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Algal metallothioneins: secondary metabolites and proteins

Nigel J. Robinson
Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, U.K.

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Abstract

Metallothioneins, MT's, are low-molecular-weight, cysteine-rich, polypeptides that complex 'soft' metal ions in thiol clusters. They are structurally diverse. Some MT's are gene products, while others are secondary metabolites. Two of the three classes of MT have been identified in algae. Eukaryotic algae possess the secondary metabolites referred to as class III MT. There is no unequivocal evidence that MT genes occur in eukaryotic algae. However, the products of MT genes have been identified in cyanobacteria. These genes and their metal regulatory elements remain to be isolated and characterized.

MT's have attracted interest from researchers involved in a wide range of disciplines including bioinorganic chemistry, biochemistry, molecular biology, physiology, toxicology, environmental science and medicine. Although, the precise physiological roles of these polypeptides remain undefined, a large number of functions have been speculated. These molecules chelate toxic trace metals, such as Cd, thereby reducing the concentration of cytotoxic, free-metal ions. Furthermore, some MT's are believed to be involved in zinc and copper homoeostasis. Future studies should reveal whether or not some of the diversity of MT structure reflects a diversity of function.

Abbreviations: $(gammaEC)_nG$ = poly(gamma-glutamylcysteinyl)glycine; MT = metallothionein; BSO = buthionine sulphoximine; mre = metal regulatory element; GSH = glutathione; GSSG = oxidized diglutathione; APS = adenosine 5′ phosphosulphate; PAPS = adenosine 3′ phosphate 5′ phosphosulphate.

Introduction

Contamination of rivers and estuaries with Pb, Hg, Cd, Zn and Cu has been documented in numerous surveys (Forstner, 1983). The toxic effects of elevated concentrations of these trace metals on aquatic organisms, including algae, are also the subjects of an extensive literature (Whitton & Say, 1975; Vymazal, 1987). Algae have been used as biological indicators to monitor toxic trace metal pollution in aquatic environments (Phillips, 1977; Whitton, 1984) and in a limited number of cases they have been applied to the purification of water contaminated with metals (Kessler, 1986). Cyanobacteria have been used to mobilize U from low grade ores in laboratory studies and this has encouraged speculation that cyanobacteria may be used in processes designed to recover valuable metals (Lorenz & Krumbein, 1985).

In some environments contaminated with toxic trace metals, metal-tolerant algae have been selected (Stokes *et al.*, 1977; Bariaud & Mestre, 1984). A variety of tolerance mechanisms have

been described. In some species that accumulate metals, metal-binding polypeptides of apparent native molecular weights of less than 14 kDa have been identified (Stokes *et al.*, 1977; Hart & Bertram, 1980; Nagano *et al.*, 1984; Gingrich *et al.*, 1986). These compounds have been compared to mammalian metallothioneins, MT's, although only very recently have any of these molecules been subject to stringent structural characterization (Gekeler *et al.*, 1988; Olafson *et al.*, 1988; Shaw *et al.*, 1989). The structure, biosynthesis and possible functions of these molecules are discussed.

Metallothionein: A note about nomenclature

Readers who are not familiar with the literature concerning MT's could find the nomenclature confusing. Different names have been used to describe the same compound and, in some cases, the same name to describe fundamentally different compounds.

The name 'metallothionein' was first given to the Cd-, Zn- and Cu-containing, S-rich protein from equine renal cortex (Kagi & Vallee, 1960). However, the recent recommendations of the committee on the nomenclature of MT's extend the definition of these compounds to include the metal-binding polypeptides which have been reported to occur in seven of the ten classes of eukaryotic algae (Weber *et al.*, 1987; Gekeler *et al.*, 1988; Shaw *et al.*, 1989). A common feature of all MT's is an abundance of Cys-Xaa-Cys sequences, where Xaa is an amino acid other than cysteine (Kagi & Kojima, 1987). These sequences are involved in binding metal ions in metal thiolate clusters (Kagi & Kojima, 1987). Another common feature of all MT's is that their synthesis increases in organisms exposed to elevated concentrations of certain trace metal ions.

Three classes of MT have been defined (Fowler *et al.*, 1987). Class I and II MT's are proteins encoded by structural genes. MT genes have been sequenced from a number of organisms including *Echinoidea* sp. (Nemer *et al.*, 1985), *Drosophila melanogaster* (Maroni *et al.*, 1986; Mokdad *et al.*, 1987), *Saccharomyces cerevisiae* (Butt *et al.*, 1984a; Butt *et al.*, 1984b), *Neurospora crassa* (Munger *et al.*, 1987), several mammals including man (Karin & Richards, 1982), but MT genes have not yet been isolated from algae. In contrast, class III MT's are secondary metabolites and hence are not directly encoded by structural genes. Class III MT's have been given the alternative names cadystin (Murasugi *et al.*, 1981; Kondo *et al.*, 1985), phytochelatin (Grill *et al.*, 1985), gamma-glutamyl metal-binding peptide (Reese *et al.*, 1988), phytometallothionein (Rauser 1987a) and poly(gamma-glutamyl-cysteinyl)glycine (Robinson & Jackson, 1986; Robinson *et al.*, 1988). Therefore, MT is a broad term covering a wide range of phenotypically related metal-thiolate polypeptides. The three classes of MT are defined:

Class I: proteins with locations of cysteine closely resembling those of equine renal MT(not identified in algae);

Class II: proteins with locations of cysteine only distantly related to those in equine renal MT (identified in the cyanobacterium *Synechococcus* TX-20; Olafson *et al.*, 1988);

Class III: non-translationally synthesized metal-thiolate polypeptides (identified in representatives of Euglenophyta, Chrysophyta, Bacillariophyta, Xanthophyta, Chlorophyta, Phaeophyta, Rhodophyta; Gekeler *et al.*, 1988; Shaw *et al.*, 1988).

