Environmental Health Perspectives
Vol. 65, pp. 77-85, 1986

Characterization of a Highly Negative and Labile Binding Protein Induced in *Euglena gracilis* by Cadmium

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The physiochemical properties and physiological significance of the cadmium-binding protein (CdBP) of the algae *Euglena gracilis* have been studied. Following *in vivo* exposure of cells to 0.4 or 1.3 μg/mL of Cd^{2+}, all the cytosolic Cd is bound to high molecular weight species. At 4.7 μg/mL, appreciable CdBP has formed in cells grown under illumination or in the dark. An analogous ZnBP could not be isolated from control or Zn-exposed (20 μg/mL) cells, but zinc and a trace of copper were bound to the CdBP when 2-mercaptoethanol (2-ME) is added to the homogenates of Cd-treated cells and the buffers used during isolation. The large pool of very low molecular weight zinc species previously reported is increased when cells are exposed to high cadmium levels. Two distinct species, BP-1 and BP-2 are resolved by ion-exchange chromatography on DEAE–Sephadex. Unusually high conductivities (25 and 40 mSiemen) are required to displace them, indicating that they are very negatively charged proteins at pH 8.6. The pH for half-titration of bound Cd^{2+} is between 5 and 6. EDTA (0.4 M) and the CdBP isolated by gel-exclusion chromatography react biphasically with pseudo-first-order rate constants of $4 \pm 3 \times 10^{-4}$ sec^{-1} and $7 \pm 2 \times 10^{-5}$ sec^{-1}. Neither form of the CdBP cross-reacts with antibodies to rat liver metallothionein (MT) antibodies. The structural, chemical, and functional differences between the *Euglena* CdBPs and mammalian MTs are discussed.

When cells are exposed to high levels of Cu (5 or 10 μg/mL), a CuBP is induced, and the very low molecular weight zinc band is depleted. Their copper-rich cytosol has a green-black color which is lost upon oxidation.

Introduction

The reactions of metals with cells are important in understanding the essential metal metabolism, environmental metal intoxication, and the mechanism of action of metallodrugs. Zinc and cadmium metabolism in *Euglena gracilis* have been studied extensively as a model for human and mammalian cells (*1–4*). These cells show characteristic zinc and cadmium antagonism which is common to most species. However, a recent examination of the biochemistry of zinc and cadmium in *Euglena gracilis* found significant differences from that of mammalian cells such as the Ehrlich ascites tumor cell (*5*).

Most striking was the finding that up to 80% of the cytosolic zinc was present as a very low molecular weight species. In mammalian cells, metallothionein-bound zinc is the physiologically active form, whereas in *Euglena gracilis*, the very low molecular weight form of zinc seems to be related to the zinc status of the cells (*5*).

Metal-inducible binding proteins in lower organisms such as fish (*6*), crustacea (*7*), and various microorganisms (*8,9*) vary greatly in chemical and physical properties among themselves and in comparison to mammalian metallothioneins (MTs). In contrast to the multiplicity of invertebrate binding proteins (BPs), MTs from numerous mammalian species have a highly conserved amino acid sequence and similar properties (*10*). Examination of the structure and chemical reactivity of BPs from lower organisms will elucidate their functions and mechanisms of reaction in metal metabolism, and by contrast will help to clarify the structure–function relationships of MTs. The Cd-inducible BP of *Euglena gracilis* was found to have a smaller apparent molecular weight and more negative charge than MTs, and to resemble MTs in having a high cysteine content and low aromatic amino acid content.

In this paper we report further investigations of the chemical properties and physiological significance of the CdBP of *Euglena gracilis* and some results on exposure of these cells to copper.

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Methods and Materials

Media

All cultures were maintained by using the modified medium of Hutner et al., with $MnCl_2$ substituted for

$MnSO_4$ and glutamic acid for ammonium glutamate as previously described (*5*). The media were sterilized by autoclaving after the pH was adjusted to 3.5 using 6 M KOH. Zinc-sufficient (Zn+) and zinc-deficient (Zn−) media were identical except for the withholding of zinc from the (Zn−) medium. (Zn+) medium contained 1 μg Zn/mL and (Zn−) medium less than 0.01 μg Zn/mL as measured by atomic absorption. Cadmium, zinc, or copper levels above normal media levels wre achieved by the addition of aqueous solutions of $CdCl_2$, $ZnCl_2$, or $CuCl_2$ either before or after autoclaving.

Cultures

Euglena gracilis (Klebs), "z" strain, #753 was purchased from the Culture Collection of Algae at the University of Texas at Austin.

Unless otherwise noted, individual experiments were begun using inoculations of stock culture cells in the log phase of growth. In experiments requiring multiple cultures, one large culture was made and then divided into 250 mL cultures in cotton-plugged 500 mL Erlenmeyer flasks and placed in a shaker bath maintained at 26 to 27°C under a 20-W GE F20T12-CW fluorescent tube approximately 40 cm overhead. Cultures grown in the absence of light ("dark-grown") were set up identically and wrapped in aluminum foil to exclude light.

Cell counts were obtained by using an American Optical Series 10 Microstar microscope at 450 × magnification with an American Optical Spencer Bright Line Phase Hemacytometer #1475 with Neubauer ruling.

Cells Exposed to Elevated Copper Levels

The effects of elevated medium copper levels upon cytosolic copper were investigated by the addition of copper as aqueous $CuCl_2$. The normal copper level in the medium is 0.064 μg/mL (1×10^{-6} M). Inoculations to 1×10^4 cells/mL were made from stock cultures and copper was then added at 10 μg/mL (1.6×10^{-4} M) or 5 μg/mL (7.8×10^{-5} M). The cells were harvested after 4 or 10 days of growth and the cytosol chromatographed over a Sephadex G-75 column.

