

NMR Studies of Crab and Plaice Metallothioneins

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Metallothioneins isolated from the hepatopancreas of the edible crab (*Cancer pagurus*) and the plaice (*Pleuronectes platessa*) after cadmium injection are predominantly cadmium proteins containing only small amounts of zinc and traces of copper.

The removal of metal ions from the two metallothioneins by EDTA was studied using proton NMR spectroscopy. The rates of removal of cadmium and zinc were monitored directly from the intensity of the resonances due to the cadmium and zinc-EDTA complexes. Nearly all the zinc present in the protein was extracted by EDTA relatively rapidly, whereas only 10 to 20% of the total cadmium was removed in at least three steps. The total (Cd + Zn) metal removed at equilibrium was 1.2 to 1.8 g-ions/mole protein. Information on conformational changes in the protein were also obtained from studying alterations in the proton resonances of the protein. This was directly correlated with removal of metal from the protein.

The coordination environments of the cadmium ions in crab metallothionein were investigated by using ^{113}Cd -NMR, and compared with ^{113}Cd -NMR spectra of rabbit liver MT-II and *Scylla serrata* MT-I.

Introduction

The edible crab and plaice both appear to produce only one metallothionein, as judged by DEAE-cellulose chromatography or gel electrophoresis (1,2), in contrast to the two isometallothioneins normally isolated from mammals and many other organisms (3).

The distribution of metal ions among the available binding sites in metallothionein and their relative accessibility and reactivity are of particular interest in view of the possible functions of metallothionein in the homeostasis of essential metals (Zn, Cu) and detoxification of others (Cd, Hg) (4). ^{113}Cd -NMR spectroscopy is used here to characterize the cadmium-binding sites in crab metallothionein and proton NMR to investigate the reactivity of the bound metal ions towards EDTA. The kinetics of metal removal from proteins such as metallothionein by EDTA can be followed by measuring the intensities of the separate ^1H resonances due to the metal-EDTA complexes. In addition, this approach allows simultaneous observations of changes in the proton NMR spectrum of the proteins themselves, which are related to changes in the tertiary structure of the protein.

Methods

Preparation of Metallothioneins

Metallothioneins were isolated from the hepatopancreas of cadmium-injected crabs (6) or from the livers of cadmium-injected plaice (2). The cadmium:zinc ratios were as follows: crab metallothionein, preparation 1, 16:1; preparation 2, 4.5:1; plaice metallothionein, 4:1.

^{113}Cd -enriched crab metallothionein was prepared either by injecting the crabs with >95% enriched ^{113}Cd , or by reconstituting the protein with ^{113}Cd *in vitro*.

Reconstitution of Crab Metallothionein with ^{113}Cd

Lyophilized cadmium-metallothionein was dissolved in 50 mL of a solution containing 50 mM succinate, 50 mM phosphate, and 50 mM mercaptoethanol and adjusted to pH 2.8 with HCl. The volume was reduced from 50 mL to about 5 mL in a pressure cell fitted with an Amicon YM2 membrane. The solution was diluted to about 50 mL and again reduced to 5 mL; this process was then repeated once more. The final filtrate contained little cadmium. The total amount of cadmium removed from the protein was determined by atomic absorption spectroscopy, and this quantity of ^{113}Cd , plus a 20% excess (prepared in the same buffer), was added back to the protein. The pH was adjusted to 7.5 at a rate of 1 pH unit every 2 min. The reconstituted protein

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was rechromatographed on a column of Sephadex G-75 equilibrated with 100 mM ammonium bicarbonate gassed with argon, and the fractions in the main cadmium-containing peak were lyophilized. No "free" cadmium eluted from the column, suggesting that the 20% excess ^{113}Cd added was still bound to the protein, or alternatively, tightly bound to the column. The latter seems unlikely, as unbound cadmium would be expected to elute eventually from Sephadex G-75. No zinc or copper was detected in this protein by atomic absorption spectroscopy.

