Chapter 1

INTRODUCTION
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Zinc is a component of many proteins and, it is involved in all aspects of metabolism. Advances in the isolation and characterization of enzymes and analysis of metals were basic to the rapid growth of the field of metalloproteins. The estimation of zinc in nanograms of proteins is now possible. Metallothionein (MT) was discovered contemporaneously with the first of zinc metalloenzymes (Margoshes and Vallee, 1957) but the lack of a readily measurable biological activity coupled with the need for sensitive methods for metal analysis stifled interest in the protein. Its existence was virtually unnoticed for a very long time and those few who were drawn by its siren call are to be commended for their vision and steadfastness. Two features of metallothionein were sufficient to sustain interest in it as a chemical and biological curiosity during the early years. One was its highly unusual amino acids composition-no aromatic or heterocyclic amino acids, while one third of its residues are cysteines-and the other was its extraordinary metal content, 7g atoms of metal per mole. Most of this metal seemed to be Cd^{2+} only added to the attraction. Moreover, it was a small protein of just 60 or so amino acids readily amenable to structural analysis. Another characteristic that eventually captured the attention of molecular geneticists is the inducibility of MT in response to heavy metals in vivo and to a great variety of metabolites such as glucocorticoids, glucagons, interleukin 1, catecholamines, progesterone,
estrogen, ethanol and interferon, thus making it a convenient target for studying the regulation of gene expression.

Although metallothionein was first isolated from equine kidney cortex, it has been found throughout the animal kingdom. Indeed, plants and fungi contain cadmium (II) thiolate oligopeptides, which are non-translationally biosynthesized, or metal-binding polypeptides only distantly related to equine renal metallothionein. Among the functions considered early are detoxification of cadmium and other heavy metals, regulation of $\text{Zn}^{2+}$ and $\text{Cu}^{2+}$ metabolism, and providing $\text{Zn}^{2+}$ for newly synthesized apoenzymes.

**Definition and Nomenclature of Metallothioneins**

Recommendations concerning the nomenclature of metallothionein were adopted by the Committee on the Nomenclature of Metallothionein appointed at the General Discussion Session of the Second International Meeting on Metallothionein and Other Low-Molecular Weight Metal-Binding Proteins in 1985. They supersede the original recommendations made by the plenum of the First International Meeting on Metallothionein and Other Low-Molecular-Weight Metal-Binding Proteins in 1978.

Historically, the term Metallothionein was introduced to designate the Cadmium, Zinc and Copper containing, Sulfur-rich protein from equine renal cortex. This protein was characterized as follows.
1. Low-molecular-weight
2. High metal content.
3. Characteristic amino acid composition (high cysteine content, no aromatic amino acid nor histidine)
4. Unique amino acid sequence (characteristic distribution of cysteiny] residues such as C-X-C)
5. Spectroscopic features characteristic of tetrahedral metal-thiolate (-mercaptide) complexes.

Prompted by the conspicuousness of these features, the designation “metallothionein” is now used as a generic term for a variety of similar metal-thiolate polypeptides. Accordingly, the following definition has been adopted. Polypeptides resembling equine renal metallothionein in several of their features can be designated as “metallothionein”.

On the basis of structural characteristics, the “metallothioneins” are subdivided into classes.

Class-I: Polypeptides with locations of cysteine closely related to those in equine renal metallothionein.

Class-II: Polypeptides with locations of cysteine only distantly related to those in equine renal metallothionein, such as yeast metallothionein.
Class-III: Atypical, nontranslationally synthesized metal-thiolate polypeptides, such as cadystin, phytometallothionein, phytochelatin, homophytochelatin etc.

The metal-free form may be designated either as apometallothionein or thionein. More specific terms, such as cadmium metallothionein or zinc metallothionein are appropriate for metallothioneins that contain only one metal. The metal prefixed to the word "thionein" should not be used to designate the metal employed for metallothionein induction. The molar metal content can be specified by a subscript, i.e.; Cd$_7$-metallothionein or Zn$_7$-metallothionein. When the metallothionein contains more than one metal, for eg:- 5-3 mol cadmium and 1-7 mol zinc/mol of PC, terms such as Cd, Zn-metallothionein, Cd, Zn-thionein, Cd$_{5,3}$, Zn$_{1,7}$-metallothionein and Cd$_{5,3}$, Zn$_{1,7}$-thionein are recommended. In contexts where specification of the metal composition is not available or is of no interest, the term metallothionein should be used.

Mammalian metallothionein are composed of approximately 61 amino acids with molecular weight of 6000-7000Da. They contain no aromatic amino acids and 20 Cys residues that co-ordinate seven divalent metal ions or 12 monovalent ions such as Cu$^+$ (Nielson and Winge, 1984) in two distinct metal clusters. The locations of the Cys residues in mammalian metallothioneins are invariant and proteins from any phyla that have similar primary structures are designated class I metallothioneins.
Class II metallothioneins are low-molecular weight Cys-rich metal binding proteins, but the distribution of Cys residues does not correspond to that in mammalian metallothioneins. These proteins have been identified in cyanobacteria, yeast, nematode Caenorhabditis elegans and a higher plant, wheat germ E₃ protein.