Cytosol Preparation

Cells were typically harvested by centrifugation at 3000*g* for 5 min. This was followed by two or three washings which consisted of resuspending the pelleted cells in a 10 mL of 0.15 M KCl per gram of cells and recentrifuging at 3000*g*. The cells were then resuspended in 0.15 M KCl, 0.05 M Tris buffer, pH 8.6, at room temperature, and sonicated in a 7 mL tube in an ice-water bath and a Sonifier Disruptor 200 for 2 min at 60% duty and 75 W of power. A 5-mL portion of homogenization medium was used per gram of cells in a 7-mL tube. The preparation was kept between 0 and 4°C after sonication and, unless otherwise noted, 5 to 10 mM 2-mercaptoethanol (2-ME) was added prior to sonication to retard oxidation of the metal-binding protein and subsequent redistribution of metals, as has been observed in mammalian systems (*11*). The sonicate was centrifuged at 40,000*g* for 20 min, resulting in a compacted pellet, a supernatant and a small, top layer consisting of fluffy material. In cells grown on high cadmium levels, the top layer has a rubberlike consistency. The middle, supernatant layer was then ultracentrifuged at 100,000*g* for 1 to 1.5 hr, and a similar set of three layers resulted. The middle layer was carefully removed and was designated the cytosol fraction. In some preparations, Cd^{2+} was added to the media before fractionation, in order to displace Zn^{2+} from the BP fractions.

Metal Analyses

Measurements of metal concentrations were made using either a Perkin-Elmer 360 or an Instrumentation Laboratories (AA/AE) 357 spectrophotometer with D_2 background correction applied for cadmium and zinc. Calibration standards were made using aqueous dilutions of 1000 μg/mL atomic absorption standards obtained from Fisher. Whenever appropriate, dilutions were made using glass distilled water.

Electron Paramagnetic Resonance

Electron paramagnetic resonance (EPR) spectra of cytosol prepared from cells grown in medium containing 10 μg/mL copper were obtained by using a Varian E-3 EPR spectrometer. The field was set at 3017 G, and 4-min scans of frozen cytosol were recorded. Diphenylpicrylhydrazyl (DPPH) was used as a marker. The frozen samples were prepared by sealing the end of a glass tube with parafilm, filling the tube with cytosol, and immersing the tube into liquid nitrogen. The solid pellet formed was then (after slight warming) transferred to an EPR tube, and the spectrum was recorded. The sample was removed from the EPR tube, allowed to thaw, refrozen, and additional spectra obtained at selected times.

Gel Chromatography

The fractionation of cytosolic species was generally accomplished by applying the sample to a 2.5 × 60 cm Sephadex G-75 column at 4 to 7°C, and eluting with 0.05 M Tris buffer, pH 8.6 (room temperature). The resulting fractions were analyzed for metal content by atomic absorption via direct aspiration into the flame. The columns were calibrated by using blue dextran (MW 2,000,000), myoglobin (MW 17,000), horse kidney or rabbit liver metallothionein (MW 6,000; apparent MW 10,000 due to its elliptical shape), $K_3Fe(CN)_6$ and Na^+. Elution positions are reported as K_d, the distribution coefficient. $K_d = (V_e - V_0)/V_s$, where V_0 is the void volume (considered here as the elution volume of blue dextran), V_e is the elution volume of the species of interest, and V_s is the volume of solvent inside the gel and available to very small molecules ($V_s = V_0 - V_{Na^+}$).

Ion-Exchange Chromatography

DEAE–Sephadex-A-25 ion-exchange chromatography was performed on the pooled, cadmium-containing, 10,000-dalton peak obtained from Sephadex columns. Early trials demonstrated that no cadmium-containing species eluted from the column until the conductivity reached at least 15 mSeiman. Consequently, the linear gradients used thereafter generally consisted of either 0.1 M to 0.4 M KCl, or 0.15 to 0.6 M KCl, in 0.05 M Tris buffer, pH 8.6, or alternately from 0.05 M Tris containing 0.2 M KCl to 0.5 M Tris containing 0.2 M KCl. Some columns were run with 10 mM ascorbic acid or 1 mM 2-ME added to the gradient buffers in efforts to stabilize the protein during this procedure. The integrity of the gradients was monitored using a Radiometer-Copenhagen CDM3 conductivity meter equipped with a type CDC 314 cell.

Reaction of EDTA with CdBP

Sephadex G-50 CdBP fractions were concentrated using ultrafiltration, reapplied to a Sephadex G-75 column, and eluted with 0.05 M Tris, pH 8.6, buffer. A series of samples was prepared that contained 0.2 mL 2 M KCl and 3.77 mL of CdBP having a Cd concentration of 4.0 μg/mL. These samples were kept at −20°C (approximately 1 week) until the reactions were performed. The reactions were initiated by the addition of sufficient aqueous EDTA solution to 1.985 mL of the CdBP sample, yielding 0.4 mM EDTA in the final reaction mixture. The blank consisted of 0.1 mL 2 M KCl, 1.885 mL of Tris buffer and 0.015 mL of the EDTA solution. The reaction mixture was then transferred to a 1-mL cuvet within 30 sec and the course of the reaction was monitored at 254 nm over time. Due to their long duration, the reactions were monitored continuously for roughly 5 hr and then periodically at later time points. Four repetitions using two independent CdBP samples were performed. The data were analyzed by plotting the log $(A_\infty - A_t)$ versus time.