^1H -NMR Measurements

^1H -NMR spectra of crab metallothionein were recorded with a Bruker AM500 spectrometer operating at 500 MHz at 298°K. Free induction decays (FIDs) were acquired into 16,384 computer points by using a 55° pulse and a 2 sec delay between consecutive pulses. Protein concentrations were between 9.5 and 1.5 mM in deuterated phosphate buffer (50 mM, pH 7.0). All solutions were purged with nitrogen. After recording an initial proton NMR spectrum, a 20-fold excess of EDTA over protein (in the same buffer) was added to the metallothionein solution, and an NMR spectrum was recorded immediately. Further spectra were recorded at frequent intervals over the next 4 to 6 hr, or until no further changes in the spectrum were observed. Protein solutions were then either stored, sealed in NMR tubes for several days and rerun, or heated at 50°C for

10 min to bring the reaction to equilibrium. Standard additions of zinc and cadmium (as $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{CdSO}_4 \cdot 7\text{H}_2\text{O}$, Spec-Pure grade (Johnson Matthey Ltd.)), were then made to give a final concentration of 1 mM to calibrate the areas of the $[\text{ZnEDTA}]^{2-}$ and $[\text{CdEDTA}]^{2-}$ peaks.

Proton NMR spectra of plaice metallothionein were recorded on a Bruker WH400 spectrometer operating at 400 MHz. In the Hahn spin-echo sequence, (90 - τ - 180 - τ - collect), a τ value of 60 msec was used and a delay of 1 sec between repetitions of the sequence. Plaice metallothionein was dissolved in D_2O and the pH (meter reading in D_2O) adjusted to 7.0. The pH was also adjusted to 7.0 for solutions of EDTA and metal ions for standard additions. A 22-fold excess of EDTA over protein was added to plaice metallothionein. The reaction was brought to equilibrium by heating at 70°C for 5 min. No protein precipitation was observed during or after heating in solutions which had been purged with nitrogen.

^{113}Cd -NMR

^{113}Cd -NMR spectra were recorded on a Bruker WH-200 operating at 44.4 MHz and ambient probe temperature. FIDs were recorded into 16,384 computer points by using a 40° pulse and a 2 sec delay between pulses, and inverse-gated proton decoupling. Spectra with reasonable signal-to-noise ratios were obtained with protein concentrations between 0.67 and 1.28 mM.

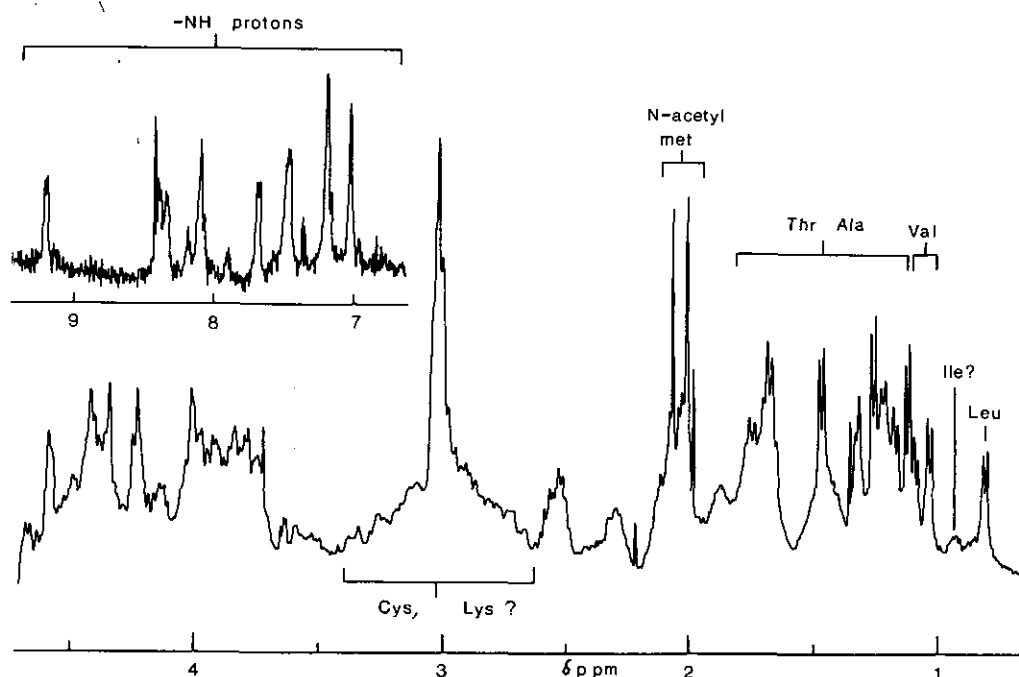


FIGURE 1. 400 MHz proton NMR spectrum of plaice metallothionein. Note the presence of resonances for some amide protons which exchange only slowly with deuterium from the solvent, and clearly resolved methyl resonances for Thr, Ala, Val and Leu.