A critical study of the literature suggests that many Cu- and Cd-binding compounds, which were isolated from algae and thought to be products of MT genes, are polypeptides which may be categorised as class III MT's. Some of these contained impurities which were incorrectly interpreted as being constituents of the polypeptide chain.

Class III Metallothionein; Poly(gamma-glutamylcysteinyl)glycine, cadystin, phytochelatin

Structure

Cd-binding polypeptides which form aggregates in the presence of Cd were first identified in

extracts from the fission yeast *Schizosaccharomyces pombe* and called cadystins (Murasugi *et al.*, 1981; Kondo *et al.*, 1985). These polypeptides are composed of the repeating dipeptide unit gamma-glutamylcysteine with a single carboxy terminal glycine residue. Using single letter abbreviations for these amino acids, the structure can be represented as $(gammaEC)_nG$. Similar polypeptides were subsequently isolated from higher plants (Bernhard & Kagi, 1985; Grill *et al.*, 1985; Robinson *et al.*, 1985) and most recently from eukaryotic algae (Gekeler *et al.*, 1988; Shaw *et al.*, 1989).

Amino acid analysis of isolated Cd-binding polypeptides from *Chlorella fusca* (Gekeler *et al.*, 1988) and *Euglena gracilis* (Shaw *et al.*, 1989) indicate that both of these algae produce $(gammaEC)_nG$. Furthermore, the Cd-complexes in these two species were demonstrated to contain inorganic sulphide, S^{2-}, (Weber *et al.*, 1987; Gekeler *et al.*, 1988; Shaw *et al.*, 1989), a characteristic of some Cd-$(gammaEC)_nG$ complexes (Murasugi *et al.*, 1983; Reese *et al.*, 1988). The polypeptides isolated from *C. fusca* were sequenced by alternating treatment with gamma-glutamyl transpeptidase and a modified Edman degradation process (Gekeler *et al.*, 1988). These polypeptides were also subjected to partial hydrolysis after performic acid oxidation and dinitrophenylation. Separation of the reaction products by two-dimensional TLC yielded patterns characteristic of $(gammaEC)_nG$. The presence of $(gammaEC)_nG$ in nine other species of eukaryotic algae has been inferred from characteristic reverse phase HPLC profiles of deproteinated extracts from Cd-exposed cells (Gekeler *et al.*, 1988). However, it remains to be demonstrated that this assay will resolve $(gammaEC)_nG$ and Cd-induced class II MT of the type found in *Synechococcus* TX-20. Class II MT of *Synechococcus* TX-20 is the most hydrophobic MT to be described and at neutral pH was resolved into seven isoforms eluting from reverse phase HPLC columns between 10 and 20% (w/v) acetonitrile (Olafson *et al.*, 1988).

Metal-binding sites

To date, published reports show only Cd- and Cu-binding to $(gammaEC)_nG$ *in vivo*, although there is indirect evidence that Hg and Ag also bind (Grill *et al.*, 1987; Wagner 1984). Loss of absorbance in the region of 250 nm at low pH, corresponding to the breaking of Cd-mercaptide bonds, has been reported for a number of isolated Cd-$(gammaEC)_nG$ complexes (Weber *et al.*, 1987; Hayashi *et al.*, 1988; Reese *et al.*, 1988). The pH at which 50% of the metal is displaced provides an estimate of the affinity of $(gammaEC)_nG$ for metals (Table 1). Polypeptides with higher 'n' values bind Cd more firmly than do smaller forms. If pH stability of binding of Zn relative to Cd for $(gammaEC)_nG$ was similar to

Table 1. The pH at which 50% of metal ions are dissociated from $(gammaEC)_nG$ complexes.

	pH of half dissociation
Euglena gracilis $(gammaEC)_nG$-S^{2-}-Cd[1]	5.7
Schizosaccharomyces pombe $(gammaEC)_nG$-Cd[2]	5.4
Schizosaccharomyces pombe $(gammaEC)_nG$-S^{2-}-Cd[2]	4.0
Schizosaccharomyces pombe $(gammaEC)_nG$-Cu[2]	1.3
Synthetic $(gammaEC)_2G$-Cd[3]	5.2
Synthetic $(gammaEC)_3G$-Cd[3]	4.8
Immobilized $(gammaEC)_nG$-Cd[4]	4.9
Nicotiana tabacum Cd-complex[5]	5.0[6]
Nicotiana tabacum Cd-complex[5]	5.4[7]
Nicotiana tabacum Cd-complex[5]	5.7[8]

[1] Weber *et al.*, 1987; [2] Reese *et al.*, 1988; [3] Hayashi *et al.*, 1988; [4] unpublished observations; [5] Reese & Wagner, 1987a; [6] plants exposed to 90 μM Cd; [7] cultured cells; [8] plants exposed to 3 μM Cd.

that observed for class I MT, then Zn binding to (gammaEC)$_n$G at physiological pH would be very weak (Reese & Wagner, 1987a). Such a weak association would be of little value in Zn sequestration or homoeostasis. Furthermore, Hg and Ag, but not Zn, affected absorbance at 250 nm of solutions containing Cd-(gammaEC)$_n$G (Wagner, 1984). This also implies inability of these polypeptides to coordinate Zn, but suggests ability to bind Hg and Ag. Isolated (gammaEC)$_n$G from *E. gracilis* contained relatively little Zn, even when the algae had been grown in media containing high Zn^{2+} concentrations (Gingrich *et al.*, 1986). Cd-(gammaEC)$_n$G can be immobilized on cyanogen bromide activated Sepharose and cycled through metal-binding and release (unpublished results). The metal-binding characteristics, K_d and Q_{max}, for a range of metals may be determined under these conditions.

