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Cadmium-Binding Component in *Escherichia coli* During Accommodation to Low Levels of This Ion

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An inducible cadmium-binding protein was isolated from *Escherichia coli* cells accommodated to 3×10^{-6} M Cd^{2+} but not from normal or unaccommodated cells. Sephadex G-100, metal chelate affinity chromatography, and disc gel electrophoresis were used in the purification procedure. The molecular weight of the Cd^{2+}-binding protein was estimated to be about 39,000 by Sephadex G-100 chromatography, making it different from the conventional, much smaller metallothionein.

Escherichia coli cells accommodate to low levels of Cd^{2+} after a long lag (9). Upon exposure to Cd^{2+}, the cells suffer extensive single-strand breakage in their deoxyribonucleic acid, and by 3 h 85 to 95% of the cells lose their ability to form colonies (8). Subsequently, deoxyribonucleic acid is repaired by ligation (7), the ability to form colonies returns, and the cells proliferate (8). Although a considerable amount of Cd^{2+} is associated with accommodated cells, this inhibiting ion does not affect the cellular level of alkaline phosphatase, a zinc metalloenzyme (9). This observation suggests that the cells have to compartmentalize the toxic ion in order to resume normal metabolic functions. In mammals, a Cd^{2+}-binding inducible protein, metallothionein, appears to serve this purpose (1), but there is no evidence for the existence of such a protein in *E. coli*. Although Mitra et al. (9) reported the presence of a high-molecular-weight Cd^{2+}-binding component(s) in accommodated cells, this component(s) was not characterized. In this research, we tried to isolate and partially characterize the component(s) to which Cd^{2+} is bound, as an initial effort toward defining the nature of the compartmentation of this toxic ion as a component of the accommodation mechanism.

(The results were taken from a thesis submitted by M.B.K. to the University of Michigan, Ann Arbor.)

MATERIALS AND METHODS

Organism and growth conditions. *E. coli* strain B was used in this work. The composition of the synthetic medium and the conditions for culture of the cells have been described previously (9). Cells taken from a culture which had been growing for 15 h were inoculated into growth medium containing 3×10^{-6} M Cd^{2+} and were grown on a shaker at 37°C.

Subcellular fractions. Cells were harvested at the midlog phase of growth, washed three times with 10^{-2} M tris(hydroxymethyl)aminomethane (Tris) hydrochloride (pH 8.0) at 4°C, and resuspended (1 g [wet weight] per 40 ml) in the same buffer. The cells were broken by passage through an Aminco French pressure cell at the maximum working pressure (2×10^4 lb/in^2), and the subcellular fractions were obtained by the method of Schnaitman (12).

Gel filtration of cytoplasmic extract by column chromatography. A cytoplasmic extract was prepared by the method of Mitra et al. (9). The soluble fraction was applied to a column (2.5 by 90 cm) of Sephadex G-100 previously equilibrated with 10^{-3} M Tris-hydrochloride (pH 8.6) containing 0.05 M NaCl. The column was eluted with the same buffer in 5-ml fractions.

Metal chelate affinity column chromatography. A column of biscarboxymethyl amino agarose loaded with $^{109}Cd^{2+}$ was prepared as described by Porath et al. (11) and was used for the affinity chromatographic separation of Cd^{2+}-binding proteins from other proteins. Before it was submitted to this step, lyophilized protein was incubated for 1 h in 0.02 M ethylenediaminetetraacetate (1 mg of protein per ml) at 22°C to remove any Cd^{2+} and then dialyzed exhaustively for 18 h in 10^{-2} M Tris-hydrochloride (pH 8.6) at 4°C. The dialyzed solution was passed through the affinity column (1 by 50 cm), and elution was started with 10^{-3} M Tris-hydrochloride (pH 8.6). After 25 fractions of 5 ml each were collected, elution was continued with 0.5 M NaCl-10^{-3} M Tris-hydrochloride (pH 8.6). The column was operated at 4°C.

