

Cadmium-Binding Proteins in *Pseudomonas putida*: Pseudothioneins

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Pseudomonas putida adapted to growth in 3 mM cadmium. The resistance mechanism involved complexation of cadmium in polyphosphate granules, changes in the structure of the cell membrane and induction of three cysteine-rich, low molecular weight proteins (3500–7000) containing 4 to 7 g-atoms per mole of cadmium, zinc, and copper. Each protein was produced during a different phase of growth, and the smallest protein (3500) was released into the environment when the cells lysed at the end of the exponential phase. The metal binding sites of the major protein were further characterized using a range of physical methods, including ^{113}Cd NMR. The properties of the bacterial pseudothioneins are compared to those of metallothioneins.

Introduction

Metallothioneins are a ubiquitous class of proteins and have been isolated from a wide range of animals and several microorganisms including yeast (1), protozoa (2), *Neurospora* (3) and cyanobacteria (4). We have recently shown that metallothionein-related proteins are present even in a member of the most primitive group of prokaryotes, the eubacteria (5,6).

Metallothioneins are implicated in metal homeostasis and detoxification (7). Bacteria have evolved a wide range of mechanisms for dealing with toxic levels of metals in their environment including methylation, precipitation, chelation, and exclusion (8,9). Cadmium resistance in *Staphylococcus aureus* has been investigated in detail and involves energy-dependent efflux of the metal (10,11).

In contrast, *Pseudomonas putida* actively accumulates cadmium from the growth medium, and the resistance mechanism appears to involve initial immobilization of cadmium in polyphosphate granules followed by the production of a series of low molecular weight cadmium-binding proteins (6): the pseudothioneins.

Such primitive organisms are likely to be useful for studying the relationship between structure and function of metallothionein-related proteins and the mechanism of their induction. Metal induction of these proteins is a topic of particular interest because of its potential use as a tool in genetic engineering.

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Isolation of Resistant Organisms and Adaptation to High Levels of Cadmium

Several organisms resistant to low levels of cadmium, copper, and zinc (0.5 mM) were initially isolated from sewage sludge. From these a strain was selected which grew in a chemically defined medium, in which >98% of

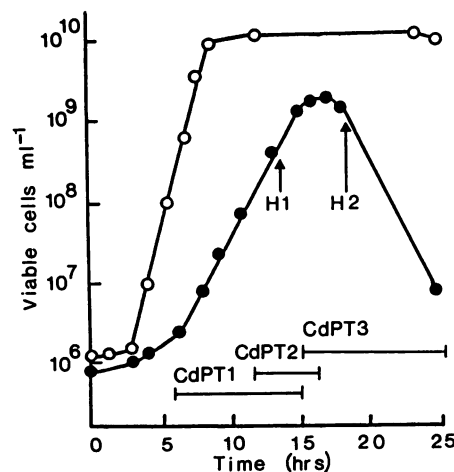


FIGURE 1. Growth curves for *P. putida*, control and cadmium-adapted cells. Control cells (○) were grown in the absence of added metals, and cadmium-adapted cells (●) in the presence of 3 mM cadmium and 60 μM zinc. The approximate time periods during which the three pseudothioneins were produced are indicated. The optimum harvest times for obtaining maximum yields of CdPT1 and CdPT2 (H1) and CdPT3 (H2) are also shown.

