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Formation and Spectroscopic Characterization of a Novel Monomeric Cadmium- and Phosphate-Containing Form of Metallothionein[†]

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ABSTRACT: The occurrence of a new monomeric cadmium and phosphate containing metallothionein (MT) form, i.e., Cd_{13}-$(P_i)_2$-MT, is reported. The preferential formation of this MT form from Cd_7-MT has been shown to occur in the presence of phosphate and free cadmium at low protein concentration and elevated temperatures. This form displays in gel filtration an apparent molecular weight of 8 900 as opposed to 12 000 for Cd_7-MT, suggesting the presence of a more globular structure. This new MT form was characterized by electronic absorption, by circular dichroism (CD), and by ^{1}H, ^{31}P, and ^{113}Cd NMR spectroscopy. The Cd_{13}-$(P_i)_2$-MT form displays at least 24 ^{113}Cd signals between 240 and 520 ppm indicating (i) the absence of the original cluster structure of Cd_7-MT, (ii) the participation of oxygen and/or nitrogen ligands besides thiolates in metal coordination, and (iii) the presence of more than one stable MT form in the sample. From homonuclear ^{113}Cd COSY and CD studies, evidence for the existence of a cluster structure was obtained. It has been demonstrated that in the Cd_{13}-$(P_i)_2$-MT form two phosphate molecules are bound noncovalently and shown that a very slow exchange with [^{32}P]phosphate in solution (half-life of approximately 56 h) takes place. In the ^{31}P NMR studies, three ^{31}P signals from protein-bound phosphate between 3.5 and 6 ppm have been observed. The ^{31}P signal at 5.7 ppm displayed a heteronuclear ^{31}P–^{113}Cd coupling (J_2 = 56 Hz) which provides evidence for direct metal–phosphate coordination. The structural and NMR features of this new MT form are discussed.

Metallothioneins (MTs)[1] constitute a class of low molecular mass proteins (6–7 kDa) characterized by their unusually high cysteine content and ability to bind with high affinity and stoichiometry d^{10} metal ions such as Zn(II), Cd(II), Cu(I), and Hg(II) (Kägi & Schäffer, 1988; Vašák & Kägi, 1983). The structural studies of this protein have focused mainly on the monomeric form containing seven bivalent metal ions like Cd or Zn. The three-dimensional solution structures of Cd_7-MT determined by two-dimensional NMR (Frey et al., 1985; Arseniev et al., 1988; Schulze et al., 1988; Messerle et al., 1990) and the crystal structure of Zn_2,Cd_5-MT (Robbins et al., 1991) indicate that in these structures the metals are organized into two adamantane-like metal–thiolate clusters containing three and four metal ions tetrahedrally coordinated by nine and eleven thiolate ligands, respectively. At present, the primary physiological function of MT is not clear. The main stream of hypotheses relate the physiological role of MT with the regulation of the metabolism of essential metals Zn and Cu and with the protection of the cell against toxic heavy metals (Cd, Hg) and other electrophilic agents (Kägi & Schäffer, 1988).

Recently, we have demonstrated that the exposure of monomeric Cd_7-MT to Cd(II) ions in the presence of phosphate results in the nonoxidative formation of MT dimers containing approximately two additional Cd(II) ions and one inorganic phosphate per monomer subunit (Palumaa et al., 1992; Palumaa & Vašák, 1992). In the present study, conditions for the formation of a novel monomeric cadmium and phosphate containing form of MT are reported. This new form of MT, containing approximately 13 Cd(II) ions and two inorganic phosphates, has been studied by electronic absorption, circular dichroism, and ^{1}H-, ^{31}P-, and ^{113}Cd NMR spectroscopy.