Gel electrophoresis. Polyacrylamide gel electrophoresis and gel staining for protein were performed by the method of Weber and Osborn (14). For determinations of radioactivity, gel slices were digested overnight in vials containing 0.5 ml of NCS solubilizer. Then, 10 ml of toluene-based scintillation fluid was added, the vials were shaken, and radioactivity was determined with a Packard liquid scintillation spectrometer.

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Analytical procedures. Protein was determined by the method of Lowry et al. (5), and cadmium levels were estimated by atomic absorption spectrometry with a Techtron AA120 instrument.

Reagents. Crystalline $3CdSO_4 \cdot 8H_2O$ was from Allied Chemicals. Carrier-free $^{109}CdCl_2$ (concentration, 2 mCi/ml) was obtained from New England Nuclear Corp. Sephadex G-100 and Sepharose 4B were purchased from Pharmacia Fine Chemicals. The disodium salt (monohydrate) of aminodiacetic acid was obtained from Aldrich Chemical Co.

RESULTS

More than 90% of the $^{109}Cd^{2+}$ added was found to be associated with accommodated cells. Cellular fractionation by the procedure described by Schnaitman (12) revealed that 80% of this Cd^{2+} was present in the cytoplasmic extract (Table 1). Two Cd^{2+}-containing macromolecular peaks were observed when the extract from accommodated cells was fractionated on a column of Sephadex G-100 (Fig. 1B). In contrast, the cytoplasmic extract from unaccommodated cells showed only the first Cd^{2+}-containing peak (Fig. 1A), although the protein profiles were not different in the two cases. In accommodated cells, peak II contained more than 60% of the total bound Cd^{2+} in the cytoplasmic fraction. Peak II was resolved by polyacrylamide gel electrophoresis into a number of components, which were stained with Coomassie blue. All of the bound Cd^{2+} appeared in one of the stained bands when the material was obtained from accommodated cells (Fig. 2B). This component contained no Cd^{2+} when peak II from nonaccommodated cells was electrophoresed (Fig. 2A).

The Cd^{2+}-binding material in peak II was separated from non-Cd^{2+}-binding protein by metal chelate affinity chromatography (Fig. 3B). This chromatographic step also separated the Cd^{2+}-binding material into at least two labeled components. Peak b (Fig. 3B) from the metal chelate affinity column was labeled in preparations from both accommodated and unaccommodated cells and therefore was probably different from the material in peak c, which was observed only in preparations from accommodated cells. Upon disc gel electrophoresis, the major protein component of peak c material (Fig. 3B) was resolved into a protein (determined by the staining method) and a Cd^{2+}-containing component (Fig. 4A); however, the protein peak did not coincide with the Cd^{2+} peak. It is possible that the level of protein present in the Cd^{2+}-containing component was too low to be detected by the staining technique. To confirm the presence of protein in the Cd^{2+}-containing component, ^3H-amino acids (i.e., protein hydrolysate) were included in the

TABLE 1. *Protein and Cd^{2+} distributions in cellular fractions of E. coli cells accommodated to 3×10^{-6} M Cd^{2+}* [a]

Fraction	Amt of protein (mg/culture)	Amt of Cd^{2+} (μg/culture)	% of Cd^{2+}	Sp act (μg of Cd^{2+} per mg of protein)
Whole cell	45.0	9.8	100.0	0.22
Cell wall	29.4	1.3	13.4	0.05
Cell membrane	11.0	0.40	3.9	0.035
Cytoplasm	5.0	7.8	79.6	1.56

[a] Subcellular fractions were obtained by the method of Schnaitman (12). Cells were harvested at the midlog phase of growth, washed, and disrupted in a French pressure cell (for details, see text).