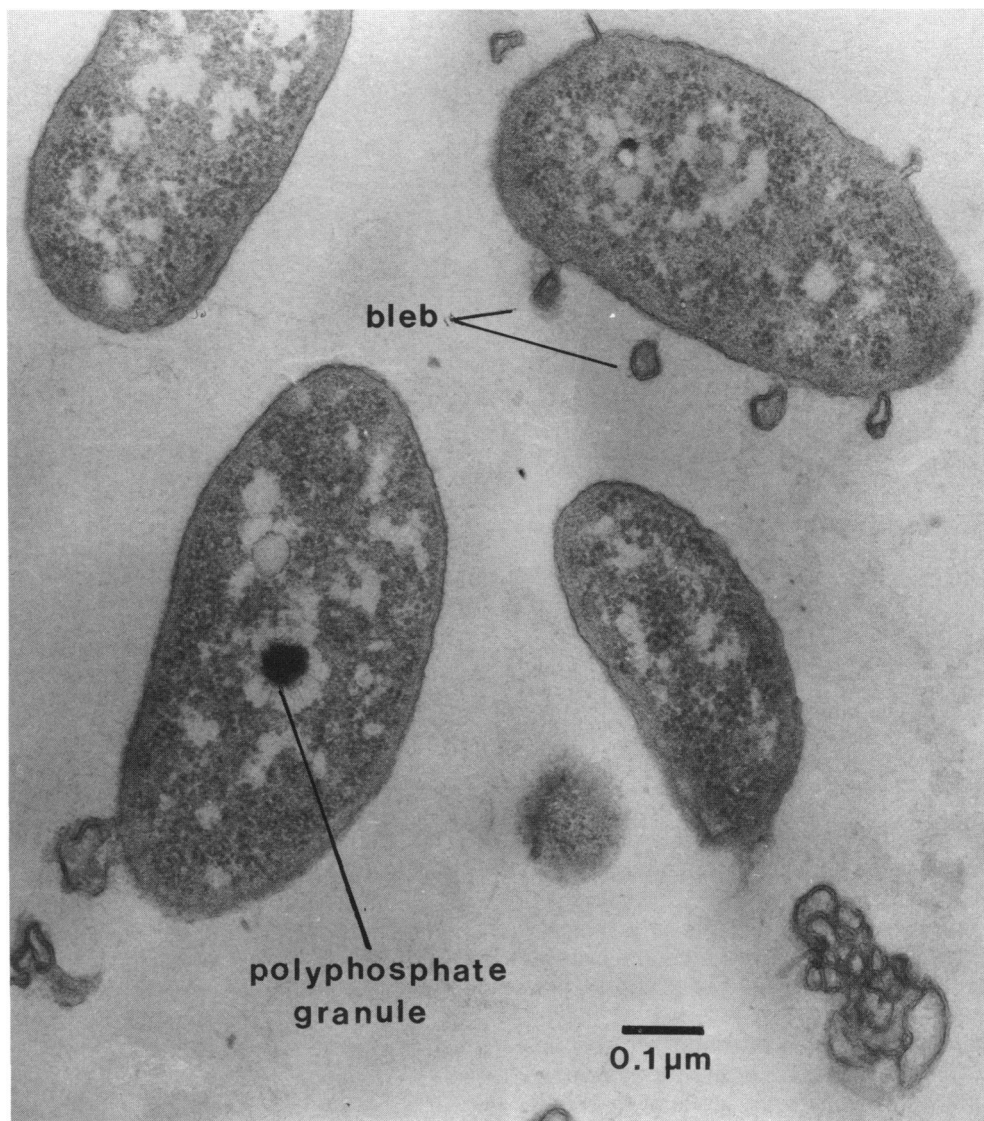


FIGURE 2. Transmission electron micrograph of lag phase cells of *P. putida*. Note the presence of black (osmium-stained) polyphosphate granules and "blebbing" of the cell membrane.

the cadmium was only weakly complexed and available to the bacteria. This organism was identified as *Pseudomonas putida* with a confidence level of 93%, but anomalously this strain of *P. putida* accumulated poly- β -hydroxybutyrate. Over a period of 8 weeks, the bacterium was repeatedly subcultured into media containing increasingly higher levels of cadmium until the bacteria had adapted to growth in 3 mM cadmium. Repeated subculturing did not result in selection of a cadmium-resistant mutant, as acquired resistance was lost when cadmium was omitted from the medium for a single subculture. Bacteria then had to "readapt" to 3 mM cadmium over a similar time course as control cells (12).

Adapted cells growing in 3 mM cadmium exhibited a long lag phase of ca. 6 hr (Fig. 1). The cell division time increased from 25 to 77 min, leading to a depressed and

prolonged exponential phase. This was followed by a brief stationary phase and then a rapid decline in the number of viable cells due to lysis (12).