Kinetic Analysis of Reactions of Metallothioneins with EDTA

As EDTA was present in excess over total metal, the data were analyzed in terms of pseudo-first-order kinetics. The data were plotted as $\log(A_\infty - A_t)$ versus time, where A_∞ and A_t are the concentrations of metal EDTA complex present at equilibrium and at time t , respectively. These concentrations were determined from peak areas. A three-step reaction was observed. The contribution of step 3 to the observed rate of step 2 was subtracted to yield a corrected rate constant for step 2.

Results and Discussion

Proton NMR Studies

The well-resolved ^1H -NMR spectrum of plaice metallothionein (Fig. 1) is typical of previously reported proton NMR spectra of metallothioneins; compare, for example, equine and human metallothioneins (5). Particularly notable is the intense resonance at 3 ppm, due mainly to CH_2 protons of Cys and Lys residues, and the two singlets at 2.02 and 2.06 ppm characteristic of a terminal *N*-acetylmethionine residue. The methyl resonances (0.9–1.7 ppm) were tentatively assigned on the basis of their chemical shifts and coupling constants. These assignments correspond well with those amino acids determined by conventional amino acid analysis. The spectrum shown in Figure 1 was obtained soon after dissolving (ca. 15 min) the protein in D_2O . Most of the amide protons had probably already exchanged with the solvent (5), but several -NH protons with slow exchange rates gave rise to resonances in the region 7 to 9.5 ppm.

The proton NMR spectrum of crab metallothionein (Fig. 2) consists of a mixture of broad and sharp resonances. In contrast to the plaice metallothionein, there are no sharp methyl resonances around 2.1 ppm from *N*-acetylmethionine or methionine. Many other methyl

resonances were again well resolved, and seven distinct doublets can be distinguished between 1.1 and 1.5 ppm. These resonances have been tentatively assigned to the $\beta\text{-CH}_3$ of Ala and the $\gamma\text{-CH}_3$ of Thr. This implies that crab metallothionein contains a total of at least seven alanine and threonine residues, each in slightly different chemical environments. Resonances for the methyl groups of valine, leucine, and isoleucine were not apparent in the proton NMR spectrum, nor were resonances from aromatic amino acids. These results are supported by amino acid analysis (6). The region around 3 ppm, tentatively assigned to CH_2 protons of Cys and Lys, is well resolved in crab metallothionein, in contrast to the same region in the spectrum of plaice metallothionein.

Resonances due to slowly exchanging amide protons were also observed in the spectrum of crab metallothionein. Four distinct resonances at 7.3, 8.6, 8.9, and 9.2 ppm were still present in the spectrum up to 5 hr after dissolving the protein in deuterated buffer. This again implies that there is a compact region of the protein which is relatively inaccessible to solvent. These -NH resonances had disappeared from the proton NMR spectrum of the same solution obtained 6 days later.

Metal Removal by EDTA

Figure 3 shows the proton NMR spectrum of a solution containing seven divalent metal ions and excess EDTA at pH 7. In metal complexes, the EDTA "acetate" protons become magnetically nonequivalent and give AB patterns. The four "ethylenic" protons remain magnetically equivalent and give rise to singlets, the chemical shifts of which are characteristic of the metal complex formed. The integrated areas of the shifted singlets are proportional to the concentrations of the metal-EDTA complexes present. The peaks for some EDTA complexes are slightly broader than others probably due to exchange reactions. Although the peaks for