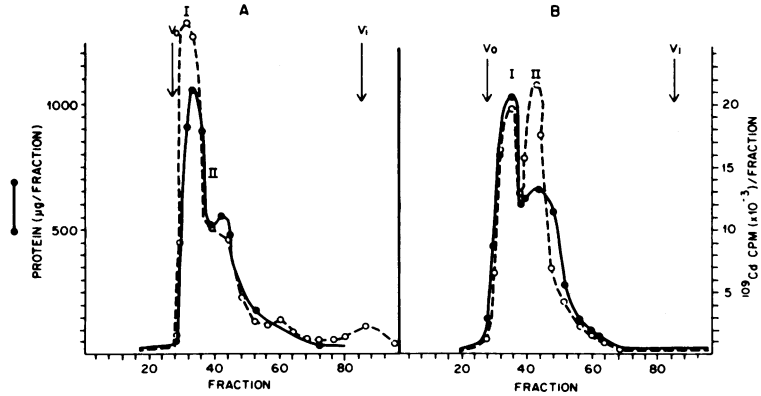

FIG. 1. *Gel filtration of cytoplasmic extracts from unaccommodated cells (A) and Cd^{2+}-accommodated cells (B) on Sephadex G-100. The cells were harvested, and cytoplasmic extracts were prepared by the method described in the text. Each high-speed supernatant was incubated with carrier-free $^{109}Cd^{2+}$ for 30 min at 37°C (13). A 10-ml portion of labeled extract (20 mg of protein, 3.5×10^5 cpm) was layered onto a Sephadex G-100 column, and the material was eluted with 10^{-3} M Tris-hydrochloride–0.05 M NaCl (pH 8.6) at 4°C. V_o, Elution volume for blue dextran 2000; V_i, elution volume for riboflavin.*

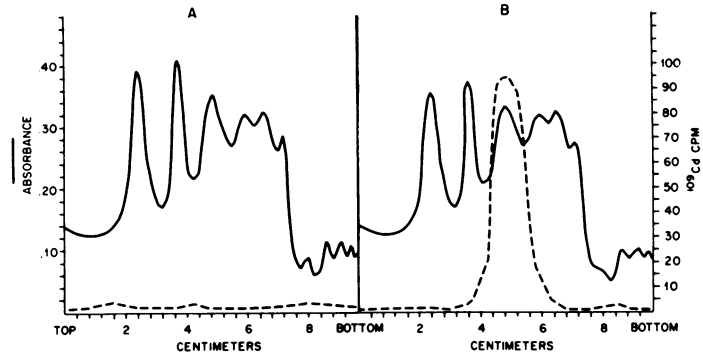

FIG. 2. *Polyacrylamide gel electrophoresis of the material from peak II from a Sephadex G-100 column separation of extracts from unaccommodated cells (A) and Cd^{2+}-accommodated cells (B). Electrophoresis was carried out in 7% polyacrylamide gels. Samples in glycerine were layered onto gels (200 μg of protein, 2 × 10^3 cpm). Gels were stained overnight with 0.1% Coomassie blue, destained by diffusion in acetic acid, and scanned at 540 nm with a Gilford gel scanner, using a 0.05-mm slit. The radioactivity in the sliced gels was measured by the methods described in the text.*