Radioimmunoassay

Samples of CdBP from Sephadex G-50 fractions and CdBP-1 and CdBP-2 from DEAE–Sephadex A-25 fractions were prepared by transferring aliquots appropriately diluted with glass-distilled water into small vials such that each vial contained 1.2 to 2.8 × 10^{-4} mole of protein. The estimations of protein were made by measuring the Zn, Cu, and Cd content and assuming the accepted mammalian values of 7 g-atoms of Zn or Cd and 10 g-atoms Cu bound per mole of metallothionein. The samples were lyophilized and sent to Syracuse University. They were redissolved in doubly distilled H_2O, and appropriate serial dilutions were made and assayed in a double-antibody radioimmunoassay (RIA) following a protocol as previously described (*12,13*). Both the ^{125}I-labeled antigen and a reference antigen were prepared from the same rat liver Cd,Zn-thionein. This isoform is known to be cross-reactive with various mammalian metallothioneins. Inverse variance weighted standard curves were developed from the data following established protocols (*14*). Changes in the sequence or in conformation affecting the principal determinants of metallothionein (*15*), and thus the affinity of the altered determinant for the primary antibody, may be evaluated in terms of either changes in slopes of the standard curves with respect to that of the reference isoform or in terms of changes in concentration of the competing antigen required to reduce the fraction bound of labeled antigen to 0.5.

Results

Isolation of CdBP

CdBP for physiochemical studies was isolated from *Euglena gracilis* grown in culture media containing 5 μg/mL of Cd^{2+} and following procedures previously described (*5*). Fractionation of the cytosol (100,000*g* supernatant fraction) over Sephadex G-75 yielded a Cd-rich band, which also contained zinc and a trace of copper under some conditions. After experimenting with a variety of isolation procedures (aerobic, N_2 atmosphere, added ascorbate or 2-mercaptoethanol), it was found that maintaining a 1 mM concentration of 2-mercaptoethanol (2-ME) in the buffers throughout the preparation yielded the most reproducible results. Rapid handling during all steps subseqeunt to the cell sonication was necessary to obtain good yields of the crude BP after gel exclusion chromatography.

Even with the most stringent precautions, loss of the zinc from the BP was observed in subseqeunt steps. Therefore, in later preparations of BP for physiochemical studies, Cd^{2+} was added to the cytosol to displace Zn^{2+} from the BP and yield a more stable all-cadmium BP preparation. The presence of zinc in the BP when 2-ME is used, but not in its absence, demonstrates that the enormous very low molecular weight pool of zinc in *Euglena gracilis* is not an artifact due to extraction by 2-ME of Zn from the BP or high molecular weight zinc pools (Table 1).

Elution of the BP from DEAE-Sephadex ion-exchange resin (Fig. 1) required high salt concentrations in the eluting gradient. Typically, 0.1 to 0.4 or 0.6 M KCl in 50 mM Tris-HCl was used. The presence of 1 mM 2-ME was necessary to prevent erratic elution behavior. Under these conditions some cadmium eluted in the column wash (≤ 30%). The remainder eluted as two CdBP bands at 22 to 25 mSiemen (BP-I) and 40 to 44 mSiemen (BP-II). The relative amounts of cadmium in the two BPs was sufficiently variable that in some cases BP-I was the major species and sometimes the minor. Temperatures between −35 and −80°C prevented degradation of these BP preparations during storage.

Relationship of BP Cytosolic to Medium Cd Levels

To assess the function of the BP, one must determine (1) whether it is constitutive or inducible or both; (2) at

Table 1. Effects of added 2-ME and Cd^{2+} on cytosolic metal distributions.[a]

Added reagents		Cytosolic Cd/μg[b]			Cytosolic Zn/μg		
2-ME/mM	Cd^{+2}/μg	HMW	BP	VLMW	HMW	BP	VLMW
0	0	29	82	—	17		>200
1	0	38	63	—	20	30	152
1	130	79	169	49	17	7.3	121

[a] Cells were grown for 6 days in media containing 5 μg Cd/L. Results are normalized for a 250 mL culture. 2-ME was added to the homogenate; Cd^{2+} to the cytosol.
[b] HMW = high molecular weight; VLMW = very low molecular weight.

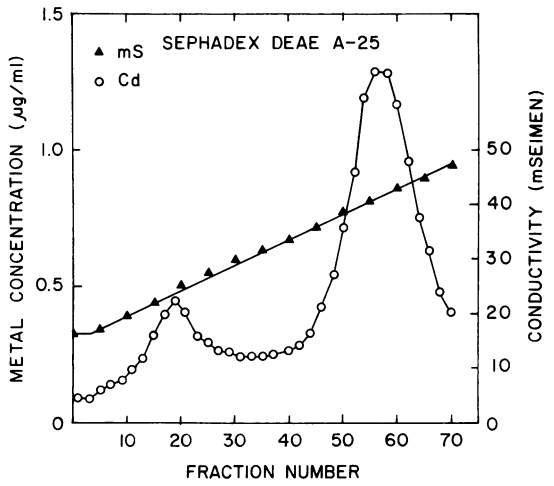

FIGURE 1. DEAE-Sephadex A-25 chromatography of CdBP from *Euglena gracilis*. The applied sample (333 μg of Cd^{2+} in 9.8 mL was eluted with a 150 to 600 mL KCl gradient buffered at pH 8.6 with 50 mM Tris-HCl and containing 1 mM 2-ME: (○) Cd; (▲) conductivity.