MATERIALS AND METHODS

Rabbit liver MT-2 was isolated from rabbits exposed to cadmium salt as described previously (Vašák, 1991a,b). The main isoform of this fraction, MT-2a, was separated by preparative HPLC as described elsewhere (Arseniev et al., 1988). The Cd_7-MT form was prepared as described (Vašák, 1991c). The protein concentration was determined spectrophotometrically by measuring the absorbance of the metal-free protein (thionein) at 220 nm in 0.1 M HCl (ϵ_{220} = 47 300 M^{-1} cm^{-1}) (Bühler & Kägi, 1979). Sulfhydryl groups were quantified with Ellmans' reagent (DTNB) in 10 mM potassium phosphate buffer, 2 M guanidinium hydrochloride, and 20 mM EDTA, pH 7.5 (ϵ_{412} = 13 600 M^{-1} cm^{-1}) (McGilvray & Morris, 1971). Cadmium contant was determined using atomic absorption spectroscopy (Instrumentation Laboratory, IL 157). The inductively coupled plasma-atomic emission spectroscopy (ICP-AES), used in determining the phosphate content, was performed by the analytical division of EMPA, Dübendorf, Switzerland (No. 143 020/15.6.1992).

In the kinetic studies, $CdCl_2$ was added to Cd_7-MT under various buffer and temperature conditions, and, at different incubation times, aliquots were applied to the gel filtration column. Gel filtration was performed using a thermostated Superdex 75 column (1 × 30 cm) (Pharmacia) attached to a Pharmacia/LKB FPLC system. The column was eluted with 20 mM Tris-HCl buffer, pH 8.0, containing 0.1 M KCl and calibrated with bovine pancreatic trypsin inhibitor (6500

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[1] Abbreviations: CD, circular dichroism; FPLC, fast protein liquid chromatography; MT, metallothionein.

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Da), ribonuclease A (13 700 Da), myoglobin (17 800 Da), chymotrypsinogen (25 000 Da), and ovalbumin (45 000 Da).

The globular MT form was prepared by 10-h incubation of 0.73 μM Cd_7-MT with 0.37 mM $CdCl_2$ or $^{113}CdCl_2$ (^{113}Cd, >95% enriched, Harwell, England) as required, in 20 mM Tris-HCl buffer containing 1 mM phosphate and 0.1 M KCl, pH 8.0, at 55 °C. Subsequently, the protein was concentrated using an Amicon ultrafiltration apparatus (YM-2 membrane, Amicon).

Exchange of inorganic phosphate in the globular MT form with [^{32}P]phosphate in solution has been studied by adding [^{32}P]phosphate (Amersham) to the solution of prepared globular MT form in the presence of 1 mM phosphate. At different incubation times an aliquot from this solution has been applied to the gel filtration column eluted with phosphate-free buffer, and the radioactivity in the fraction of globular MT has been measured by a β-counter (Kontron BETAmatic II).

The absorption measurements were performed on a Cary 3 UV–visible spectrophotometer. A Jasco spectropolarimeter (model J-500) interfaced with an IBM PS/2 computer was used for CD measurements. ^{1}H, ^{113}Cd, and ^{31}P NMR spectra were recorded on a Bruker AMX-600 spectrometer. Chemical shifts in ^{113}Cd and ^{31}P NMR spectra are reported in parts per million (ppm) relative to 0.1 M $Cd(ClO_4)_2$ and phosphoric acid (85%), respectively. All NMR samples contained 10% ^2H_2O to provide the field-frequency lock and were measured in 5-mm NMR tubes. The ^{31}P and ^{113}Cd NMR spectra were acquired using 60° and 80° pulses, relaxation delays of 1 and 0.3 s, and acquisition times 98 and 20 ns, respectively. The absence of signal saturation in the ^{113}Cd NMR spectrum has been confirmed by its comparison with those acquired using relaxation delays up to 1.5 s. ROESY, EXSY, and COSY experiments were performed using standard pulse sequences.

RESULTS

In our previous study on cadmium- and phosphate-dependent dimerization of Cd_7-MT, it has been demonstrated that at low protein concentrations the formation of a new chromatographic peak with a lower apparent molecular weight compared to Cd_7-MT is observed (Palumaa and Vašák, unpublished experiments). However, under the conditions of low protein concentrations and room temperature, this form represents only a minor chromatographic component (Figure 1B). In the present study, attempts were made to increase the yield of this novel MT form thus facilitating its characterization. For this purpose the effects of temperature and protein concentration on the cadmium- and phosphate-induced rearrangement of Cd_7-MT have been studied.