Adapted cells had an increased zinc requirement compared to control cells, and additional zinc was actively accumulated from the medium (12). When the concentration of zinc in the growth medium was increased (from 0.6 μ M) to 60 μ M, the cell division time of adapted cells decreased to 54 min, but the rate of cell lysis increased (Fig. 1). A similar increase in the zinc concentration had no effect on the growth of control cells. Bacteria used for the extraction of metal-binding proteins were grown in media containing 3 mM cadmium and 60 μ M zinc.

Increased resistance to cadmium was accompanied by major changes in cell morphology. Adapted cells produced large "blebs" on the membrane (Fig. 2), were more

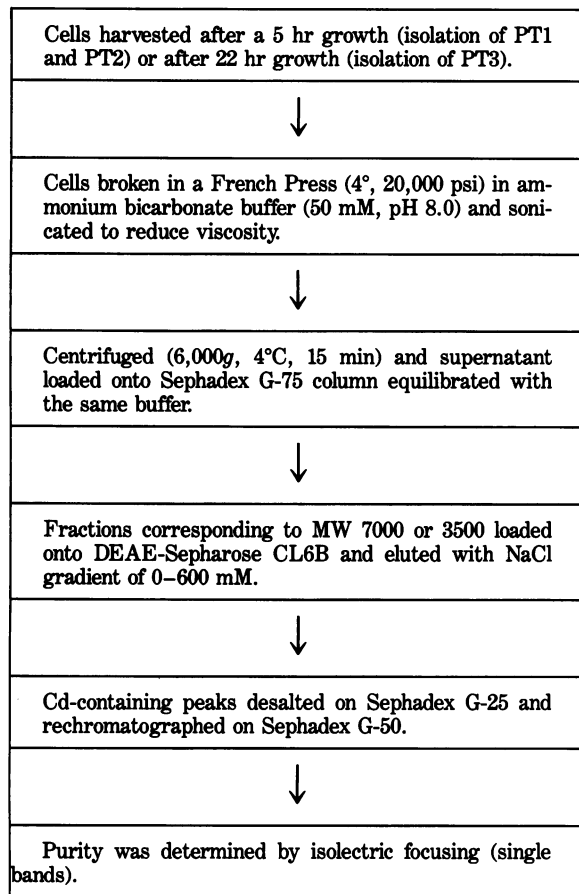


FIGURE 3. Purification protocol for pseudothioneins.

rounded than control cells, and were nonmotile. Removal of cadmium from the culture medium resulted in a reversion to rod-shaped, motile cells and the disappearance of blebs on the membrane, after a single overnight culture. The change in the morphology of the membranes of cadmium-adapted cells may be related to the large amount of cadmium localized in the cell envelope. Cadmium-adapted bacteria were more sensitive to certain antibiotics, including aminoglycosides, cyclic polypeptides, and chloramphenicol (12). This is presumably related to the observed changes in membrane structure. The outer membrane of Pseudomonads is normally thought to play an important role in excluding these antibiotics from the cell (13).

Table 1. Characteristics of pseudothioneins.

| Protein | Phase of growth | MW ^a | pI ^b |
|---------|------------------|-----------------|-----------------|
| CdPT1 | Exponential | 7239 | 8.3 |
| CdPT2 | Late exponential | 7216 | 3.5-4.5 |
| CdPT3 | Stationary | 3815 | 5.2 |

^a Minimum molecular weight from amino acid analysis.

^b Isoelectric points (pI) were determined by isoelectric focusing, except for CdPT2 (which did not stain with Coomassie blue) and its pI is based on the NaCl concentration required for elution from DEAE-Sepharose CL6B.

Metal-Binding Proteins

Bacteria resistant to 3 mM cadmium actively accumulated cadmium from the growth medium. At the end of the exponential phase the total cellular concentration of cadmium was 4.5 to 9 mM, of which ca. 60% was localized in the cell envelope. During the long lag phase the cytoplasmic cadmium was associated with polyphosphate granules and high molecular weight proteins (MW > 70,000). Toward the end of the lag phase, when the polyphosphate had been metabolized, the cadmium was associated with both high and low molecular weight proteins (MW 7000). After 10 hr growth, ca. 30% of the cytoplasmic cadmium was bound to low molecular weight proteins. Three cadmium-binding proteins were isolated