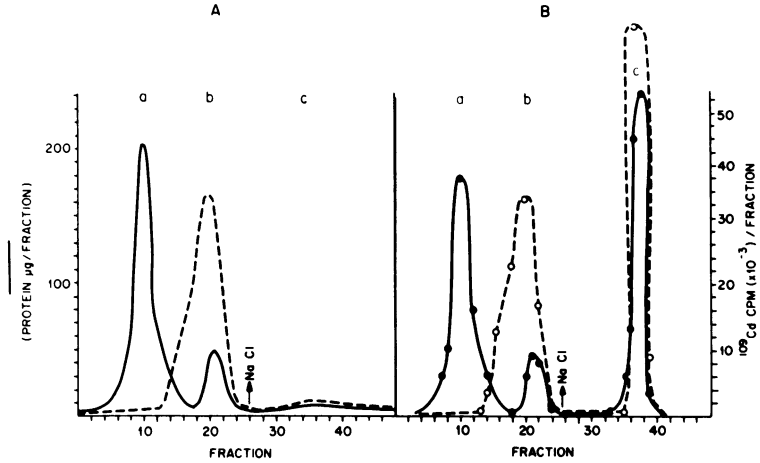

FIG. 3. *Metal chelate affinity chromatography of peak II from a Sephadex G-100 column separation of extracts from unaccommodated cells (A) and Cd^{2+}-accommodated cells (B). The lyophilized material from peak II of a Sephadex G-100 column was applied (8 mg of protein) after incubation for 1 h with 0.02 M ethylenediaminetetraacetate and overnight dialysis against 10^{-2} M Tris-hydrochloride (pH 8.6) buffer. Material was eluted with 10^{-3} M Tris-hydrochloride (pH 8.6) and then with 10^{-3} M Tris-hydrochloride containing 0.5 M NaCl (pH 8.6) at 4°C. Fractions (5 ml) were collected, and radioactivity was measured.*

growth medium, and the cells were allowed to accommodate to Cd^{2+}. When the same isolation procedure and disc gel electrophoresis were used, the ^3H-amino acids were shown to be incorporated into a major protein peak which contained no $^{109}Cd^{2+}$; most of the $^{109}Cd^{2+}$ was present with a minor peak of ^3H (Fig. 4B). The molecular weight of the Cd^{2+}-binding protein was estimated to be about 39,000 by a semilogarithmic plot of K_{av} values calculated from elution of this material, as well as purified samples of ribonuclease A, chymotrypsinogen A, and ovalbumin from Sephadex G-100 (2). The K_{av} for the Cd^{2+}-binding protein was 0.30, whereas the K_{av} for ovalbumin was 0.27 and the K_{av} for chymotrypsinogen was 0.42.

DISCUSSION

Exposure of *E. coli* to 3 × 10^{-6} M Cd^{2+} in minimal medium results in nicking of the deoxyribonucleic acid and an abnormally long lag phase, but once deoxyribonucleic acid repair and proliferation begins, the doubling time (55 min) and the final size of the culture do not differ from control values (9). Estimates of the amount of Cd^{2+} which was associated with Cd^{2+}-accommodated cells indicated the presence of 0.7 μg of Cd^{2+} per mg of cellular protein when 350 μg of