Metal ions are coordinated to a cluster containing several (gammaEC)$_n$G molecules in a metal-(gammaEC)$_n$G aggregate (Reese *et al.*, 1988). Two different forms of Cd-(gammaEC)$_n$G aggregate are produced. One form contains acid labile S in the cluster. The S is present as reduced sulphide, S^{2-} (Murasugi *et al.*, 1983). Aggregates containing S^{2-} have both higher affinity and capacity for Cd (Reese *et al.*, 1988). However, higher average 'n' values have also been detected in the (gammaEC)$_n$G present in S^{2-} containing aggregates. Evidence that increased Cd-capacity of S^{2-} containing aggregates is due, at least in part, to the presence of S^{2-} and not exclusively due to the apparent increase in 'n' values, was obtained with isolated Cd-(gammaEC)$_n$G from *E. gracilis* (Shaw *et al.*, 1988). Removal of S^{2-} from dissociated Cd-(gammaEC)$_n$G of *E. gracilis* limited the amount of Cd which re-associated (Shaw *et al.*, 1989). S^{2-} has not been identified as a component of isolated Cu-aggregates. Furthermore, the pH of half dissociation of Cu ions indicates a much higher affinity for Cu than for Cd (Table 1).

Function

Metal detoxification

There is evidence that (gammaEC)$_n$G detoxifies Cd and excess Cu in some cells (Jackson *et al.*, 1987). Furthermore, there is indirect evidence that these polypeptides detoxify excess Ag (Grill *et al.*, 1987). Although synthesis of (gammaEC)$_n$G has been shown to increase following exposure of cells to metals other than Cd, Cu and Ag (Grill *et al.*, 1985; Grill *et al.*, 1987), there is no evidence that other metals form complexes with (gammaEC)$_n$G *in vivo*. In *Scenedesmus actutiformis* and *C. fusca*, (gammaEC)$_n$G synthesis has been shown to increase following exposure to Cd, Pb, Zn, Ag, Cu and Hg (Gekeler *et al.*, 1988). However, at present only Cd and Cu have been demonstrated to be bound to such compounds in extracts from algal cells. Furthermore, while Cd induces (gammaEC)$_n$G synthesis in *E. gracilis*, cytosolic Zn is found primarily in a low molecular weight peak distinct from Cd-(gammaEC)$_n$G (Gingrich *et al.*, 1984; Shaw *et al.*, 1989). Based upon the stucture of (gammaEC)$_n$G, binding of some metals other than Cu and Cd seems likely, but this needs to be confirmed.

Much of the data implicating (gammaEC)$_n$G in the detoxification of Cd and supra-optimal concentrations of Cu has been obtained in studies of *S. pombe* and higher plant cells. Cell lines of *Datura innoxia* selected for resistance to different concentrations of Cd show a direct correlation between level of Cd-resistance and maximal accumulation of Cd-(gammaEC)$_n$G (Jackson *et al.*, 1987). Buthionine sulphoximine (BSO) is a specific and potent inhibitor of the enzyme gamma-glutamylcysteine synthetase (EC 6.3.2.2), which is involved in (gammaEC)$_n$G synthesis. Exposure of cells to this inhibitor leads to inhibition of (gammaEC)$_n$G synthesis (Reese & Wagner, 1987b). In a number of species, dramatic synergism has been observed between Cd and BSO in the inhibition of growth. This synergism has been directly correlated with decreased levels of (gammaEC)$_n$G (Steffens *et al.*, 1986; Grill *et al.*, 1987; Reese & Wagner, 1987b). Under

these experimental conditions, reduction in Cd-(gammaEC)$_n$G leads to a corresponding increase in the amount of Cd eluting at the total volume of gel permeation columns, coincident with the elution position of free Cd (Reese & Wagner, 1987b). Free Cd is cytotoxic and inhibits growth. BSO has not yet been demonstrated to increase sensitivity to metals in any algae. Mutants of *S. pombe*, unable to make (gammaEC)$_n$G, are Cd-sensitive (Mutoh & Hayashi, 1988). Furthermore, mutants unable to produce aggregates of Cd-(gammaEC)$_n$G containing S^{2-} also show reduced resistance to Cd. In this context, it would be of interest to examine Cd resistance in mutants of *Chlorella pyrenoidosa* which are deficient in enzymes involved in the generation of S^{2-} (Schiff & Fankhauser, 1981). *C. pyrenoidosa* mutant Sat_2^-, is deficient in organic thiosulphate reductase, while $Sat_{1,3,6}^-$ are deficient in adenosine 5′ phosphosulphate (APS) sulphotransferase. Therefore, all of these mutants are unable to grow on media containing SO_4^{2-} without supplements (Abrams & Schiff, 1973). These *C. pyrenoidosa* mutants may be analogous to the class of Cd-sensitive *S. pombe* mutants which are unable to produce the higher molecular weight form of Cd-S^{2-}-(gammaEC)$_n$G aggregates.

