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PROKARYOTIC METALLOTHIONEIN: PRELIMINARY CHARACTERIZATION OF A BLUE-GREEN ALGA HEAVY METAL-BINDING PROTEIN

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SUMMARY - A cadmium inducible metal-binding protein has been isolated from cadmium exposed Synechococcus sp. blue-green algae. Amino acid analysis indicated a high half-cystine concentration and an apparent minimum molecular weight of 8100. The isoelectric point was determined to be 4.3. Together with electrochemical characteristics these findings constitute the first conclusive evidence for the existence of metallothionein in a prokaryotic organism.

The toxicological and physiological significance of the heavy metal-binding protein metallothionein has become an area of significant research encompassing investigators from clinical and environmental sciences (1,2,3). Of fundamental importance to the latter group is the question of phylogenetic distribution in the environment. Metallothionein is well characterized in vertebrates (4), invertebrates (5), and eukaryotic microorganisms (6,7) but no conclusive evidence exists for the presence of this protein in prokaryotes. MacLean et al (8) have reported the presence of a cadmium and zinc-binding material in the fresh water blue-green alga Anacystis nidulans, but no attempt at further characterization was made. Investigations using bacteria were not successful in elucidating the presence of metallothionein (9). This study has utilized a marine blue-green alga Synechococcus sp. in an attempt to verify the presence of metallothionein in a prokaryotic organism.

MATERIALS AND METHODS

Synechococcus sp. Naegeli, 1849, blue-green alga, was provided by the Roche Research Institute of Marine Pharmacology, Dee Why, New South Wales (Strain RRIMP N1). This species was isolated from a tidal pool near Sydney and is apparently identical to Coccochloris elabens Drouet and Daily, 1956. Cells were maintained in axenic culture on slopes of Guillard's f medium (10)

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modified by use of ferric citrate and EDTA in place of ferric sequestrene. Organisms were grown in 20 l of f medium broth containing 5 x 10^{-5} M $CdCl_2$. Sequential cultures grown in 1 x 10^{-5}, 2.5 x 10^{-5} and 5 x 10^{-5} M $CdCl_2$ were necessary to produce cadmium tolerant cells prior to initiating the 20 l culture. The large scale culture was grown in a 27° incubator under fluorescent light (50w/m^2) with stirring and slow addition of filtered air. Forty microcuries of carrier free $^{109}CdCl_2$ (New England Nuclear, Boston) was introduced three days before harvesting. Cells were concentrated by centrifugation in a constant flow centrifugation system and broken in a French press at 0° in the presence of 0.5 M Tris/HCl pH 8.6 and a further 10 microcuries of ^{109}Cd added. The product was centrifuged at 0° for 10 min at 12,000 x g and the supernatant applied directly to a 5 x 100 cm Sephadex G-75 column. Subsequent isolation and characterization was conducted as described earlier (5) except that the Brdička differential pulse polarographic procedure was performed as described by Paleček and Pechan (11) with the following modifications. The surface active agent maximum suppressor was found unnecessary and the scan was routinely made from -1.35 to -1.6 volts at 5mv/sec.

RESULTS AND DISCUSSION

Growth of the Synechococcus sp. blue-green algae was found to be inhibited by cadmium chloride unless cells were pre-exposed to metal levels less than 5 x 10^{-5}M. Cadmium tolerant cells were therefore produced by stepwise subculturing into higher cadmium concentration medium and the 12,000 x g supernatant from cadmium tolerant cells applied to a Sephadex G-75 column (Fig. 1). The effluent absorbance was monitored at 250 nm and fractions were assayed for sulphydryl containing proteins using the Brdička procedure. Bound ^{109}Cd, was also determined. The material eluting in the 10,000 molecular weight range had a marked 250 nm absorbance, contained ^{109}Cd and was the only peak in the profile with a high sulphydryl concentration. Since these four characteristics are typical of metallothioneins, material present in the peak was pooled and applied directly to an ion-exchange column for further purification.

