin for all considerations and experiments, so result and observation lead to a conclusion which is as follows-



The predominant structural transitions of myoglobin in the presence of increasing concentration of HTL which finally leads to the protein aggregation have been monitored by different spectroscopic techniques. Prior to onset of the protein aggregation, HTL induces significant changes in the tertiary structures of myoglobin. As concluded by ThT binding there was some aggregation of protein but no amyloid formation. The study provides useful insights to what could be the scenario in cellular environment in hyperhomocysteinemic condition where the cellular and serum Hcy levels are highly elevated. N-homcysteinylation of myoglobin would render the protein non-functional protein which could lead to other associated muscles complications.

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