Effect of Temperature. The effect of temperature between 25 and 55 °C was studied at the following component concentrations: 3.68 μM protein (Cd_7-MT), 0.37 mM cadmium, and 1 mM phosphate. The representative results presented in Figure 1 reveal an increasing yield of the low apparent molecular weight MT form (peak 3) with increasing temperature. Concomitantly, a decrease of the yield of MT dimers (peak 2) was observed. The peak 1 represents the monomeric Cd_7-MT form.

Effect of Protein Concentration. In these experiments the concentration of cadmium (0.37 mM) and phosphate (1 mM) in the incubation mixtures at 45 °C was kept constant and the concentration of Cd_7-MT was varied. The results summarized in Figure 2 show that by lowering the protein concentration the yield of the low apparent molecular weight MT form (peak 3) increases while the yield of MT dimers (peak 2) decreases.

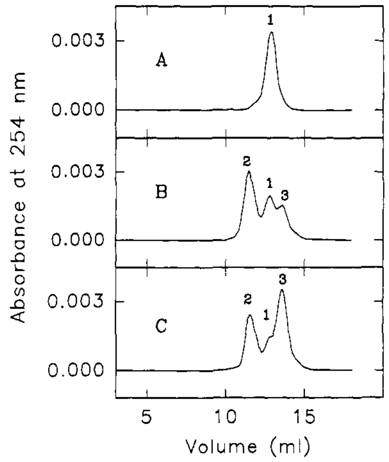

FIGURE 1: Effect of temperature on the gel filtration profiles of 3.68 μM Cd_7-MT incubated with 0.37 mM $CdCl_2$ in 20 mM Tris-HCl, 1 mM phosphate, and 0.1 M KCl at pH 8.0. (A) Cd_7-MT in the absence of free Cd, (B) 25 °C, incubation time 20 h, and (C) 45 °C, incubation time 3 h (Superdex 75 column, 10 °C; 20 mM Tris-HCl, 0.1 M KCl, pH 8.0, as elution buffer).

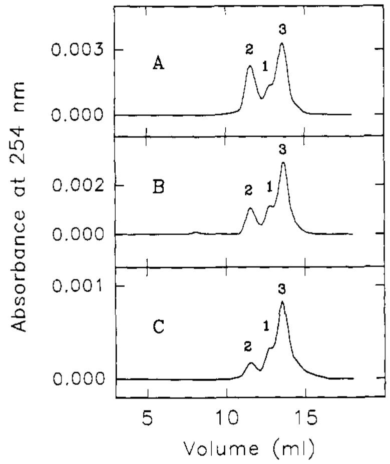

FIGURE 2: Effect of protein concentration on the gel filtration profiles of Cd_7-MT incubated for 3 h with 0.37 mM $CdCl_2$ in 20 mM Tris-HCl, 1 mM phosphate, and 0.1 M KCl, pH 8.0, at 45 °C. (A) 3.68 μM Cd_7-MT, (B) 1.47 μM Cd_7-MT, and (C) 0.74 μM Cd_7-MT (Superdex 75 column, 10 °C; 20 mM Tris-HCl, 0.1 M KCl, pH 8.0, as elution buffer).

On the basis of the results presented above, a nearly complete formation of the low apparent molecular weight MT form could be obtained at low protein concentration and elevated temperature. In the ensuing studies the new MT form was prepared using the following conditions: 0.37 mM $CdCl_2$ was added to 0.73 μM Cd_7-MT in 20 mM Tris-HCl buffer containing 1 mM phosphate and 0.1 M KCl, pH 8.0, and the mixture was incubated for 10 h at 55 °C. Under these conditions a yield of the low apparent molecular weight MT form of more than 90% was obtained. Subsequently, the protein was concentrated at 4 °C by ultrafiltration and purified at 10 °C by gel filtration on the preparative Superdex 75 column using phosphate-free elution buffer. On the basis of further chromatographic studies, this new MT form was found to be stable at 10 °C in 20 mM Tris-HCl buffer containing 0.1 M KCl, pH 8.0, over a time period of at least 24 h. This purified form was used for analytical and spectroscopic characterization.