Constitutive function

It has been argued in relation to the function of class I MT, that toxic trace metals are not present at high, or fluctuating concentrations in most biotopes and would not exert a sufficiently strong selection pressure to cause the widespread existence of a specialized detoxification system (Karin, 1985). This argument could also be proposed for (gammaEC)$_n$G. Furthermore, these polypeptides have been detected, albeit in small amounts, in cells grown in the absence of elevated concentrations of trace metals (Steffens *et al.*, 1986). Increased accumulation of (gammaEC)$_n$G occurs very rapidly following exposure to elevated concentrations of metals (Grill *et al.*, 1986). In one higher plant cell line, increased accumulation of (gammaEC)$_n$G was detected as early as 5 min after exposure to Cd (Robinson *et al.*, 1988). This induction response has been demonstrated to be largely insensitive to cycloheximide (Scheller *et al.*, 1987; Robinson *et al.*, 1988). These data demonstrate that enzymes involved in (gammaEC)$_n$G biosynthesis are constitutively produced in the absence of elevated levels of trace metals. This suggests either a constitutive function for (gammaEC)$_n$G, or an alternative constitutive function for the biosynthetic enzymes.

Is (gammaEC)$_n$G involved in assimilatory SO_4^{2-} reduction?

Association of acid-labile S with (gammaEC)$_n$G has encouraged speculation that this polypeptide may be involved in assimilatory SO_4^{2-} reduction (Steffens *et al.*, 1986). There are two distinct patterns of assimilatory SO_4^{2-} reduction, one using APS as the nucleotide sulpho donor and the other using adenosine 3′ phosphate 5′ phosphosulphate (PAPS) as the donor (Schiff, 1983). The former pattern occurs in most O_2 evolving photosynthesizers, including all eukaryotic algae and some prokaryotic cyanobacteria. Within the cyanobacteria, *Spirulina platensis, Synechococcus* 6301 and *Synechocystis* 6719 have all been demonstrated to use the latter pattern involving PAPS. In contrast, *Plectonema* 73110 uses APS as the donor and therefore is more closely related to the eukaryotic algae in this respect (Schmidt, 1977).

Investigations of organisms possessing the pathway involving APS have concluded that the sulpho group of APS is transferred, via a specific APS sulphotransferase, to a low molecular weight thiol carrier (Schiff, 1983). Evidence for such a carrier is, however, indirect and includes the inability to detect free intermediates such as SO_3^{2-} and S^{2-}. Also, it includes the ability of molecules containing thiol groups to interact with the sulphotransferase in reconstruction experiments. The requirement for such a carrier may have coincided with aerobiosis. Free SO_3^- and S^{2-}

react with O_2, therefore carrier bound intermediates which are not autoxidizable may be required in aerobes (Schiff, 1983). In *Chlorella* spp. glutathione, GSH, was thought to act as a sulpho-carrier in this pathway (Tsang & Schiff, 1978). However, other molecules such as $(gammaEC)_nG$ could also be active, and may interact with the enzyme *in vivo*. Furthermore, the material isolated as the carrier from *Chlorella pyrenoidosa* showed co-electrophoresis with oxidized glutathione, GSSG, and was only identified as glutathione based on the presence of Glu, Cys and Gly. The ratio of these three amino acids was not determined. Therefore, it is conceivable that the material identified as GSSG could have been $(gammaEC)_nG$ (J.A. Schiff, pers. comm.).

A sulpho-acceptor which is known to be structurally similar to GSH but with a higher molecular weight than GSH, has been identified in higher plants (Schiff, 1980). Steffens *et al.* (1986) observed that $(gammaEC)_nG$ possesses the necessary requirements to act as the sulpho-acceptor. Unfortunately, it may be difficult to test whether $(gammaEC)_nG$ is the principal physiological S^{2-} carrier due to the apparent lack of specificity of APS sulphotransferase *in vitro*. This enzyme has recently been purified from *Euglena* spp. Unless $(gammaEC)_nG$ were to show some unique properties in its interaction with APS sulphotransferase, incubation of the isolated enzyme with purified $(gammaEC)_nG$ might simply demonstrate that this molecule is another thiol capable of interacting with the enzyme (J.A. Schiff, pers. comm.). However, reports that acid labile S is often associated with isolated $(gammaEC)_nG$ supports the hypothesis that this molecule may be the physiological S^{2-} carrier. The acid labile S associated with $(gammaEC)_nG$ from *Euglena gracilis* and from *S. pombe*, was demonstrated to be present as reduced S^{2-}, which can be quantified by standard assays (Murasugi *et al.*, 1983; Weber *et al.*, 1987). Is this S^{2-} generated by the action of organic thiosulphate reductase?

In the pathway for assimilatory SO_4^{2-} reduction in eukaryotic algae, S associated with the carrier is reduced to S^{2-} by the action of organic thiosulphate reductase, at the expense of reduced ferredoxin (Schiff, 1983). In *Euglena* sp. NADPH is the reductant rather than reduced ferredoxin (Brunold & Schiff 1976). Subsequently, the S^{2-} is donated to o-acetyl serine to form cysteine and acetate (see Fig. 1). The carrier is then recycled. Implications for this proposed synonymity between $(gammaEC)_nG$ and the physiological S carrier are discussed in some of the following sections. An alternative source of S^{2-} is produced by sulphite reductase, an enzyme which will bring about the reduction of free SO_3^- to S^{2-}, but whose function is otherwise unknown (von Arb & Brunold, 1986).