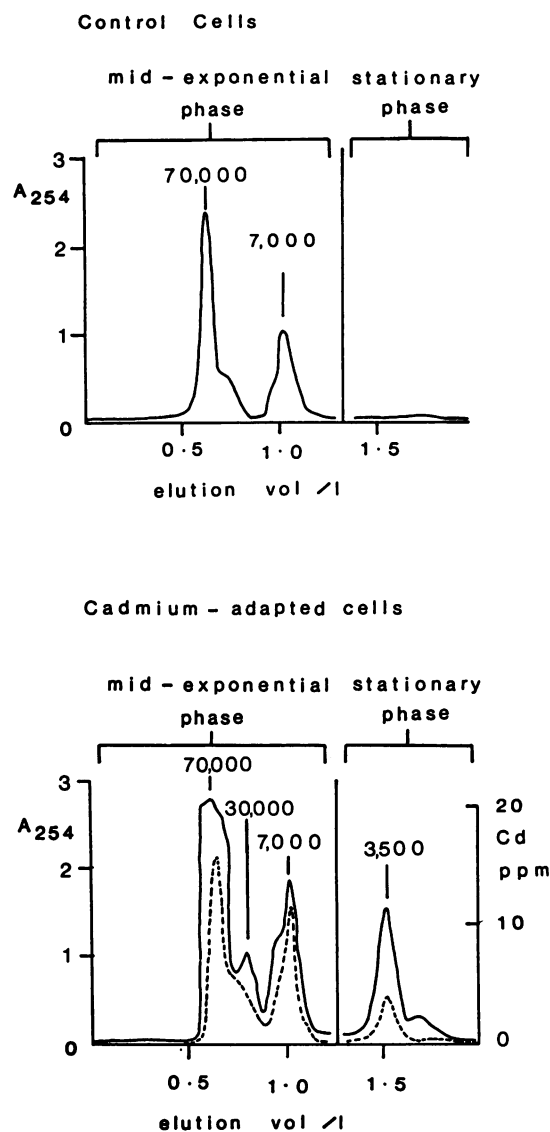


FIGURE 4. Gel-permeation chromatography cytoplasmic extracts from control and cadmium-adapted cells. Extracts from cells harvested either in mid-exponential phase or stationary phase were chromatographed on a Sephadex G-75 column (3 x 90 cm) equilibrated with ammonium bicarbonate buffer (50 mM, pH 8.0).

at different phases of growth (Table 1). CdPT1 and CdPT3 were about three times more abundant than CdPT2. CdPT3 was released into the growth medium when the cells lysed.

The protocol used for the isolation and purification of the three CdPTs is shown in Figure 3. In cadmium-adapted cells a greater proportion of the total cellular protein was found in the low molecular weight fraction (Fig. 4). In addition, during the late exponential and stationary phase a peak attributable to a new, very low molecular weight material was present in the G-75 chromatogram. This material could not be isolated from control cells, which suggests the induction of low molecular weight protein(s) by cadmium.

Further purification of G-75 fractions using DEAE-Sephrose (Fig. 5) showed that CdPT1, CdPT2, and CdPT3 were induced and were not present in detectable quantities in control cells grown in the absence of cadmium or zinc.

In common with mammalian metallothioneins the bacterial proteins contain cadmium, copper, and zinc. The copper content of the proteins is markedly reduced when the bacteria are grown in copper-depleted (Chelex-treated) medium, and the cadmium and zinc contents are correspondingly increased (Table 2). However, CdPT3, as isolated, does not appear to contain zinc.

CdPT1 and CdPT3 undergo facile oxidation in air. The oxidized proteins are yellow-brown, whereas the native proteins are colorless. Oxidation was prevented by purging solutions with nitrogen. Rechromatography of partially oxidized CdPT1 on Sephadex G-50, indicated the presence of small amounts of higher molecular weight bands, probably dimers and trimers.