The new MT form eluted with an apparent molecular weight of 8900 on the Superdex 75 column. Cadmium analysis revealed the presence of 13.1 ± 0.9 metals in this MT form and, on the basis of phosphate determination using plasma

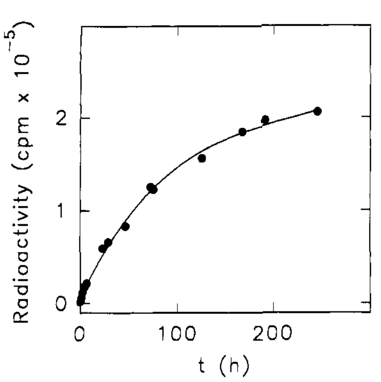

FIGURE 3: Exchange of Cd$_{13}$-(P$_i$)$_2$-MT bound phosphate with [^{32}P]-phosphate in solution (1 mM phosphate, 20 mM Tris, 0.1 M KCl, pH 8.0, 25 °C). The MT sample was prepared as described under Materials and Methods.

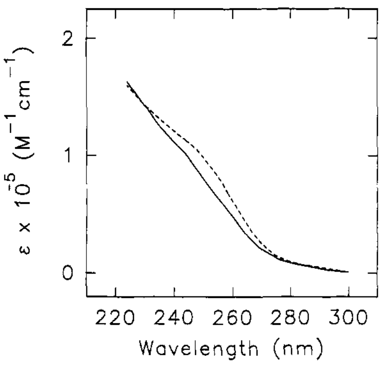

FIGURE 4: Electronic absorption spectra of Cd$_7$-MT (- - -) and of Cd$_{13}$-(P$_i$)$_2$-MT (—) (20 mM Tris-HCl, 0.1 M KCl, pH 8.0).

emission spectroscopy, the presence of 1.7 ± 0.4 phosphates per protein was determined. From this point on, the new MT form will be denoted as Cd$_{13}$-(P$_i$)$_2$-MT.

The Stability of Cd$_{13}$-(P$_i$)$_2$-MT toward Complexing Agents. The stability of Cd$_{13}$-(P$_i$)$_2$-MT toward EDTA and 2-mercaptoethanol was investigated in 20 mM Tris-HCl buffer containing 0.1 M KCl, pH 8.0, at 25 °C. Upon the addition of 1 mM EDTA to the new MT form, the original chromatographic peak of this species converted within 1 h into a single chromatographic peak with a retention time identical to that of Cd$_7$-MT. The corresponding CD spectrum confirmed the formation of the latter species. Similar chromatographic behavior was also observed upon the addition of 1 mM 2-mercaptoethanol to Cd$_{13}$-(P$_i$)$_2$-MT. These results demonstrate the absence of oxidation in the new MT species and rule out MT fragmentation as the reason for the observed decrease of its apparent molecular weight.

Phosphate Exchange in Cd$_{13}$-(P$_i$)$_2$-MT. The time dependence of the exchange of the protein-bound phosphate with [^{32}P]phosphate in solution is presented in Figure 3. It reveals that [^{32}P]phosphate incorporation is an extremely slow process occurring, under the conditions studied, with a half-life of about 56 h. [^{32}P]Phosphate, incorporated into the protein, was completely released from the protein after a 1-h incubation of Cd$_{13}$-(P$_i$)$_2$-MT with the complexing agents EDTA or 2-mercaptoethanol. These results provide evidence for the noncovalent nature of phosphate binding in Cd$_{13}$-(P$_i$)$_2$-MT and suggest direct binding to the protein bound metal ions.