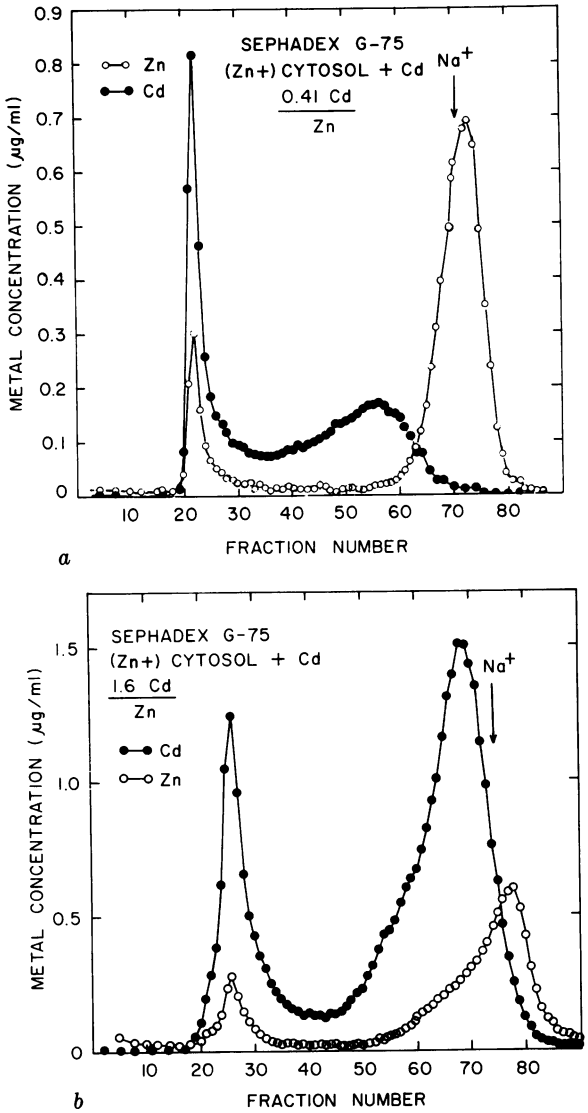

FIGURE 2. Sephadex G-75 chromatography of cytosol after *in vitro* addition of (*a*) 0.4 Cd/Zn and (*b*) 1.6 Cd/Zn, based on total cytosolic zinc content. 50 mM Tris-HCl buffer, pH 8.6; (*a*) 2.0 mL sample and 4.0 mL fractions; (*b*) 5.0 mL sample and 3.8 mL fractions.

what levels of Cd^{2+} does induction occur; and (3) whether the presence of the BP is toxic or protective to the cell. The ability to bind cadmium is the only known function and the most unique and easily determined property of the BP. Therefore, the presence of BP in cells grown in Cd-free media was sought by addition of Cd^{2+} to the cytosol before fractionation. When the added Cd^{2+} was 40% of the total cellular zinc, a substantial amount of Cd bound to high molecular weight species (0.097 μg/10^8 cells). A distinct Cd band at K_d = 0.67, close to the normal elution profile of CdBP, was present (Fig. 2*a* and Table 2) and contained most of the cadmium. When the Cd/Zn ratio was 1.6, the high molecular weight Cd species remained constant, while the band at 0.67 became a shoulder on a large very low molecular weight band of Cd, K_d = 0.88 (Fig. 2b). Interestingly, the amount of high molecular weight cadmium is comparable to the amount found after *in vivo* exposure to high levels of Cd^{2+} in the media. The formation of the band at K_d = 0.67 suggests the presence of apo-BP in the cell cytosol. When cells were grown in media containing 0.4, 1.3, and 4.7 μg/mL of Cd^{2+}, the cytosol of the cells from the two lower concentrations contained no measurable amounts of CdBP (Fig. 3). Only at the highest level did the Cd Appear in the BP fractions. At the lowest levels of Cd^{2+}, the cytosolic Cd appears exclusively in the high molecular weight bands, which have Cd/Zn mole ratios of 0.1 and 0.4. There is clearly no CdBP present in the cytosol. For the higher concentration, which yields the CdBP, the mole ratio is 0.9. (Note that Fig. 3 is plotted as μg/mL and not in molar units.) Possible explanations of these observations are (1) the BP may only be inducible by high levels of Cd^{2+} in the cell or external media or (2) the BP may have a weak affinity for Cd^{+} and may bind it only

Table 2. Distribution of cytosolic zinc and cadmium (μmole/10^8 cells) of *Euglena gracilis* grown in media with controlled cadmium levels and after *in vitro* addition to homogenates.

Cadmium added		Cadmium, μmole/10^8 cells			Zinc, μmole/10^8 cells		
Media, μg/mL	Homogenate, Cd/Zn	HMW	BP	VLMW	HMW	BP	VLMW
0.4	0	0.01	a	a	0.8	a	0.21
1.3	0	0.03	a	a	0.07	a	0.27
4.7	0	0.11	0.33	a	0.12	a	—
0	0.46	0.097	0.10	a	0.023	a	0.28
0	1.6	0.11	0.31	a	0.031	a	0.19

[a] Below the limits of detection.