Figure 6 shows a comparison of the amino acid composition of the pseudothioneins and human liver MTII. All three pseudothioneins have a lower Cys content than metallothionein (12–23% compared to 33%). Therefore, all the metals cannot occupy MS_4 sites, where M is Cd, Zn, or Cu and S is thiolate sulfur from cysteine. The Ser and Lys contents are also correspondingly lower in the pseudothioneins. The bacterial proteins have a higher content of Glx, Ala, Val, and Leu than metallothionein. It is evident from the amino acid compositions that the three pseudothioneins are distinct proteins, and not fragments of the same protein. Both CdPT1 and CdPT2 contained aromatic amino acids, arginine and leucine, which are rarely found in mammalian metallothioneins. CdPT1 had higher levels of Val and Leu compared to CdPT2 and

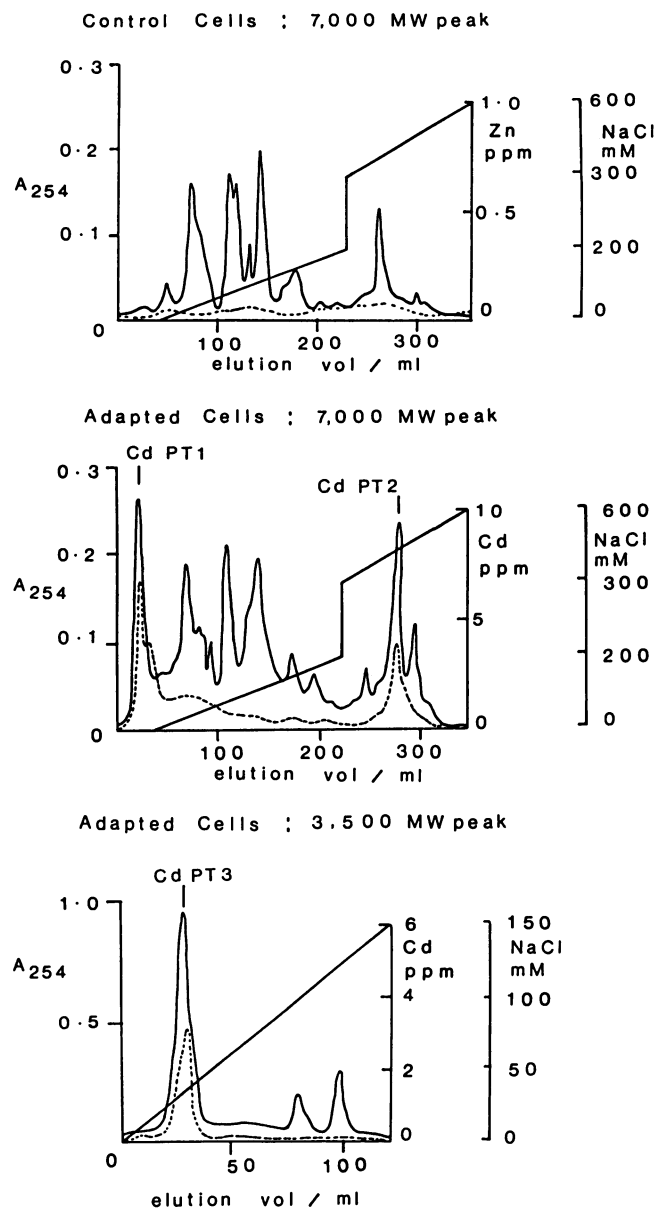


FIGURE 5. Ion exchange chromatography of G-75 fractions from control and cadmium-adapted cells. Cadmium-containing fractions corresponding to molecular weight of 7000 or 3500 were applied to a Sepharose CL6B column (equilibrated with the same buffer) and eluted with the NaCl gradients shown.

Table 2. Metal contents of pseudothioneins (6).

| Pseudothionein | Metal content per mole ^a | | | | | | | |
|----------------|-------------------------------------|-----|-----|-------|-------------------------------------|-----|-----|-------|
| | Standard medium | | | | Copper-depleted medium ^b | | | |
| | Cd | Zn | Cu | Total | Cd | Zn | Cu | Total |
| CdPT1 | 4.2 | 0.9 | 1.8 | 6.9 | 4.8 | 1.9 | 0.3 | 7.0 |
| CdPT2 | 1.2 | 2.0 | 2.0 | 5.2 | 2.3 | 1.8 | 1.1 | 4.2 |
| CdPT3 | 3.0 | — | 1.5 | 4.5 | 3.5 | — | 0.7 | 4.2 |

^a Metal contents per mole protein were determined on accurate weights of salt-free protein by flame atomic absorption spectroscopy, uncorrected for residual water content and based on molecular weight in Table 1.