Electronic Absorption and Circular Dichroism Studies. The electronic absorption and circular dichroism spectra of Cd$_{13}$-(P$_i$)$_2$-MT together with corresponding spectra of Cd$_7$-MT are shown in Figures 4 and 5, respectively. Comparison of absorption spectra of the two MT forms reveals the absence of the pronounced shoulder at 250 nm in the spectrum of Cd$_{13}$-(P$_i$)$_2$-MT (Figure 4). However, the pronounced tailing of this spectrum suggests that a contribution from this feature still exists. The most prominent features of the CD spectrum of Cd$_{13}$-(P$_i$)$_2$-MT (Figure 5) are two oppositely signed CD bands at (+)231 and (−)246 nm with an inflection point at about 240 nm. In the low-energy region of the CD spectrum, the strong CD band of Cd$_7$-MT at (+)260 nm is markedly reduced in intensity, and an additional CD band at (−)275 nm is discerned. The latter band occurs in the spectral region where only tailing in the corresponding absorption spectrum is observed.

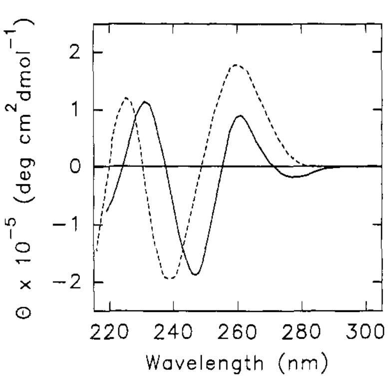

FIGURE 5: Circular dichroism (CD) spectra of Cd$_7$-MT (- - -) and of Cd$_{13}$-(P$_i$)$_2$-MT (—) (20 mM Tris-HCl, 0.1 M KCl, pH 8.0).

As has been shown previously, the pronounced shoulder in the absorption spectrum of Cd$_7$-MT at 250 nm is due solely to CysS–Cd(II) charge-transfer bands (Kägi & Vallee, 1961). By Gaussian analysis, this shoulder has been resolved into three metal-induced bands. The lowest energy band at 250 nm was assigned to the first CysS–Cd(II) charge-transfer transition of the bridging thiolate ligands (Vašák et al., 1981). In the absorption spectrum of Cd$_{13}$-(P$_i$)$_2$-MT, the blue-shift of the absorption edge of Cd$_7$-MT is indicative of the participation of other ligands in cadmium coordination (Plocke & Kägi, 1992). This conclusion is supported by the similarity of the absorption spectra of Cd$_{13}$-(P$_i$)$_2$-MT to that of a glutathione containing cadmium phytochelatin where mixed-ligand metal ligation has been proposed (Plocke & Kägi, 1992).

In the CD spectrum of Cd$_7$-MT the two oppositvely signed CD bands at (+)260 and (−)238 nm have been assigned to excitonically split transitions of bridging thiolates (Willner et al., 1987). In the CD spectra of Cd$_{13}$-(P$_i$)$_2$-MT above 240 nm a similar CD profile, although red-shifted, is still present, suggesting that an excitonic coupling involving juxtaposed bridging thiolate ligands also occurs in this MT form. In analogy with previous studies on short-chain cysteine–peptide–cadmium models (Willner et al., 1987), the additional (−)-275-nm CD bands of Cd$_{13}$-(P$_i$)$_2$-MT can belong to the same excitonic system when different geometrical constraints are considered (Willner et al., 1992). Accordingly, the presence of excitonic type interactions in the CD spectrum of Cd$_{13}$-(P$_i$)$_2$-MT indicates the presence of bridging thiolates and consequently of a cluster structure.

NMR Studies of Cd$_{13}$-(P$_i$)$_2$-MT. In order to gain insight into the structural properties of Cd$_{13}$-(P$_i$)$_2$-MT, this form was subjected to detailed multinuclear, i.e., ^{1}H, ^{31}P{^{1}H}, and ^{113}Cd-{^{1}H} NMR investigations.