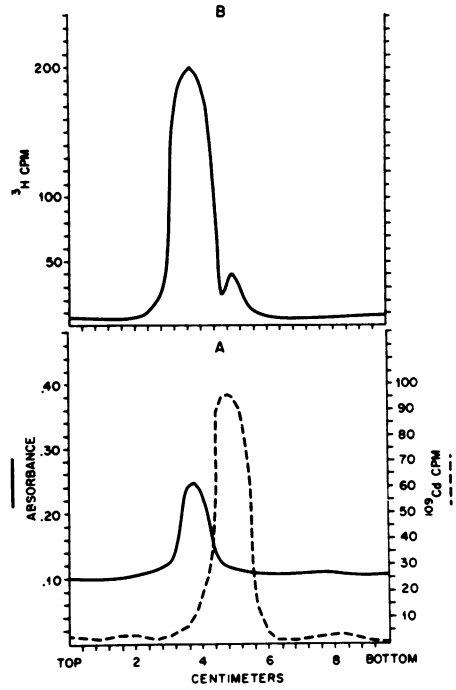

FIG. 4. *Polyacrylamide gel electrophoresis of the material in peak c from the metal chelate affinity column. (A) Cells were grown in a medium containing 3 × 10^{-6} M Cd^{2+} (A) or in a medium containing 3 × 10^{-6} M Cd^{2+} plus 1 μCi of ^{3}H-protein hydrolysate (B). The cells were harvested at the midlog phase of growth. Cytoplasmic extracts were prepared and applied to a Sephadex G-100 column as described in the legends to Fig. 1 and 2. The protein of peak II was collected and lyophilized. After preparation of apoprotein as described in the text, this fraction was applied to the metal chelate affinity column prepared as previously described. Peak c from the metal chelate affinity column was collected and, after dialysis, was submitted to 7% polyacrylamide gel electrophoresis. Protein and radioactivity levels in the gel slices were determined as described in the text. In (A) absorbance represents a densitometric analysis of protein.*

Cd^{2+} was present in 150 ml of medium at the time of cell inoculation (9). Resumption of cell proliferation even though the cells contained a considerable amount of Cd^{2+} must mean that the cells could control their internal environment, possibly by binding Cd^{2+} with cellular macromolecules.

Specific binding proteins for Cd^{2+} are known in vertebrates (4), invertebrates, and eucaryotic microorganisms (10). The specific Cd^{2+}-binding protein known as metallothionein is characterized by (i) a low molecular weight, (ii) a high cysteine content, (iii) the absence of aromatic amino acids, and (iv) an affinity for other metals, such as mercury, zinc, and copper. Recently, the presence of metallothionein has been reported in procaryotic organisms, such as a blue-green alga (*Synechococcus* sp.) (10). Investigations using bacteria have not been successful in detecting the presence of metallothionein (10), although a considerable amount of Cd^{2+} was found in *E. coli* (9; F. I. MacLean, O. J. Lucis, Z. A. Shaikh, and E. R. Jansz, Fed. Proc. 31:699, 1972) grown in the presence of low levels of Cd^{2+}.

The estimation of Cd^{2+} levels by atomic absorption spectrometry in different subcellular fractions prepared by the French pressure cell technique indicated that 80% of the Cd^{2+} added was present in the cytoplasmic extract (Table 1). It has been observed by fractionation of cytoplasmic extracts obtained from cells grown in the presence of 3 × 10^{-6} M Cd^{2+} plus $^{109}CdCl_2$ by gel chromatography that cadmium is associated with at least two species of macromolecules. A similar result was obtained upon fractionation of an in vitro-labeled cytoplasmic extract from unlabeled Cd^{2+}-grown cells. Both in vivo and in vitro labeling experiments have suggested the presence of a high-molecular-weight Cd^{2+}-containing component that is synthesized during accommodation.

This report describes the presence of a Cd^{2+}-binding high-molecular-weight component in accommodated cells, which was not found in unaccommodated or normal cells. More than 60% of the total bound Cd^{2+} in the cells was associated with this component. It is possible that this Cd^{2+}-binding component is also present at an extremely low level in unaccommodated or normal cells but that its synthesis is increased in the presence of Cd^{2+}. The role of this component may be to sequester the Cd^{2+} present in the cells in order to allow the cells to resume normal metabolic functions. A determination of the localization of the Cd^{2+}-binding component(s) should aid in our understanding of the nature and biological significance of these proteins, and such an effort is in progress. A high-molecular-weight (30,000) component having a very high affinity for Cd^{2+} has also been reported in rat testes (3). Lucis et al. (6) reported that cadmium in mammary tissue is associated with a high-molecular-weight protein which is different from the metallothionein present in the livers, kidneys, and placentas of rats.

The material eluted from the metal chelate affinity column in peak c included a protein which did not contain $^{109}Cd^{2+}$. This was not surprising since the solution originally passed through the column was alkaline, a condition which favored less selective absorption than would have been the case at a lower pH (11). The $^{109}Cd^{2+}$-binding protein in peak c is the most interesting. However, the non-Cd^{2+}-containing

labeled protein was also found only in accommodated cells and therefore may be involved in the accommodation process. It would also be of interest to determine whether the appearance of these proteins represents a general response to heavy metals.

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