In 1985, it was reported that the major Cd²⁺ ligands in Cd²⁺-intoxicated plant cells are composed of poly (γ-glutamyl-cysteinyl) glycine (Grill, 1985; Grill et al., 1985; Bernhard and Kagi, 1985; Robinson et al., 1985). These polypeptides and other γ-glutamyl isopeptides in which glycine is either absent or substituted with β-alanine, are designated as class III metallothioneins (Kojima, 1991). These compounds were first identified and characterized in the fission yeast Schizosaccharomyces pombe and termed cadystins (Murasugi et al., 1981; Kondo et al., 1984). Similar polypeptides are subsequently purified from plant cell cultures and termed phytochelatins (PC) (Grill et al., 1985). From this it is clear that class III metallothioneins differ markedly from class I and II metallothioneins. They are enzymatically derived and are most commonly composed of poly (γ-glutamyl-cysteinyl) glycine, (γ-EC)ₙG where n=2-11 depending on the organism, although the most common forms have n=2-4 (Grill et al., 1986 a). However, class III metallothioneins isolated from the Fabaceae contains β-alanine in the C-terminal position and these species produce predominantly homoglutathione
(γ-glutamylcysteinyl-β-alanine) rather than glutathione (γ-glutamylcysteinyl glycine) (Grill et al., 1986 b).

Metal complexes containing these γ-glutamyl isopeptides have apparent native molecular weight ranging from 2,000-10,000Da depending upon the source and method of isolation and include multiple polypeptides in cluster (Steffens, 1990; Rauser, 1990). Sulphide is sometimes present in Cd²⁺ complexes in varying amounts but it has not been found in copper complexes (Steffens, 1990; Rauser, 1990). High molecular weight sulphide-containing complexes show enhanced affinity for metals (Reese and Winge, 1988). The structure of such complexes is of interest, being composed of a CdS quantum semiconductor crystallite core surrounded by polypeptides (Dameron et al., 1989). Sulphide containing complexes have been described in Candida glabrata (Mehra et al., 1988), tomato (Reese et al., 1992) & a selenium – tolerant wild mustard (Brassica juncea) (Speiser et al., 1992 a). Metal-tolerant Silene vulgaris (Verkleij et al., 1990) and cell cultures of Datura innoxia (Robinson et al., 1990) incorporate greater amounts of S²⁻ into these complexes than their less tolerant counterparts.

**Biosynthesis**

Structural similarities between glutathione (GSH) and class III metallothioneins suggest that the latter are synthesized from the former or its precursors. In vivo experiments demonstrate a significant reduction of
free glutathione upon exposure of plant cell cultures to Cd$^{2+}$ (Rauser, 1990; Steffens, 1990). Pulse-chase experiments, where the cellular glutathione pool is tagged with $^{35}$S, show loss of radioactivity from glutathione with a concomitant increase in radiolabelled class III metallothioneins (Berger et al., 1989). Treatment of cell cultures with buthionine sulfoxamine, a potent inhibitor of γ-glutamylcysteine synthetase, results in the loss of metal tolerance and an inability to synthesize class III metallothioneins (Grill et al., 1987). In addition, mutants of the fission yeast deficient in enzymes of glutathione synthesis are unable to produce class III metallothioneins and are hypersensitive to Cd$^{2+}$ (Mutoh and Hayashi, 1988).

There are several alternate pathways that might produce class III metallothioneins from glutathione or γ-glutamylcysteine. It has been reported that class III metallothioneins are synthesized from glutathione by the enzyme γ-glutamylcysteine dipeptidyl transpeptidase (phytochelatin synthese) in *Silene cucubalus* cell suspension culture (Grill et al., 1989). The molecular weight of the native protein was reported to be 95,000Da; the protein is composed of four subunits, each with a molecular weight of approximately 25,000Da. Enzyme activity is dependent upon the presence of metal ions. Addition of EDTA or apopeptides to reaction mixtures terminate the synthesis (Loeffler et al., 1989). The mechanism of
biosynthesis requires two glutathione molecules or one glutathione and a previously synthesized phytochelatin molecule. The transfer of the \(\gamma\)-glutamyl cysteine moiety of glutathione to another glutathione or to a previously synthesized phytochelatin does not require ATP.

\[
\gamma\text{-ECG} + (\gamma\text{-EC})_n - G \rightarrow (\gamma\text{-EC})_{n+1} - G + G
\]