^{31}P NMR. ^{31}P{^{1}H} spectra of Cd$_{13}$-(P$_i$)$_2$-MT (10 mM) containing either the 95% enriched ^{113}Cd ($I = {}^1/_2$) isotope or mainly the ^{112}Cd ($I = 0$) isotope (natural abundance) are shown in Figure 6. Both spectra reveal three rather broad ^{31}P phosphate resonances between 3.5 and 6 ppm in addition to

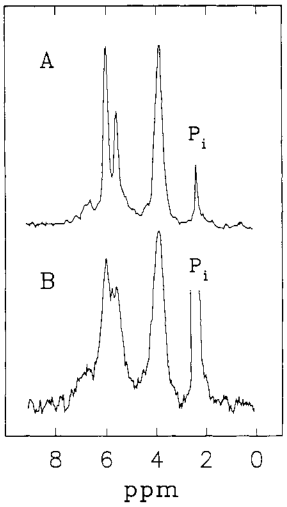

FIGURE 6: Proton-decoupled 242.9-MHz ^{31}P NMR spectra of Cd$_{13}$-(P$_i$)$_2$-MT containing (A) mainly the ^{112}Cd isotope (natural abundance) or (B) the 95% enriched ^{113}Cd isotope in 20 mM Tris-HCl and 0.1 M KCl, pH 8.0, at 10 °C (25 000 transients). The concentration of the protein in both samples was 10 mM.

the ^{31}P resonance of free phosphate (2.3 ppm). The latter resonance increased in intensity when the same spectrum was rerecorded after 7 days (the sample was stored at −20 °C), indicating a slow phosphate dissociation. The remaining three broad ^{31}P resonances are thought to originate from the protein-bound phosphate. In the ^{31}P NMR spectrum of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT, the ^{31}P resonance at 5.7 ppm is split into a doublet. A single ^{31}P resonance is observed with the MT form containing the NMR inactive ^{112}Cd isotope. Accordingly, this splitting can be assigned to a heteronuclear scalar ^{31}P–^{113}Cd coupling (J_2 = 56 Hz), thus providing evidence for a direct phosphate–cadmium interaction. However, although no evidence for heteronuclear coupling has been observed for the other two ^{31}P resonances of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT, a similar interaction with a coupling constant unresolved in our studies cannot be ruled out. The presence of at least three different phosphate resonances for the two protein-bound phosphate molecules in Cd$_{13}$-(P$_i$)$_2$-MT implies that at least one phosphate exists in two different relatively stable states. In order to obtain information about a possible phosphate exchange, the ^{31}P-EXSY (two-dimensional phosphorous exchange spectroscopy) spectra of Cd$_{13}$-(P$_i$)$_2$-MT were recorded using 100- and 300-ms mixing times. Under these conditions, no cross-peaks, due to exchange, were detected. This finding is in agreement with our [^{32}P]phosphate exchange studies (see above) and indicates, moreover, the absence of fast exchange among the protein-bound phosphates.

^{113}Cd NMR. The ^{113}Cd{^{1}H} NMR spectrum of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT (10 mM) is shown in Figure 7. In this spectrum the ^{113}Cd resonances occur between 675 and 240 ppm. On the basis of our previous ^{113}Cd NMR studies on Cd- and phosphate-induced MT dimers, the four weak resonances between 615 and 675 ppm can be attributed to this species (Palumaa et al., 1992). The presence of about 10% of this form in the NMR sample of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT after NMR measurements was confirmed by gel filtration of a small aliquot of this sample. Thus, the ^{113}Cd resonances of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT occur between 521 and 240 ppm. In this region at least 24 ^{113}Cd resonances can be distinguished. As in the ^{31}P NMR studies, more resonances than expected are also observed in the ^{113}Cd NMR spectrum of ^{113}Cd$_{12}$-(P$_i$)$_2$-MT, indicating the presence of more than one state of the molecule. In order to learn more about the structure of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT, a homonuclear ^{113}Cd COSY experiment was conducted. Unfortunately, due to very short T_2 relaxation times of ^{113}Cd resonances (less than 10 ms), causing an excessive line broadening, only a limited number of cross-peaks have been resolved in the COSY spectrum (data not shown). However, all resolved ^{113}Cd resonances of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT (Figure 7) exhibit one or more cross-peaks being diagnostic of scalar ^{113}Cd–^{113}Cd couplings through bridging ligands, thus ruling out the presence of isolated ^{113}Cd sites. The demonstrated participation of bridging ligands in metal binding strongly suggests the presence of a cluster structure in the new MT form.