Only partial purification was realized after DEAE cellulose chromatography with elution of rather poorly defined broad peaks. The radioactive, sulphydryl containing material was pooled and after concentration and desalting, subjected to isoelectric focusing as described earlier (5) (Fig.2). Discounting the 250 nm absorbing ampholyte artifacts indicated on the profile, only a single 250 nm absorbing peak with a pI of 4.3 and bound ^{109}Cd, was observed. This isoelectric point is very similar to those reported for

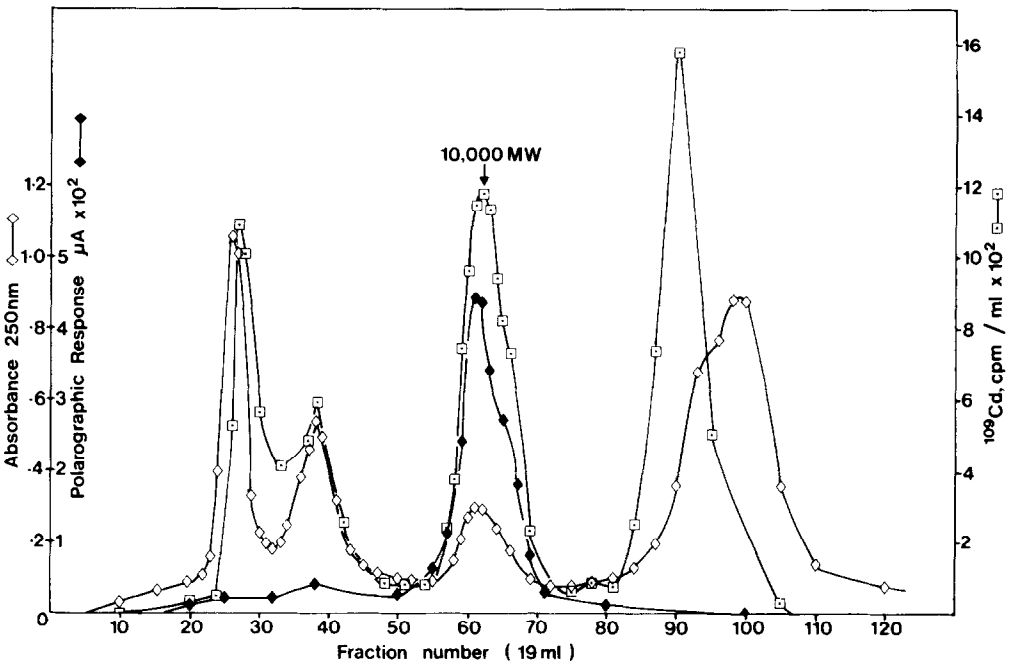


Fig. 1. Sephadex G-75 column chromatography of 12,000 x g supernatant of blue-green algae cell extract (20 g wet weight of cells).