Metal ion homoeostasis

Evidence that mammalian MT is inducible by Zn and Cu, coupled with observed changes in MT concentration during early development and physiological stress, reveal that this molecule has a role in the metabolism of essential metals in mammals (Hamer, 1986; Bremner, 1987). By analogy, it could be speculated that $(gammaEC)_nG$ may play a similar fundamental role in metal ion homoeostasis in eukaryotic algae. However, it is first necessary to demonstrate that essential metals bind *in vivo*. Certainly, $(gammaEC)_nG$ binds avidly to Cu (Table 1). Therefore, it is possible that these polypeptides could serve as a degradable storage form in the homoeostasis of Cu. Demonstration of Zn-binding is of particular importance in this context, since Zn availability could regulate a cells metabolic and proliferative status (Karin, 1985). Zn-requiring enzymes are active in many biological processes such as DNA replication, RNA transcription, energy metabolism, protein synthesis and protein degradation. Studies of $(gammaEC)_nG$ isolated from higher plants suggest that it is likely to only weakly associate with Zn *in vivo* (Reese & Wagner, 1987a). Furthermore, an examination of the biochemistry of Zn and Cd in *Euglena gracilis* found Cd bound to $(gammaEC)_nG$, but the majority of the Zn present in a very low molecular weight pool (Gingrich *et al.*, 1984; Shaw *et al.*, 1989). Exposure to Zn did not induce synthesis of

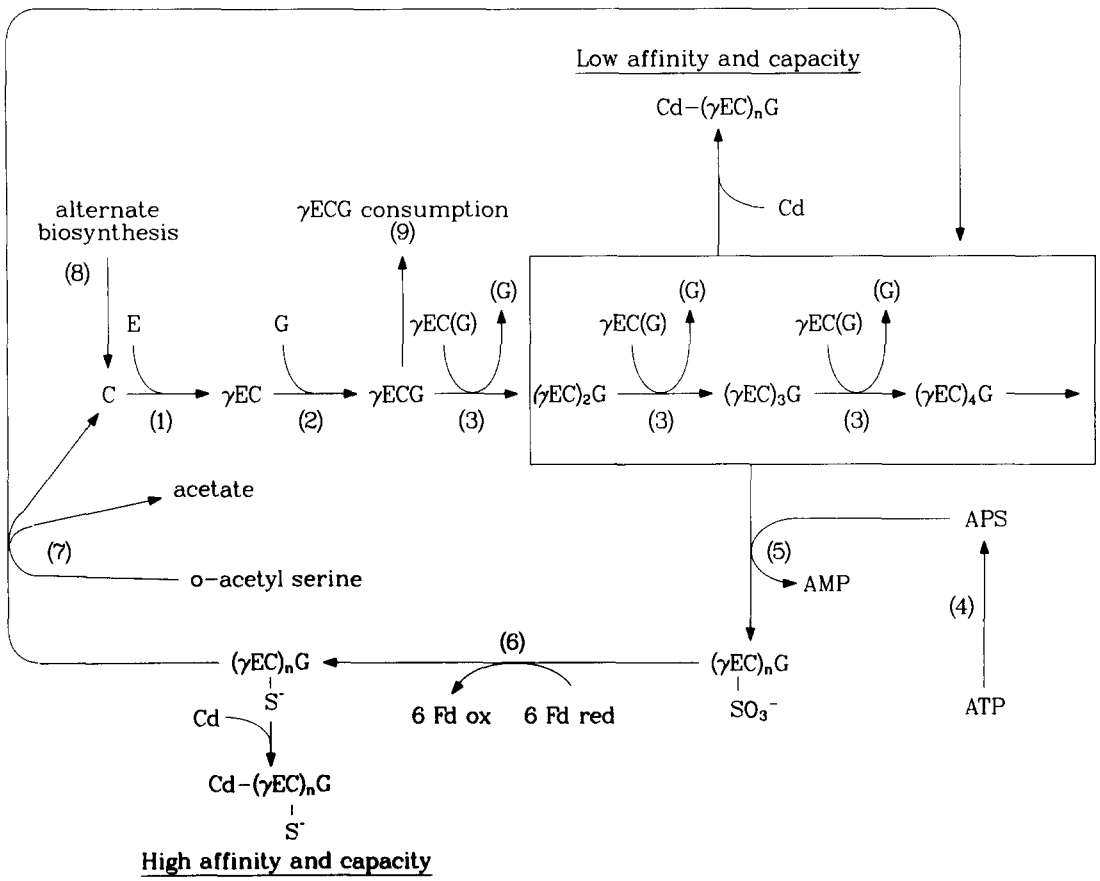

Fig. 1. A theoretical unified scheme linking assimilatory sulphate reduction and (gammaEC)$_n$G biosynthesis. A possible alternative source of S^{2-} to that shown above for the formation of Cd-S^{2-}-(gammaEC)$_n$G aggregates, is generated by the action of non-organic sulphate reductase. The enzymes involved in this theoretical cycle are; 1, gamma-glutamylcysteinesynthetase (EC 6.3.2.2.), 2, glutathione synthetase (EC 6.3.2.3), 3, uncharacterized enzyme(s) assumed to be (gammaEC)$_n$G synthetase, 4, ATP sulphurylase (EC 2.7.7.4), 5, APS sulphotransferase, 6, ferredoxin dependent organic thiosulphate reductase (EC 1.8.7.1), 7, o-acetyl L-serine sulphydrolase (EC 4.2.99.8), 8, alternative pathways for cysteine biosynthesis, 9, pathways that consume glutathione.

Zn-(gammaEC)$_n$G in *E. gracilis* (Weber *et al.*, 1987; Shaw *et al.*, 1989).

Glutathione analogues; the detoxification of free radicals

The structural similarity between (gammaEC)$_n$G and glutathione, GSH, suggests that these two molecules may be functional analogues. The possibility that (gammaEC)$_n$G could be involved in the detoxification of free radicals has encouraged researchers to investigate the possible role of these polypeptides in resistance to ionizing radiation.