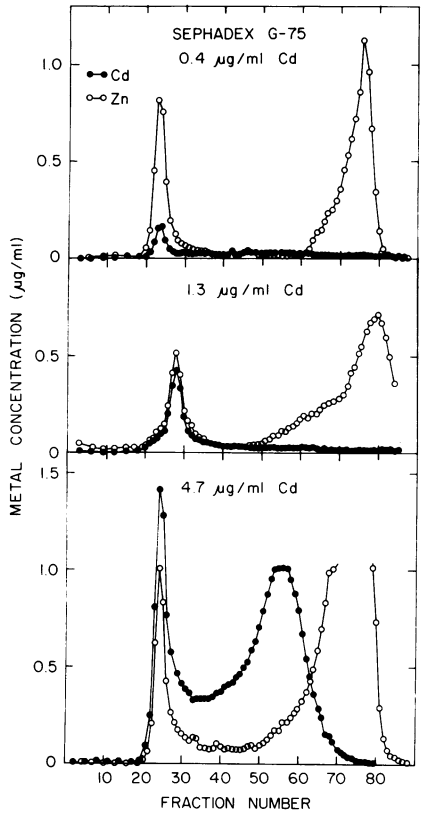

FIGURE 3. Sephadex G-75 chromatographic profiles of cytosol of cells exposed for 6 days to 0.4 (top), 1.3 (middle), and 4.7 (bottom) μg Cd/mL added to the media. For each sample equal amounts of cytosolic protein were applied. Fraction volumes 4.4 (top), 3.9 (middle), and 4.1 mL (bottom). Conditions as in Fig. 2.

after stronger binding sites in the hmw fractions are saturated.

Reaction with EDTA

Recent work on mammalian MTs has established that these proteins are dynamic and reactive entities (*11,16–18*) in contrast to the earlier view that they are inert metal-sequestering agents (*10*). A variety of ligands, competing metals, and electrophiles are able to effect displacement of metals from metallothioneins. Since the *Euglena gracilis* BP appeared to be more labile than MT and to differ in characteristic properties such as size and charge, EDTA was used to probe its reactivity. Rate constants for the CdMT reaction with EDTA are known (*16*).

Samples of the Sephadex G-75 BP preparation were reacted with a 0.4 mM EDTA solution. Since the EDTA is present in large excess, pseudo-first-order kinetic treatment was used to determine the reaction order with respect to cadmium bound to the protein. The semilogarithmic plots of absorbance versus time yielded two steps in pseudo-first-order rate constants of $4 \pm 3 \times 10^{-4}$ sec^{-1} and $7 \pm 2 \times 10^{-5}$ sec^{-1}. Approximately one-third of the cadmium reacts in the fast step and two-thirds in the slow step. Thus both steps have a dependence on the CdBP:

$$\text{rate}_f = k_f[\text{CdBP}]$$

and

$$\text{rate}_s = k_s[\text{CdBP}]$$

These data establish that EDTA reacts more rapidly with the CdBP than the mammalian MT, for which the pseudo-first-order rate constant under comparable conditions (1 mM EDTA) shows a single step with a rate constant of $2–4 \times 10^{-6}$ sec^{-1}.

The slow step observed here is an order of magnitude faster than for mammalian MTs and the fast step is two orders of magnitude faster. These data provide striking quantitative confirmation of the qualitative observation of the labilty of Cd in this protein.

pH Titrations

The thiol group of cysteine residues, which are expected to play a major and possibly exclusive role in metal binding, lose their protons upon binding cadmium. Thus, pH titrations can be used to assess the strength of metal binding:

$$\text{CdBP} + n\text{H}^+ \overset{K_b}{\rightleftharpoons} \text{H}_n\text{BP}^{+n-2} + \text{Cd}^{2+}$$

For mammalian MTs, the half-titration point (i.e., the pH at which half the bound metal is displaced by protons) is 4.5 for Zn^{2+} and 3.2 for Cd^{2+} (*19*). Aliquots of the BP were subjected to progressively smaller pH values and quickly fractionated at that pH (Fig. 4). By pH 5–6, the half-titration point was reached. At pH 3, which is close to the half-titration point of MT, the cadmium is completely displaced from the BP.

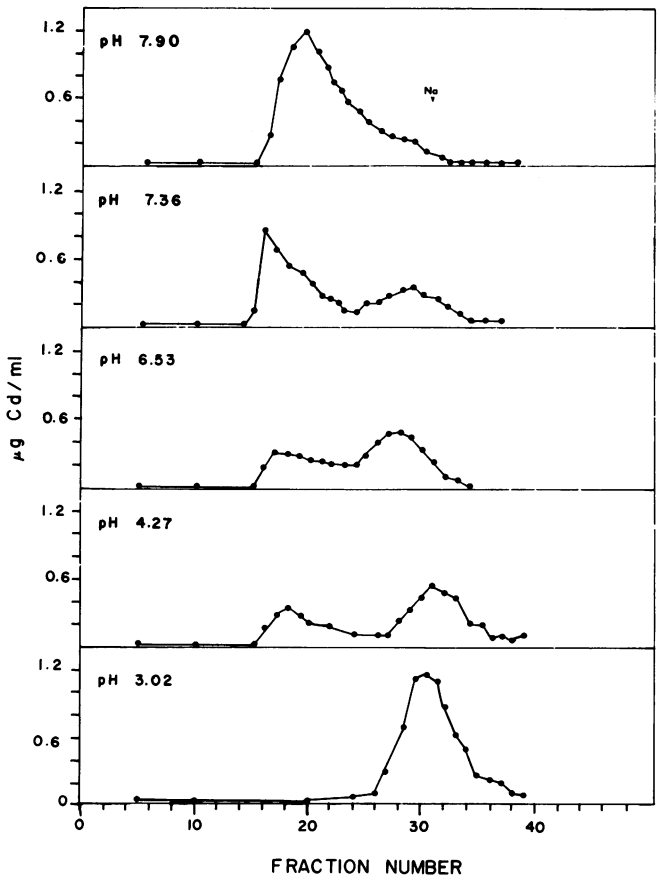

FIGURE 4. Effect of pH on CdBP. Aliquots (1.0 mL) of CdBP were adjusted to the pH of the column, incubated 10 min, then chromatographed over Sephadex G-25 using 100 mM phosphate buffer.