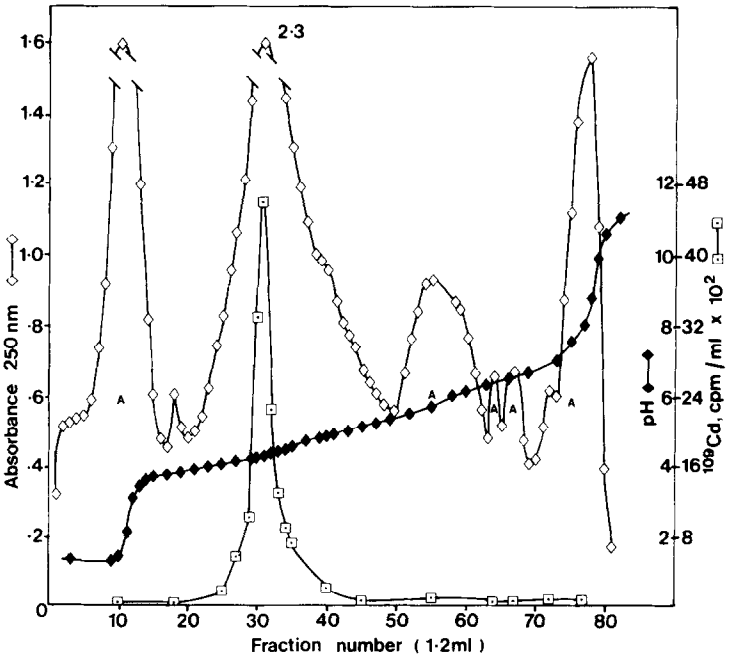


Fig. 2. Isoelectric focusing profile of ^{109}Cd containing, sulphydryl rich, DEAE cellulose fractions. The ampholyte gradient was pH 3.5-7.0 maintained at 5°. Ampholyte artifacts absorbing at 250 nm are indicated by the letter A.

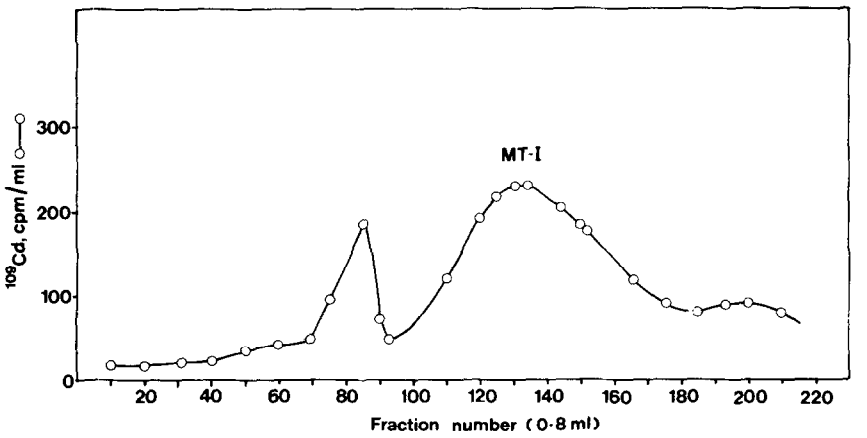
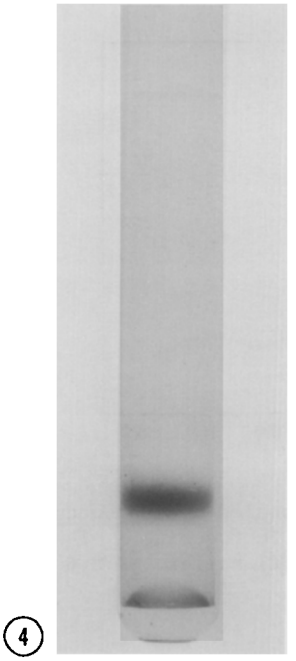


Fig. 3. DEAE-cellulose ion exchange chromatography of pooled Synechococcus sp. metallothionein fractions isolated by isoelectric focusing. The linear gradient (.05 - .5 M) was developed with Tris/HCl buffer, pH 8.6 in a total elution volume of 2 l.

eukaryotic metallothioneins (5,12). However, as found with crustacean metallothioneins (5), disc gel electrophoresis of the electrofocused material resulted in two protein bands. Subsequent chromatography of the isoelectric focused material on DEAE-cellulose allowed isolation of a major ^{109}Cd-binding protein (termed MT-1 (Fig. 3)). A smaller peak, eluting later, was poorly resolved from the preceding major fraction and found too contaminated for further characterization. Similarly the first peak to elute, marking the start of the linear gradient, was found to be impure. However, disc gel electrophoresis of the major MT-1 fraction resulted in a single band as shown in Figure 4 and the ultraviolet absorption spectrum was seen to be very similar to previously isolated metallothioneins (5) (Fig. 5). The characteristic high 250/280 nm ratio for these proteins was evident.