Biosynthesis

(gammaEC)$_n$G are secondary metabolites and not post-translationally modified proteins (Robinson *et al.*, 1988). Mutants of *S. pombe*, deficient in the activity of either gamma-glutamylcysteine synthetase or GSH synthetase are unable

to make $(gammaEC)_nG$ (Mutoh & Hayashi, 1988). Therefore, both of these enzymes are required for $(gammaEC)_nG$ synthesis. Mutants have also been identified which have normal activities of both of the above enzymes, but are also incapable of $(gammaEC)_nG$ synthesis (Mutoh & Hayashi, 1988). Such mutants are assumed to be deficient in the unidentified final enzyme(s) in the pathway, putative $(gammaEC)_nG$ synthetase(s). A final class of Cd-sensitive mutants also occur which are capable of producing $(gammaEC)_nG$, but are unable to produce larger complexes, presumably because of inability to introduce S^{2-}. Therefore, these mutants only assemble low Cd-affinity and Cd-capacity aggregates devoid of S^{2-}, and are Cd-sensitive.

After exposure to Cd, shorter forms of $(gammaEC)_nG$ are accumulated prior to longer forms (Grill *et al.*, 1986). The sequence of appearance of these polypeptides is consistent with the suggestion that $(gammaEC)_{n-1}G$ is a precursor for the synthesis of $(gammaEC)_nG$. However, pulse chase data imply that in the presence of Cd, a large pool of $(gammaEC)_nG$ is not available for the synthesis of $(gammaEC)_{n+1}G$ (Berger *et al.*, 1988). $(gammaEC)_nG$ could be produced by condensation of GSH molecules with the release of glycine residues, or by the sequential addition of gamma-glutamylcysteine moieties to a single terminal GSH molecule. Figure 1 illustrates these alternatives and also represents a unified scheme linking $(gammaEC)_nG$ biosynthesis and assimilatory SO_4^{2-} reduction. This is based upon the hypothesis that $(gammaEC)_nG$ is a S^{2-} carrier in assimilatory SO_4^{2-} reduction.

The enzyme which catalyses the formation of $(gammaEC)_nG$ has not been characterized. The development of an *in vitro* assay for this enzyme is necessary. However, present attempts to conduct cell-free synthesis of $(gammaEC)_nG$ have not been successful. One possible reason for the failure of such assays is that the enzyme may be compartmentalized. Biosynthesis of GSH occurs both inside chloroplasts and within the cytosol (Hell & Bergmann, 1988). However, in most eukaryotic algae, assimilatory SO_4^{2-} reduction is confined to the chloroplast (Schiff & Fankhauser, 1981). It has been proposed that the inability of heterotrophically cultured algal cells to lose these plastids is due to the localization of assimilatory S and N reduction in chloroplasts. One exception to this statement is *Euglena* spp. which can lose their chloroplasts (Schiff & Fankhauser, 1981). In *Euglena* spp. the enzymes of assimilatory SO_4^{2-} reduction are localized within mitochondria. According to the hypothetical scheme shown in Fig. 1, the apopolypeptide $(gammaEC)_nG$ may therefore be localized within the chloroplast, or the mitochondria in *Euglena* spp. A first step in the development of an assay for the cell-free synthesis of $(gammaEC)_nG$ may be the isolation of these organelles.

After exposure to Cd, GSH pools are depleted coincident with $(gammaEC)_nG$ synthesis (Grill *et al.*, 1986; Rauser, 1987b), suggesting that increased synthesis of $(gammaEC)_nG$ is regulated at some point in the pathway after GSH. This initial response is known to be regulated at a post-translational level. Prolonged exposure to metal may elicit increased transcription of genes encoding enzymes involved in $(gammaEC)_nG$ synthesis. A number of Cd-induced sequences have been identified in cells producing $(gammaEC)_nG$, but it has not yet been established that any of these correspond to enzymes involved in $(gammaEC)_nG$ synthesis (Jackson *et al.*, 1989).

Mechanisms of tolerance to supra-optimal concentrations of trace metals

Adaptation to tolerate supra-optimal concentrations of toxic trace metals has been described in a wide range of algae (De Filippis & Pallaghy, 1976; Stokes *et al.*, 1977; Butler *et al.*, 1980; Hart & Bertram, 1980; Bariaud & Mestre, 1984). Mechanisms of tolerance to metals with different chemical properties often differ. Furthermore, there are likely to be a number of alternative ways that a species may achieve a particular adaptation and a different species may have a different set of

alternatives. Clearly, modification of the metabolism of $(gammaEC)_nG$ could represent one alternative mechanism of metal tolerance. Some possible ways in which $(gammaEC)_nG$ metabolism could be modified are:

1. Increased activity of enzymes involved in $(gammaEC)_nG$ biosynthesis;
2. increased activity of enzymes responsible for S^{2-} saturation of metal-$(gammaEC)_nG$ complexes;
3. modified compartmentation of one of the components, $(gammaEC)_nG$, S^{2-}, or metal;
4. modified rates of $(gammaEC)_nG$ turnover.

At present, modification of the metabolism of $(gammaEC)_nG$ has not been correlated with increased tolerance to toxic trace metals in any algae.

Class II metallothionein from Synechococcus

Structure

Class II MT from *Synechococcus* TX-20 is the only prokaryotic MT to have been isolated and characterized. The sequences of *Synechococcus* TX-20 MT (Olafson *et al.*, 1988) and equine renal MT (Kojima *et al.*, 1979) are shown for comparison:

Synechococcus TX-20	T S T T L V K C A C E P C L C
	N V D P S K A I D R N G L Y Y
	C C E A C A D G H T G G S K G
	C G H T G C N C
Equine MT-1A	M D P N C S C P T G G S C T C
	A G S C K C K E C R C T S C K
	K S C C S C C P G G G C A R C
	A Q G C V C K G A S D K C S C
	C A

The amino acid sequence of cyanobacterial MT has been compared with a selection of eukaryotic MT sequences including *Scylla serrata, Saccharomyces cerevisiae, Echinoidea* sp. and human MT-2. Sequences were aligned allowing for breaks and conservative amino acid replacements. The statistical significance of the alignments of the cyanobacterial and eukaryotic MT's was determined (Olafson *et al.*, 1988). Despite the high frequency of cysteine residues increasing the probability of chance cysteine alignments, the data base comparisons indicated that there was no significant homology between class II MT of *Synechococcus* TX-20 and any eukaryotic class I MT, or the class II MT's of either *Echinoidea* sp. or *Saccharomyces cerevisiae*. Also, class II MT of *Synechococcus* TX-20 did not show sequence homology with the MT-like E_c protein from wheat germ (Olafson *et al.*, 1988; Hofmann *et al.*, 1984).