^b Standard medium treated with Chelex 100 resin.

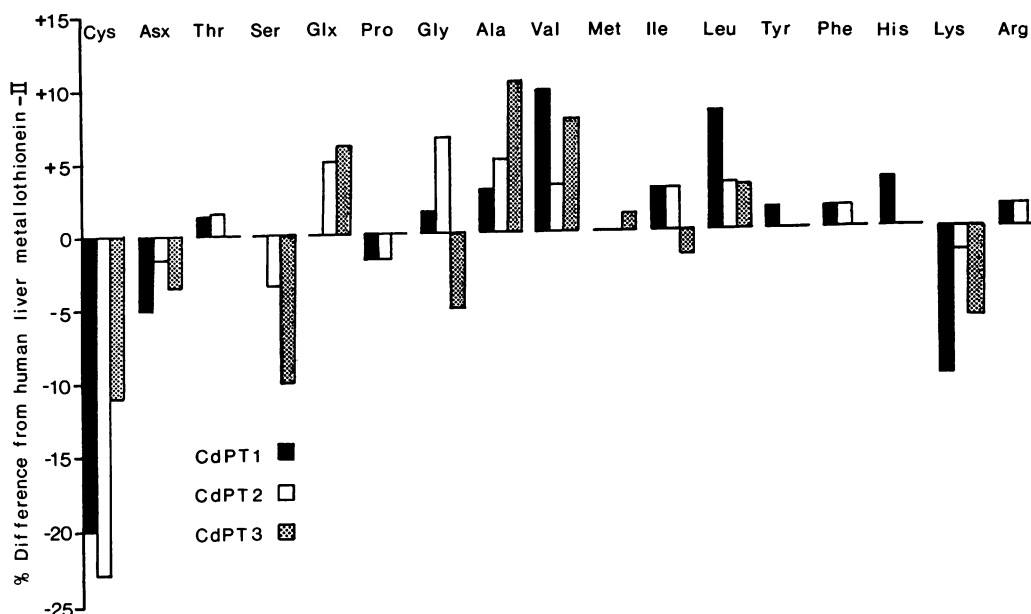


FIGURE 6. Comparison of the amino acid compositions (%) of the three pseudothioneins and human liver MTII.

CdPT3. CdPT2 had the highest Gly content and was also rich in Glx. Of the three proteins CdPT3 was the most similar to previously reported metallothioneins and contained no aromatic amino acids, Arg or Ile and only one residue of Leu. However, CdPT3 had a lower Ser and Lys content than metallothionein and was very rich in Glx.

Characterization of CdPT1

The metal binding sites of CdPT1, the major pseudothionein produced during the exponential phase, were probed in more detail. This protein contained a total of approximately 7 g-atoms of metal per mole: 4 Cd, 2 Zn, and 1 Cu.

The circular dichroism spectrum of CdPT1 showed the presence of 5 bands between 230 and 350 nm (Table 3). The positive Cotton effect at 260 nm is common to many mammalian metallothioneins containing cadmium alone, or cadmium and zinc (Table 3) (14). The high-wavelength bands are present in Cu(I)-containing metallothioneins, such as bovine fetal and chicken liver metallothioneins (15).

Native CdPT1 did not give an ESR spectrum, suggesting that copper is present as Cu(I) or alternatively, an antiferromagnetically coupled Cu(II) pair. When the

Table 3. Circular dichroism bands for CdPT1 compared with those of rabbit liver MTI (14) and chicken liver MTI (15).

| Pseudothionein-I (4Cd, 2Cu, 1Zn) | Rabbit liver MTI (7Cd) | Chicken liver MTI (10Cu) |
|-------------------------------------|---------------------------|-----------------------------|
| (-) 238 | (-) 233 | (-) 220 |
| (+) 260 | (+) 259 | (+) 260 |
| (-) 275 | — | — |
| (+) 292 | — | (-) 290 |
| (-) 315 | — | (-) 310 |
| — | — | (+) 350 |

protein was allowed to oxidize, an ESR spectrum typical of Cu(II) was obtained (12).