This reaction is strictly dependent on the presence of heavy metal ions. The formation of the stable metal phytochelatin (PC) complex certainly shifts the reaction in the forward direction and thus prevents the release of toxic metals. The enzyme is self regulated in that the reaction product, the PC, chelate the PC synthase-activating metal and thus terminate the enzymatic reaction. Addition of metal, metal complexing amount of metal-free PC\(_2\) or PC\(_7\) in the presence of Cd\(^{2+}\) immediately stops the ongoing chain elongation reaction. (Loeffler et al., 1989). There are several reports of rapid synthesis of class III metallothioneins being insensitive to inhibitors of de novo protein synthesis in plant cell culture exposed to Cd\(^{2+}\) (Scheller et al., 1987; Robinson et al., 1988) indicating that the enzymes responsible for the synthesis of phytochelatins and their precursors are constitutive in cells in the absence of excess metal ions. Furthermore, enzyme activity was detected in cell-free extracts from cultures not exposed to elevated metal ion concentrations (Grill et al., 1989). The enzyme was activated by the following metal ions. The order of
efficiency is as follows: \( \text{Cd}^{2+} > \text{Ag}^+ > \text{Pb}^{2+} > \text{Cu}^{2+} > \text{Hg}^{2+} > \text{Zn}^{2+} > \text{Sn}^{2+} > \text{Au}^{3+} > \text{As}^{4+} > \text{In}^{3+} > \text{TI}^{3+} > \text{Ge}^{2+} > \text{Bi}^{3+} > \text{Ga}^{3+} \). Thirty-nine other elements of the periodic table did not activate the enzyme.

**Localization**

There are convincing proofs that the vacuole of the plant cell is the major site for transient accumulation of Cd-PC complexes. The location of these polypeptides was determined following isolation of protoplasts and vacuoles from leaves of \( \text{Cd}^{2+} \)-exposed seedlings. Both phytochelatins and \( \text{Cd}^{2+} \) were found in the vacuoles proving that vacuole is the ultimate storage site for heavy metals and most likely initially at least in the form of a PC complex (Vögeli – Lange and Wagner, 1990). Ortiz et al. (1992) indicated that there is a specific transporter, designated HMT 1, required for the accumulation of high molecular weight \( \text{CdS} \) complexes in the vacuole of yeast \( S. \text{pombe} \) cell. A \( \text{Cd}^{2+} \) sensitive mutant of \( S. \text{pombe} \), designated \( \text{LK} \ 100 \), was isolated that accumulated less of these complexes than the wild type. \( \text{LK100} \) cells transformed with hmt1 cDNA suggest that its product is similar to ATP-binding cassette (ABC)-type membrane transport proteins. The transport may be energized by the hydrolysis of ATP. Sub cellular fractionation of extracts from \( S. \text{pombe} \) containing an hmt1-lac2 fusion indicated that the encoded fusion protein is localized in the vacuolar membrane. It is not clear that which of the components of the
complex (Cd$^{2+}$, S$^2$ polypeptides or their precursors) is transported into the vesicle.

Structure

The PC molecules form complexes with heavy metals of atomic weight in range 2.5 to 3.6 KDa. These compounds cannot be crystallized and their NMR spectra are extremely complex. Extended X-ray absorption fine structure (EXAFS) is used to find their primary structure (Strasdeit et al., 1991). The observed complex shows Cd-thiolate co-ordination with a Cd-S bond length of $2.52 \pm 0.02\text{Å}$. The EXAFS measurement excluded the participation of the carboxylate groups in the complexion. These carboxylate groups of the PC molecules are directed towards the outside of
the complex. This explains the extreme hydrophilic surfaces and the polyanionic character with its extreme water solubility. A structural model for the complex \([\text{Cd}_3 (\text{PC}_3)_4]\) is:

\[
\begin{array}{c}
\text{S} \quad \text{S} \quad \text{S} \\
\text{Cd} \\
\text{S} \quad \text{S} \quad \text{S}
\end{array}
\]

**Importance of *Eichhornia crassipes* in Metal Detoxification**

The water hyacinth (*Eichhornia crassipes*) is perhaps one of the most commonly cited species for phytoremediation of polluted waters (Gupta, 1980; McDonald and Wolverton, 1980). The plant has a rapid growth rate and can hyperaccumulate nutrients (Cornwell et al., 1977) as well as heavy metals (Wolverton, 1975; Wolverton et al., 1975).

The water hyacinth has a number of problems that tend to hinder its commercial use. The first of these is that in many countries it is a noxious weed that has choked large areas of waterways. For e.g. the backwaters and other waterways of Kerala are rendered difficult for transport and other uses by thickly free-floating water plants.
As pointed out by Kay et al. (1984), much of the research on the water hyacinth has been faulty insofar as estimates of biomass production have been carried out in the field in unpolluted waters, whereas uptake experiments are usually carried out in the laboratory where there is concomitant, but unmeasured, reduction in biomass.

Little is known about the mechanism of phytoremediation by the plant. Our preliminary aim was to check the hyperaccumulation of heavy metals by the plant. Because it reveals a positive result, purification of the metal-binding complexes were done leading to HPLC analysis and immobilization studies.

**Summary of the aims:-**

1. **Biochemical Investigations:** to check the ability of the plant *Eichhornia crassipes*, with a free floating root system, in taking up of heavy metals, including synthesis of PCs.

2. **Purification of PCs:** PCs from heavy metal stress Cd$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$.

3. **Immobilisation:** The metallothionein immobilized to check a metal ion substitution and to suggest application purpose.

4. **HPLC:** HPLC analyses of the peptides, splitting into apothioneins in presence of trifluoroacetic acid (TFA).

5. **In vitro Studies:** PC synthase purification and in vitro syntheses of PCs were done.