Polarographic analysis of the MT-1 fraction by use of the Brdička reaction for sulphydryl groups indicated a molecule of high sulphydryl concentration with a wave peak potential at -1.455 V and wave height of 245A/mole protein. The latter values agree well with values for rat liver MT-2 of -1.450 V and 221 A/mole protein respectively. Further analysis of the blue-green alga protein by cyclic voltametry indicated marked similarity



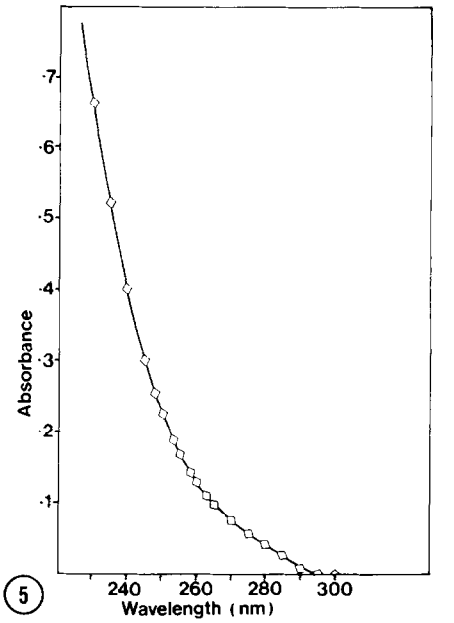


Fig. 4. Polycrylamide disc gel electrophoresis of Synechococcus sp. metallothionein MT-I isolated by ion exchange chromatography.

Fig. 5. Ultraviolet absorption spectrum of MT-I in 10 mM Tris/HCl, pH 8.6

with crustacean and mammalian metallothioneins (13). Recent investigation of this procedure has indicated that metallothioneins have very marked cyclic voltamograms and differ considerably from non-metal complexed, sulphydryl or disulphide containing proteins.

Preliminary metal determination and amino acid analysis was also conducted on purified MT-1. Results of the metal determination indicated the presence of cadmium, copper and zinc, as is frequently observed in eukaryotic metallothioneins (14) (Table 1). Levels of metal were lower (2.24 g atoms/mole protein) than those previously reported for higher organisms and are likely attributable to acidic isoelectric focusing conditions. However, this result is consistent with the observed amino acid composition (Table 2) which indicated 15% half-cystine content - again lower than that reported for eukaryotic metallothioneins (5). Since only sufficient purified protein for a single amino acid analysis was available, it is not possible at this time

Table 1: Heavy Metal Concentration in *Synechococcus sp.* Metallothionein

	gram atoms/mole protein
Cadmium	1.28
Copper	0.64
Zinc	0.32
Total	2.24

Table 2: Amino Acid Composition of *Synechococcus sp.* Metallothionein

	Residues/Molecule [c]	Nearest Integer
Cys A [a]	10.8	11
Asx	4.6	5
Thr	4.2	4
Ser	6.4	6
Glx	8.2	8
Gly	9.8	10
Ala	8.2	8
Val	3.4	3
Met [b]	1.0	1
Ile	2.2	2
Leu	2.6	3
Tyr	1.3	1
Phe	1.1	1
His	4.0	4
Lys	5.1	5
Arg	-	-
Pro	2.1	2

Apparent Minimum MW = 8115

a. Cysteic acid
b. Determined as methionine sulphone
c. Assuming Met = 1

to rely heavily on differences in amino acid composition between the prokaryote and eukaryotic metallothioneins. For example, in light of the ultraviolet spectrum it is considered likely that the single aromatic residues are contaminants. Further work is in progress to confirm these results. The amino acid composition obtained, however, indicated a single

methionine, a large half-cystine concentration and elevated lysine and serine levels. These results, together with the reported physical and electrochemical characteristics provide conclusive evidence that metallothioneins or proteins similar to metallothioneins exist in the prokaryotic blue-green algae.

Initial experiments have provided evidence that like eukaryotic metallothioneins, the prokaryotic protein is induced in the presence of cadmium. Organisms kept in culture for one month in 2.5×10^{-5} M $CdCl_2$, produced approximately 50 ng metallothionein/mg wet cells as determined polarographically. A similar quantity (100 mg wet weight) of control cells, not exposed to cadmium, produced no measurable metallothionein. Thus the blue-green algae metallothionein has physiological as well as physico-chemical properties in common with the eukaryotic proteins. Work in progress is directed towards elucidation of the toxicological, physiological and phylogenetic significance of this protein in organisms.

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