The stoichiometry of protons bound per Cd^{2+} displaced is not known, and therefore, the binding constant cannot be calculated quantitatively, but the qualitative result, the much higher pH of the half-titration point, indicates a much weaker thermodynamic interaction between the BP and cadmium. Thus, the *Euglena gracilis* BP has an affinity for Cd that is much lower than the value of $K_b = 7 \times 10^{14}$ estimated for CdMt (*19*).

Radioimmunoassay

Isolated samples of CdBP-I and CdBP-II, which were shown to give only single bands by polyacrylamide gel-electrophresis, were subjected to competitive radioimmunoassay according to published methods (*12,13*). In this technique, a radiolabeled ^{125}I-rat liver MT and the increasing amounts of the competing ligand are assayed with antibodies raised against rat liver MT. The results of assaying Euglena CdBP-I and CdBP-II and, as a control, a mixture of rat liver MT-I and MT-II is shown in Table 3 and reported as the fraction Y of bound ^{125}I-labeled MT for various concentrations of competing ligand. The amount of the rat liver MT required to achieve $Y = 0.50$ was 0.94 pmole as determined by regression analysis. In contrast, using 100 pmole of CdBP-I or CdBP-II yielded $Y = 0.89$ and $Y = 0.98$, respectively. Thus, the CDBPs do not effectively compete with the radiolabeled antigen, and there is negligible cross-reactivity of the Euglena BPs with antibodies to mammalian MTs.

CdBP Synthesis in Dark-Grown Cells

Euglena are heterotrophic organisms which can grow under illumination or in the dark. Because much of the previous work on zinc deficiency in this organism was done in the dark, we also examined their ability to generate CdBP when grown under these conditions. Figure 5 shows the CdBP band which results from 6 days of growth with Cd^{2+} (5 μg/mL) in the media and without illumination. The zinc and cadmium distribution of control and cadmium-exposed cells are compared in Table 4. The cadmium in the high molecular weight band (180 nmole/10^8 cells) was marginally more than that observed in light-grown cells, and 85% of the cadmium (960 nmole/10^8 cells) was in the CdBP band. No cadmium was detected in the low molecular weight region. The distribution of zinc was altered, compared to the control cells, by a dramatic increase in the very low molecular weight zinc pool, 105 versus 1080 nmole/10^8 cells. Figure 4 shows that a similar but not so dramatic increase occurred with illuminated cells grown at comparable Cd levels.

Cytosolic Copper

Experiments were undertaken to determine whether high levels of copper in the media would act like cadmium, inducing a BP, or like zinc, appearing as a very low molecular weight species. Cells were grown for 10 days in media containing 10 μg/mL of added Cu^{2+} and attained a density of 5×10^6 cells, comparable to the density of cells in normal media after 5 to 6 days (*5*). Three bands of copper are evident after fractionating the cytosol over Sephadex G-75 (Fig. 6). The copper bound to the high molecular weight fractions greatly exceeded the zinc content: 180 versus 15 nmole/10^8 cells. The biggest pool of copper (300 nmole/10^8 cells), however, was in the region characteristic of metal-binding proteins and accounted for 43% of the cytosolic copper. A third major band was present in the very low molecular weight region and accounted for 32% of the copper. No zinc was bound to the CuBP and only a very small amount was present in the very low molecular weight region.

In additional experiments where cells were grown for 4 days on media containing 5 to 10 μg/mL of added copper, the concentration of copper in the high molecular weight region again greatly exceeded the zinc content, and the majority of the zinc was in the BP region. There was, however, no very low molecular weight copper band, and the very low molecular weight zinc was depleted in the cells on 5 μg/mL of copper and absent from those on 10 μg/mL.

Thus, copper does apparently induce and bind to some species of cytosolic protein similar in size and elution to the CdBPs. However, unlike cadmium-exposed cells, in which the very low molecular weight peak of zinc is com-

Table 3. Radioimmunoassays: fraction of ^{125}I-labeled rat MT-I angiten bound (Y) as a function of competing ligand.[a]

Competing agent	Y at competing antigen amount, pmole					
	0.1	1	10	100	1000	10000
CdBP-I			0.98		0.89	0.88
CdBP-II					0.98	0.99
CdBP (G-50)					1.03	1.03
50/50 Rat MT-I and MT-II	0.87	0.49	0.41			

[a] Values of responses with 50/50 mix of Rat MT-I and MT-II as competitor derived from standard curve [linear-log regression relating to response Y to Q (log pmole competing antigen)]: $Y = 0.490–0.377\ Q$. SEM of Y values greater than 0.90 is ± 2%.