The proton NMR spectrum (Fig. 7) of CdPT1 consisted of contrasting regions: a broad aliphatic region (0.5–4.5 ppm) and a much sharper aromatic region (5.5–8.4 ppm). The reason for this is not clear, but it is possible that the aromatic residues may be in a relatively mobile region of the protein. The broadening of the aliphatic region may be due to protein aggregation or to the presence of paramagnetic Cu(II) ions. Peaks for Phe, Tyr, and two His residues have been tentatively assigned in the aromatic region on the basis of their chemical shifts, coupling constants, phase modulation in spin-echo ^1H NMR spectra, and shifts of ^1H resonances during pH titrations. The two sets of His resonances occur at higher frequency than would be expected for the free amino acid, perhaps indicative of their coordination to metal ions.

The ^{113}Cd NMR spectrum of CdPT1 (Fig. 8) (with ^{113}Cd in natural abundance 12.3%) was compared with that of rabbit liver MTII ($^{113}\text{Cd}_7$) (16). The bacterial protein has four sharp resonances at 476, 483, 604, and 615 ppm. The two higher frequency resonances are within the chemical shift range observed for cadmium-metallothioneins (600–680 ppm) (17) and appear to be typical of CdS_4 sites. These resonances may be shifted to lower frequency by their proximity to Cu(I) sites. Briggs and Armitage (18) found similar shifts in calf liver $\text{Cd}_{3.8}\text{Cu}_{2.6}\text{MTI}$, with ^{113}Cd resonances at 668.2, 629.2, 624.6, 612.3, 606.2, 600.8, and 590.4 ppm. The peaks at 483 and 476 ppm are compatible with CdS_2ON sites (19).

A speculative model for the metal binding sites of $\text{Cd}_4\text{Cu}_2\text{Zn}_1\text{PT1}$, which at present appears to account for much of the data described above is shown in Figure 9. The metal cluster incorporates two Cu(I)(Cys) $_2$ sites, two Cd(Cys) $_4$ sites and two Cd(Cys) $_2$ (His)(Glu) sites.