Spectroscopic data obtained for Zn-MT, and MT substituted with Cu, from *Synechococcus* TX-20, suggests that the prokaryotic protein may have a metal-thiolate cluster similar to that of eukaryotic MT, but in a single domain (Olafson *et al.*, 1988). Most eukaryotic MT's have two metal-binding domains designated alpha and beta (Hamer, 1986). However, the Cu-containing class I MT's of *Neurospora crassa* and *Agaricus bisporus* are exceptions. Both are shorter than mammalian class I MT's and correspond to the beta-binding domain of the latter group of proteins. Unlike the cyanobacterial MT, the locations of the cysteine residues in the two fungal MT's match the location of these amino acids at the amino terminus of mammalian MT (Kagi & Kojima, 1987). Such evidence suggest that these two fungal MT's have common ancestry with mammalian MT and hence their designation as class I MT's.

Using reverse phase HPLC, a large number of variants of *Synechococcus* TX-20 MT were resolved. However, these could not be attributed to the presence of major isoproteins as assessed by either amino acid analysis or amino acid sequencing of the isoforms (Olafson *et al.*, 1988). This protein possesses six long chain aliphatic residues and two aromatic amino acid residues, making this the most hydrophobic MT to have been characterized. The presence of aromatic amino acids is also a distinguishing feature, since these are absent from other MT's causing characteristic low absorbance at 280 nm (Kagi & Kojima, 1987; Olafson *et al.*, 1988).

Regulation of synthesis

Mammalian MT genes are regulated by a wide range of factors and possess a complex array of elements determining basal MT expression and responses to a number of metals, hormones and other factors associated with acute stresses (Palmiter, 1987). By contrast, regulation of fungal MT genes is less complex with the metal regulatory elements, mre's, of *S. cerevisiae* and *N. crassa* responding only to elevated concentrations of Cu (Hamer, 1986). These fungal mre's have been isolated and extensively characterized. Details of the regulation of cyanobacterial MT genes await the isolation of these sequences. However, increased synthesis of class II MT in *Synechoccocus* TX-20, following exposure to Cd and Zn, is regulated at a transcriptional level (Olafson, 1984; Olafson, 1986). The regulatory elements are not responsive to Cu.

The recent sequencing of a cyanobacterial MT provides the opportunity to produce oligodeoxynucleotides which can be used to isolate the corresponding gene from genomic DNA libraries. Transformation protocols are available for non-filamentous cyanobacteria (Dzkelzkalns *et al.*, 1988). Therefore, the mre's which determine expression of this gene may also be studied. This could provide a useful model system for the study of environmentally modulated gene expression in a prokaryote. Furthermore, there are a number of potential applications for a prokaryotic mre.

Function

It is unclear whether or not cyanobacterial class II MT performs vital functions other than the detoxification of Cd and Zn. The fact that synthesis is transciptionally regulated by Zn and that the protein binds to Zn, *in vivo*, is significant. Therefore, it can be speculated that this protein may perform a vital function in Zn homoeostasis, as proposed for mammalian MT (Karin, 1985). However, unlike mammalian and fungal MT's (Hamer, 1986; Olafson, 1986), this protein does not appear to play a role in either Cu homoeostasis or Cu detoxification.

Some mammalian cell cultures resistant to supra-optimal concentrations of trace metals show either modified regulation of MT genes or MT gene amplification (Crawford *et al.*, 1985; Durnam & Palmiter, 1987). In *S. cerevisiae* the *CUP1* gene encodes class II MT (Butt *et al.*, 1984a; Karin *et al.*, 1984). Cu-resistant strains of *S. cerevisiae* which contain ten or more tandem duplications of the *CUP1* gene have been isolated (Karin *et al.*, 1984; Fogel & Welch, 1982). *Synechococcus* TX-20 MT binds Cd and Zn, but not Cu and therefore amplification of class II MT genes in this cyanobacterium would lead to a different spectrum of metal tolerance to that observed in yeast. In the cyanobacterium *Anacystis nidulans* protection against Cd toxicity was correlated with production of an MT-like protein (Maclean *et al.*, 1972). However, this molecule awaits detailed characterization. At present, there is no uniquivocal evidence of modified expression of class II MT genes conferring resistance to supra-optimal concentrations of toxic trace metals in any algae.