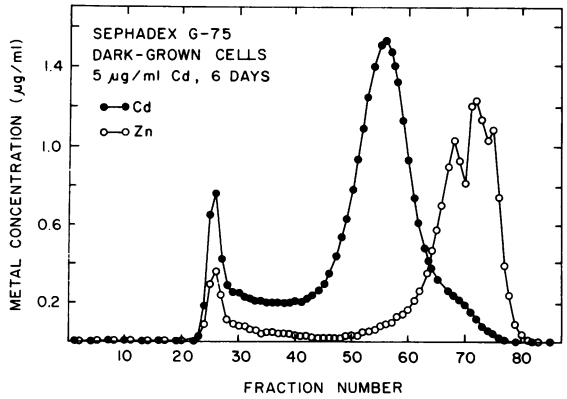

FIGURE 5. Sephadex G-75 chromatographic profile of (○) cytosolic Zn and (●) Cd from cells grown without illumination for 6 days in media containing 5 μg Cd/mL. Applied sample: 2.5 mL. Fractions 3.4 mL. 50 mM Tris-HCl buffer, pH 8.6.

parable to or larger than that for control cells, the very low molecular weight zinc band is diminished by copper exposure.

An unexpected observation in the course of this study was that the cytosol of the copper-exposed cells was greyish-black in color. This contrasts with the light green color of the cells grown in normal media or with 5 μg/mL of Cd. On exposure of the cytosol to air, the color fades, and eventually the green color of normal cells is obtained. The EPR spectra of frozen samples of the grey-black cytosol are almost featureless, suggesting that Cu(I) is present as the sample is oxidized by air exposure, with concomitant loss of the grey-black color, a characteristic signal for Cu(II) is apparent after 1 hr and very intense after 5 hr. The loss of the black color is apparently due to oxidation of some unidentified Cu(I) species to copper (II).

Discussion

As metal-binding proteins in lower organisms are investigated in greater detail, it becomes increasingly clear that they are not simply homologues of mammalian MTs, but a diverse and complex array of small proteins and peptides, varying in structure, chemical reactivity and physical properties. For example, in rainbow trout (*Salmo gairdneri*), four binding proteins have been identified: two resemble MT and are present only after injecting cadmium into the fish and two are present after exposure to environmental cadmium but do not resemble MTs (*6*). The blue-green algae Synechoccus (sp) and the green alga *Chlorella ellipsoidea* each had only a single form of BP (*8,20*), in contrast to the inevitable two (and possibly more) isoproteins of mammalian MTs. Oligomeric cadmium-containing aggregates of cysteine-rich penta- and heptapeptides (cadystin A and B, respectively) have been isolated from the fission yeast *Schizosaccharomyces pombe* after cadmium exposure (*9*). Thus, the results presented here demonstrating significant differences in physiological behavior, structure, and chemical reactivity between the Euglena BP and mammalian MTs are not unexpected.

Turning first to its physiological significance, the BP could be isolated with an appreciable zinc content only after exposure to high levels of cadmium (> 4.7 μg/mL) and with the use of 2-ME in the preparation. Following this finding, we repeated our earlier efforts to isolate a ZnBP (*5*) by exposing the cells to 20 μg/mL of zinc in the media and using 2-ME to stabilize any resulting ZnBP during the preparation. The results, however, were negative. While the possibility of losing zinc from the BP during isolation attempts cannot be unambiguously eliminated, the variety of conditions employed, including an anaerobic atmosphere of N_2 previously reported (*5*), are sufficiently varied and stringent to support the conclusion that in Euglena the very low molecular weight zinc pool is a real phenomenon. Under zinc deficient conditions the very low molecular weight Zn is analogous in behavior to zinc MT of Ehrlich ascites cells (*5*). At present, there is no evidence to support a role for the BP in zinc metabolism. The formation of CdBP when media cadmium lev-

Table 4. Comparison of cytosolic metal distribution for control and Cd-exposed cells grown in the dark.

Media Cd, μg/mL	Zinc, nmole/10^8 cells			Cadmium, nmole/10^8 cells		
	HMW	BP	VLMW	HMW	BP	VLMW
0[a]	66	c	105	—	—	—
5[b]	120	c	1080	170	960	c

[a] Harvested on day 6.
[b] Harvested on day 7.
[c] Below the limits of detection.

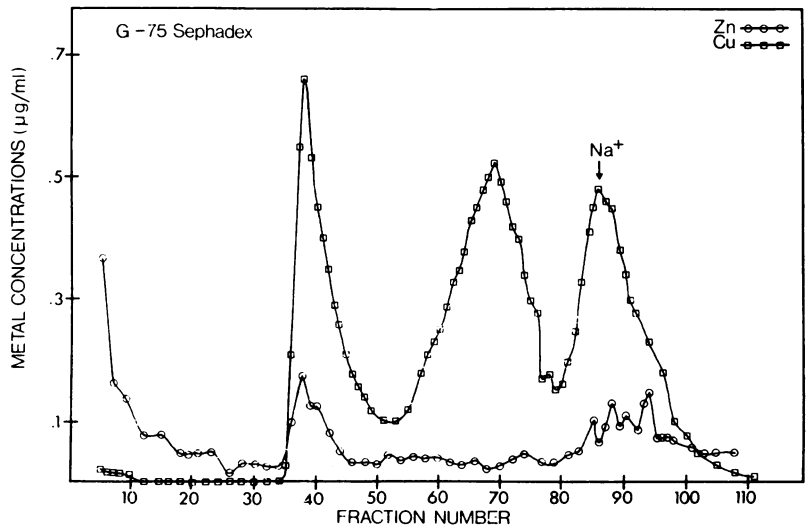

FIGURE 6. Sephadex G-75 chromatographic profile of (□) Cu and (○) Zn in the cytosol of *Euglena gracilis* grown for 10 days in media containing 10 μg Cu/mL. 50 mM Tris-HCl buffer, pH 8.6, was used to elute the column.