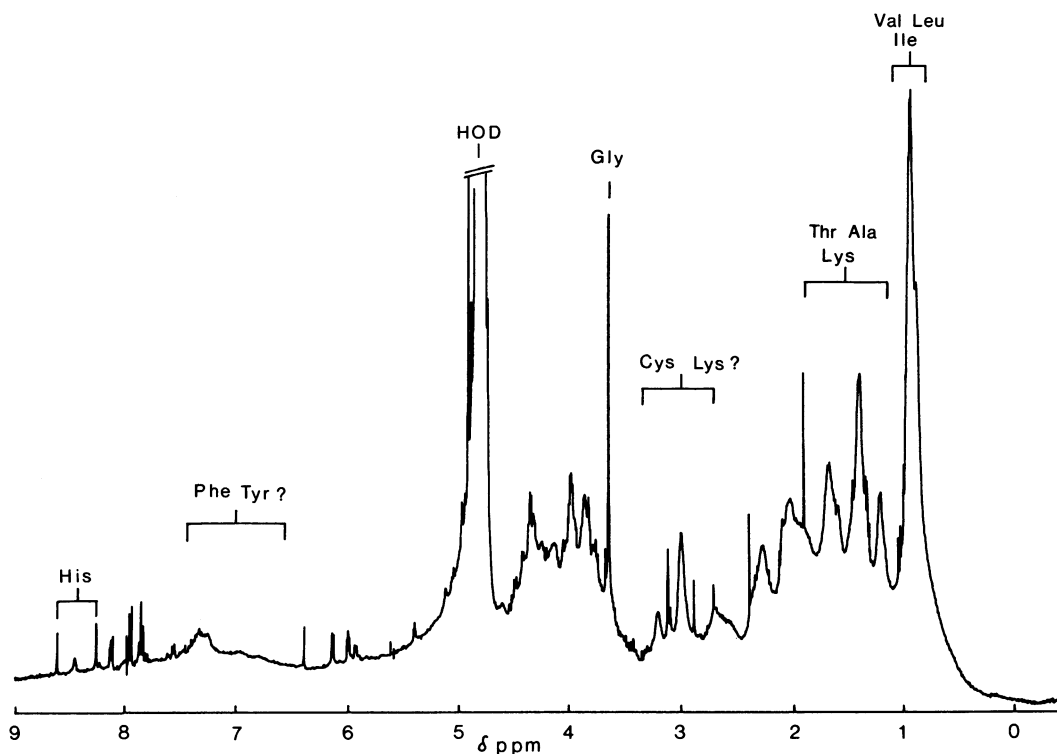


FIGURE 7. Proton NMR of CdPT1 at 400 MHz. The purified protein was dissolved in deuterated ammonium bicarbonate (20 mM, pH 8.0), and the spectrum was recorded at 294°K into 32k computer points using a 70° pulse and a 3.4 sec delay between consecutive pulses. Chemical shifts are in ppm from TSP (d_4 sodium 3-trimethylsilylpropionate).

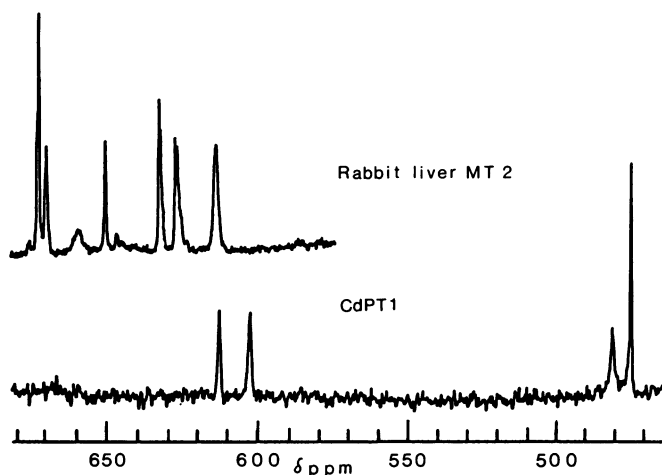


FIGURE 8. ^{113}Cd NMR spectrum of $(\text{Cd}_4\text{Cu}_2\text{Zn}_1)\text{CdPT1}$ and rabbit liver (Cd_7) MTII. The proton decoupled (inverse gated) ^{113}Cd NMR spectrum of CdPT1 (^{113}Cd in natural abundance) was recorded at 44 MHz at 298°K into 16k computer points using a 70° pulse and a 1.5 sec recycle time. It is a result of 46,700 pulses. Chemical shifts are referenced to $\text{Cd}(\text{ClO}_4)_2$. The pseudothionein has two peaks in the same region as a typical mammalian metallothionein (17).

The isolation of pseudothioneins adds important information to our knowledge of the occurrence of low molecular weight metal-binding proteins. Although the bacterial proteins resemble metallothioneins, there are

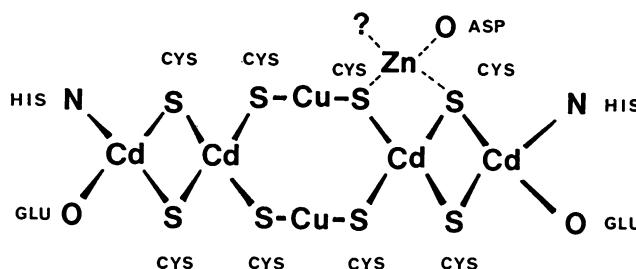


FIGURE 9. A model for the metal-binding sites of $(\text{Cd}_4\text{Cu}_2\text{Zn}_1)\text{PT1}$ (8).

several significant differences, especially in their lower cysteine content. This implies that amino acids other than Cys are involved in metal binding. The lower Cys content and presence of aromatic amino acids in the pseudothioneins are also characteristics of a metallothionein isolated from Cyanobacteria (20).

Further investigation of the pseudothioneins should provide an insight into the mechanism of induction of metal-cluster proteins and the relationship between their structure and function. However, the work described here also suggests that the adaptation of *P. putida* to high cadmium concentrations involves a complex series of other processes in addition to the production of pseudothioneins.

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