Metallothionein genes or not: an evolutionary dichotomy

It has been suggested that $(gammaEC)_nG$ (class III MT) may be a primitive precursor of class I and II MT (Vallee, 1987). It remains to be established whether or not the phylogenetic distribution of these molecules will support this suggestion. More detailed descriptions of the functions of these compounds are also required. For example, if $(gammaEC)_nG$ is a S^{2-} carrier, then its distribution might coincide with the distribution of the pathway of assimilatory SO_4^{2-} reduction involving organic thiosulphate reductase. Consistent with this, $(gammaEC)_nG$ has not yet been isolated from mammals, which rely on the organisms they eat for a supply of reduced S metabolites, but has been identified in a range of eukaryotic algae, higher plants and the primitive fission yeast *S. pombe*. In this context, it is of interest that $(gammaEC)_nG$ has not yet been identified in cyanobacteria and class II MT has been characterized in *Synechococcus* TX-20. In

this cyanobacterium, assimilatory SO_4^{2-} reduction uses PAPS as the S donor rather than APS, but in common with the APS pathway of eukaryotic algae, a sulpho-carrier molecule still appears to be involved in the process (Schmidt, 1977). Furthermore, yeasts also possess the PAPS pathway and (gammaEC)$_n$G has been identified in *S. pombe* (Schiff & Fankhauser, 1981; Murasugi *et al.*, 1981; Hayashi *et al.*, 1988). It will be of interest to establish the structure of any Cd-induced metal-binding polypeptides in other species of cyanobacteria, such as *Plectonema* 6719, which used APS as the sulpho-donor (Schmidt, 1977).

The proposal that Zn homoeostasis may be the primary function of class I MT, in mammals, has been discussed (Karin, 1985). This has also been proposed as a possible function of class II MT in *Synechococcus* TX-20 (Olafson *et al.*, 1988). However, in *Euglena gracilis* the majority (> 80%) of the cellular Zn occurs in a low molecular weight peak distinct from Cd-(gammaEC)$_n$G (Gingrich *et al.*, 1984). This implies that (gammaEC)$_n$G may not be involved in Zn homoeostasis. This may also be true for other species and it has been proposed that Zn may only weakly bind to (gammaEC)$_n$G *in vivo* (Reese & Wagner, 1987a). Therefore, the presence of (gammaEC)$_n$G in a particular species does not mean that class I or class II MT genes would be absent *a priori*. However, the presence of (gammaEC)$_n$G in eukaryotic algae (preventing accumulation of high concentrations of free metal ions in the cytoplasm) may prevent the activation of MT gene mre's in response to any metals that bind to (gammaEC)$_n$G. Only basal expression of MT genes may occur in these species (Thurman & Tomsett, unpublished observation). This could account for the inability of researchers to isolate such proteins from species which produce (gammaEC)$_n$G. It is necessary to establish whether MT genes and (gammaEC)$_n$G are mutually exclusive. If this were true, then it would either suggest that there is an alternative mechanism of Zn homoeostasis in species which produce (gammaEC)$_n$G, or argue against the proposal that Zn homoeostasis is a primary function of class I or class II MT. Similar arguments could also be proposed for fungal MT's which only bind Cu *in vivo*.

Prospective

The induction of synthesis of class I MT in animals and class I and II MT in fungi, has provided useful model systems for the study of environmentally modulated gene expression (Hamer, 1986; Palmiter, 1987). Furthermore, there are applications for MT genes and their promoters (Hamer, 1986; Butt & Ecker, 1987). MT gene promoters have been attached to a variety of other genes. The expression of these genes is then regulated by changes in the concentration of trace metals. Increased production of MT can confer resistance to metals. Therefore MT genes can be used as either selectable markers in screening protocols or as reporter genes to monitor promoter function in transformed organisms. The *CUP1* gene is already widely used for such purposes in yeast. An approach for isolating a class II MT gene and its mre's from *Synechococcus* TX-20, has been discussed. These sequences may be useful for the manipulation of prokaryotic genes, as other MT genes and promoters have been used in eukaryotes.

Genes involved in (gammaEC)$_n$G metabolism may be isolated from *S. pombe* using recently identified mutants (Mutoh & Hayashi, 1988). Genomic DNA libraries produced in these mutants can be screened for complementation. Transformants showing restored metabolism will be carrying the genes of interest. Subsequently, such sequences can be used to identify homologous suquences in eukaryotic algae. The expression of such genes could then be examined in metal-tolerant and metal-sensitive eukaryotic algae. It has not yet been demonstrated that metal-induced synthesis of (gammaEC)$_n$G in eukaryotic algae is regulated at a post-translational level, as established for higher plants. If this were demonstrated, then it could be inferred that genes encoding enzymes involved in (gammaEC)$_n$G metabolism do not possess mre's.

The structure of (gammaEC)$_n$G has been used as a model to produce synthetic genes that encode

protein analogues of these small metal-binding polypeptides which differ from $(gammaEC)_nG$ in containing only alpha-carboxamide bonds (Robinson *et al.*, 1988). Associated with appropriate expression vectors these genes may confer metal tolerance. Micro-organisms and algae containing such constructs, may be used either to accumulate metals in effluent treatment or applied to other bioprocessing applications which require growth in the presence of supraoptimal concentrations of trace metals. A prokaryotic mre included in such constructs may allow expression of the synthetic gene to be modulated in concert with changes in the concentration of trace metal ions, although it remains to be established whether a *Synechococcus* mre would function in any other organism. In this context, it should be noted that concern has been expressed regarding the use of plasmids, rather than stable integrations into the genome, to engineer special organisms to recover toxic elements from industrial waste water. It is considered likely that the toxic conditions found in industrial waste water could cause plasmid losses from organisms, even though they may have been shown to function extremely well in the laboratory (Wood & Wang, 1983).

$(gammaEC)_nG$ has been immobilized in a functional form to produce columns that remove metals from solution. There may be applications for such columns in metal removal. The structure of $(gammaEC)_nG$ has also been used as a model for the chemical synthesis of biomimetic ligands to perform equivalent functions (Furlong *et al.*, 1988). Unlike proteins, $(gammaEC)_nG$ is resistant to degradation by peptidases and is therefore more amenable to such applications. Finally, it is possible that metal-specific ligands could be of use in the production of metal-specific biosensors for detecting elevated concentrations of metals in both critical chemical processes and for monitoring aquatic environments.

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