els were high, but its absence at 1 μg/mL or less of Cd^{2+} are indicative of a role during acute cadmium exposure, but not during low level, chronic exposure. This result could reflect induction of Cd^{2+} only at very high media and intracellular Cd^{2+} levels. Alternatively, it may result from a low affinity of the BP for Cd^{2+}. When intracellular concentrations are low, cadmium is bound almost exclusively to high molecular weight species. It only appears in the BP fractions after a level of ca. 11 μmole Cd/10^8 cells has been achieved. In support of the proposal that the BP has a low affinity for Cd^{2+} is the finding that *in vitro* addition of Cd^{2+} leads to a saturation of high molecular weight binding sites at levels of Cd^{2+} (10–11 nmole/10^8 cells) similar to those achieved *in vivo*, and to the presence of Cd in the BP fraction. These data suggest that the BP is constitutive and may have other biological functions or at least be involved in other equilibria which render it less available to bind intracellular Cd^{2+}.

The thermodynamic, kinetic and structural properties observed here substantiate and extend our previous findings (*5*) that the Euglena BP is smaller and more negatively charged than metallothioneins. Table 5 compares and contrasts the properties of the BP and mammalian MTs. Clearly, although the BP is a cysteine-rich metal-binding protein, it has a unique set of chemical and physical properties and is not simply a homologue of the MTs.

Whereas half the cadmium of MT is displaced at approximately pH 3, that point is reached by pH 5 to 6 for the BP (Fig. 4), indicating that much lower concentrations are necessary to compete for the Cd-binding sites. Thus, the pH titration provides direct chemical evidence that the equilibrium constant, K_b for the reaction

$$Cd^{2+} + H_nBP \overset{K_b}{\rightleftharpoons} CdBP + nH^+$$

is lower than the value 7×10^{14} which is characteristic of mammalian MTs (*19*).

Similarly, the EDTA reaction provides evidence of increased kinetic lability for the CdBP. Extraction of Cd from CdMT or Cd,ZnMT by 1 mM EDTA results in a pseudo-first-order rate constant of $2–4 \times 10^{-6}$ sec^{-1}, which accounts for over 90% of the total reaction (*16*). The slow and fast reactions of the Euglena BP, comprise two-thirds and one-third of the cadmium removed and are 10 and 100 times faster, respectively. These differences in the rates of reaction must arise from substantial structural changes between the MT and the CdBPs. The two steps for the Euglena BP may be due either to the two forms of BP, one more labile than the other, or to two classes of binding sites present in both BP-I and BP-II.

Table 5. Comparison of Euglena BP and mammalian metallothioneins.

Property	Euglena CdBPs	Metallothioneins
Apparent molecular weight	~8000	10,000 daltons
Molecular weight	?	6000
DEAE elution (conductivity mS)	20–40 mS	6–10 mS
Cysteine content	High	33–34%
1/2 titration pH	5–6	3–4
EDTA pseudo-first-order rate constants (% reacting) [EDTA]	4×10^{-4} sec^{-1} (~33%) 7×10^{-5} sec^{-1} (~67%) [0.4 mM EDTA]	Very fast (10%) $2–4 \times 10^{-6}$ sec^{-1} (90%) [1 mM EDTA]
Inducibility	High (Cd^{2+})	Low (Cd^{2+})
Forms	CdBp-I and CdBP-II	MT-I and MT-II
Cross-reactivity to rat liver MT antibody	No	Yes

Further studies to resolve this question are in progress.

The radioimmunoassay of BP-I and BP-II against rat liver metallothionein antibodies indicated essentially no cross-reactivity. The antigenic sites of metallothionein are sequential. The immunodominant site involves residues 20–26; a secondary site involves residues 1–5 at the (acetylated) amino terminal. The antigenicity of various metallothioneins is not significantly altered by dramatic structural changes such as replacing zinc or cadmium with gold (*15*) or by removal of metals and oxidation of the thiols to cysteic acid groups (*18*). Thus, the absence of cross-reactivity of the Euglena BP-I and BP-II to the antibodies cannot be attributed to conformational differences and most likely indicates that the antigenic site is not present. Interestingly, the immunodominant determinate sequence includes two to three lysines, and the absence of these and perhaps other lysines from the BP sequence may contribute to the increased negative charge found by ion-exchange chromatography.

The high conductivity required for DEAE elution, the lower apparent molecular weight, and the absence of cross-reactivity with MT antibodies all point to substantial differences in amino acid sequence for the BPs. One would expect on this basis that the sites of metal binding would differ from those of the three- and four-metal cluster (*21*) of MT. Alterations in the metal-binding structures should give rise to changes in the equilibrium binding constants for Cd and to the rates of reactions with competing metal ligands, as have been reported here.

Finally, changes in the structure and reactivity may indicate a physiological role of the CdBP in Euglena that is very different from that of MT in mammalian kidney and liver. The failure of Cd^{2+} to bind to the BP upon low level (< 1 μg Cd/mL) exposure supports the hypothesis of altered function. Also, the failure of zinc to bind, except in the presence of bound cadmium, may also reflect another function. Clearly, continuing efforts to unravel the structure, chemical reactivity and function of the BP are warranted not only by their intrinsic interest, but also because of the light they can shed on MT structure and function in relation to human metal metabolism and health.

The authors (CFS, DHP, DJG and DNW) wish to acknowledge support from the NIEHS Aquatic Biomedical Research Center, (ES-01985) and the UWM Graduate School. The radioimmunoassays (JSG) were performed with support from NIH (NIEHS 01629).

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