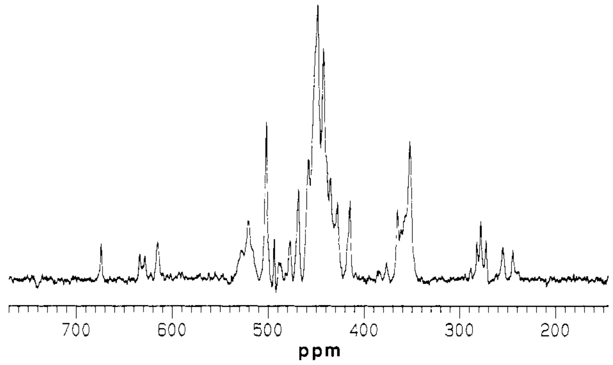

FIGURE 7: Proton-decoupled 133.1-MHz ^{113}Cd NMR spectra of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT in 20 mM Tris-HCl and 0.1 M KCl, pH 8.0, at 10 °C (200 000 transients). The concentration of the protein was 10 mM.

^{1}H NMR. The one-dimensional ^{1}H spectrum of ^{113}Cd$_{13}$-(P$_i$)$_2$-MT revealed very broad proton resonances (up to 25 Hz) compared to that of the Cd$_7$-MT form (ca. 5 Hz) (data not shown). This feature is consistent with short T_2 relaxation of the proton resonances. In this case, short T_2 relaxation may originate either from the high molecular weight (slow tumbling), from paramagnetic impurities, or from chemical exchange processes associated with a conformational fluxionality of the structure. Unfortunately, no evidence for a chemical exchange has been obtained from a ROESY experiment recorded with 20-ms mixing time. Moreover, MT aggregation as the reason for short T_2 relaxation could be excluded as a 10 times diluted NMR sample (monomeric based on gel chromatography) showed similar line widths. Furthermore, on the basis of the sharpness of the ^{1}H resonances originating from Tris buffer in the same sample of Cd$_{13}$-(P$_i$)$_2$-MT, the effect of paramagnetic impurities as the cause for short T_2 relaxation could also be ruled out.

DISCUSSION

The results presented here provide the first evidence for the formation of a monomeric MT form containing more than seven Cd(II) ions and inorganic phosphate, i.e., Cd$_{13}$-(P$_i$)$_2$-MT. This novel MT form develops from Cd$_7$-MT in the presence of free Cd(II) ions and phosphate in parallel with the dimerization of MT. Our results reveal that the formation of Cd$_{13}$-(P$_i$)$_2$-MT is favored against MT dimers at low protein concentration. As under these conditions the bimolecular dimerization of MT (Palumaa and Vašák, unpublished experiments) is unfavorable, this behavior points toward an intramolecular mechanism of this reaction. The latter requires a rearrangement of the protein structure reflected most directly in the gel filtration behavior of the Cd$_{13}$-(P$_i$)$_2$-MT form compared to that of Cd$_7$-MT. Thus, the substantially more metal-loaded Cd$_{13}$-(P$_i$)$_2$-MT form surprisingly shows a lower apparent molecular weight (8900) compared to that of Cd$_7$-

MT (12 000). Since, on the basis of the crystal structure, the standard form of MT containing seven divalent metal ions has an elliptical shape (Robbins et al., 1991), we infer that the Cd_{13}-$(P_i)_2$-MT form acquired a more globular shape. This conclusion is consistent with the close similarities of the analytical molecular weight of Cd_{13}-$(P_i)_2$-MT of 7760 and the apparent molecular weight of 8900 derived from elution of this species on a gel filtration column calibrated with globular proteins.

A number of structural features of the Cd_{13}-$(P_i)_2$-MT form can be derived from the present spectroscopic studies. First of all, both ^{113}Cd and ^{31}P NMR spectra display more resonances than expected from the number of bound metals and phosphates. This fact suggests the presence of more than one stable form of MT in the samples studied, which complicates the interpretation of the spectroscopic results. However, as the ^{113}Cd signals of Cd_{13}-$(P_i)_2$-MT are located between 240 and 520 ppm, the original cluster structure of Cd_7-MT (resonances between 620 and 680 ppm) (Otvos & Armitage, 1980) is absent in the new MT form. The former range of chemical shifts is in line with different modes of metal binding compared to Cd_7-MT and suggests further that besides sulfur also oxygen and/or nitrogen ligands participate in the metal coordination (Summers, 1988). Moreover, the large spread of the observed resonances (over 300 ppm) indicates widely differing coordination spheres of the bound metals. This is in contrast to the structure of Cd_7-MT, where all Cd(II) ions are tetrahedrally coordinated to thiolate ligands. However, although no detailed information as to the metal organization in Cd_{13}-$(P_i)_2$-MT is currently available, both the homonuclear ^{113}Cd COSY and the CD studies provide evidence for the presence of a cluster structure.

A striking feature of this new MT form is the binding of two molecules of inorganic phosphate. It should be noted that specific phosphate binding also occurs with the cadmium-induced dimeric MT form but is absent in Cd_7-MT (Palumaa & Vašák, 1992; Palumaa and Vašák, unpublished experiments). In the Cd_{13}-$(P_i)_2$-MT form, two phosphate molecules are bound noncovalently and show a very slow exchange with phosphate in solution. This behavior is consistent with phosphate encapsulation within the protein structure. The ^{31}P NMR results provide evidence that at least one phosphate is directly coordinated to cadmium. The binding of phosphate is directly connected with increased metal loading of Cd_7-MT and will by charge neutralization add substantially to the overall stability of the highly positively charged Cd_{13}-$(P_i)_2$-MT form.

The origin of unusually short T_2 relaxation times observed in all NMR experiments of Cd_{13}-$(P_i)_2$-MT should also be discussed. In general, very efficient T_2 relaxation may arise from chemical exchange, long molecular correlation times, and paramagnetic impurities as well as from rather specific relaxation mechanisms like chemical shift anisotropy (CSA) (Noggle & Schirmer, 1971; Neuhaus & Williamson, 1989). Although CSA can be ruled out for the proton spectra as line widths were found to be very similar in the 300- and 600-MHz ^{1}H NMR spectra of Cd_{13}-$(P_i)_2$-MT, it might be a contributing factor in the ^{113}Cd and ^{31}P NMR spectra. Moreover, protein aggregation as well as the presence of paramagnetic impurities as reasons for short T_2 relaxation could be ruled out (see above). We have noted, however, that in the previous ^{1}H NMR studies on small inorganic cages, i.e., $M_4(SR)_{10}$ [M = Zn(II), Cd(II), Co(II), Fe(II)] a kinetic lability of the metal–thiolate bonds has been found responsible for the nonrigidity of these clusters (Hagen et al., 1982). The demonstrated exchange process in this work has been interpreted in terms of an intramolecular rearrangement of the thiolate ligands accompanied by a temporary rupture of Me(II)–S bonds. In view of these results, we conclude that the observed very short T_2 relaxations in Cd_{13}-$(P_i)_2$-MT are caused most probably by chemical exchange brought about by a conformational fluxionality of the structure. The reason why our NMR experiments failed to detect these processes remains unknown. Unfortunately, due to the limited thermodynamic stability of this MT form at elevated temperatures, the temperature-dependent NMR studies commonly applied in such cases could not be performed.

Taken together, in the presented results as well as in our previous studies on cadmium-induced MT dimers (Palumaa & Vašák, 1992; Palumaa et al., 1992), we have demonstrated that besides the well-characterized Cd_7-MT form, MT forms with higher cadmium loading can exist. A typical feature of these new MT forms is the additional binding of inorganic phosphate. The existence of multiple stable metalloforms of Cd(II)-MT with different metal loading indicates a great degree of structural adaptability of MT in response to various conditions, e.g., protein, metal, and phosphate concentrations. The functional significance of this structural adaptability of MT as well as the physiological relevance of the multiple MT forms remains to be established.

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