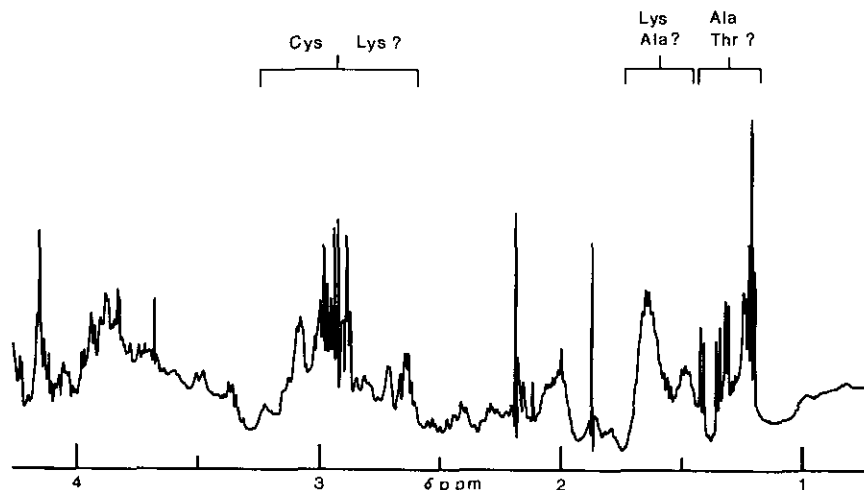


FIGURE 2. 500 MHz proton NMR spectrum of crab metallothionein. A Gaussian resolution enhancement was applied to the FID before Fourier transformation. Note apparent absence of resonances for *N*-acetylmethionine and valine.

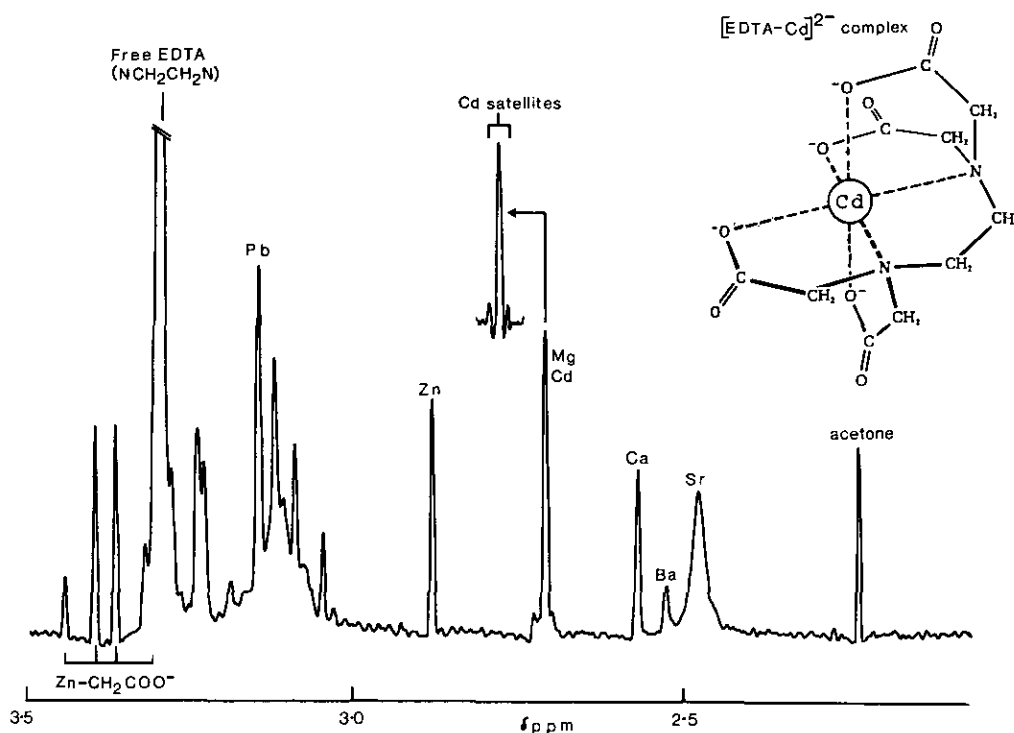


FIGURE 3. 400 MHz proton NMR spectrum of a solution of seven divalent metal ions in the presence of excess EDTA. The chemical shift of the singlet resonances for the ethylenic protons are characteristic of different metal EDTA complexes.

the ethylenic protons of the Cd- and Mg-EDTA complexes (2.76 ppm) overlap, a Gaussian resolution en-

hancement (Fig. 3, insert) resolves the Cd satellites (due to ¹¹³Cd and ¹¹¹Cd, total natural abundance 25%).

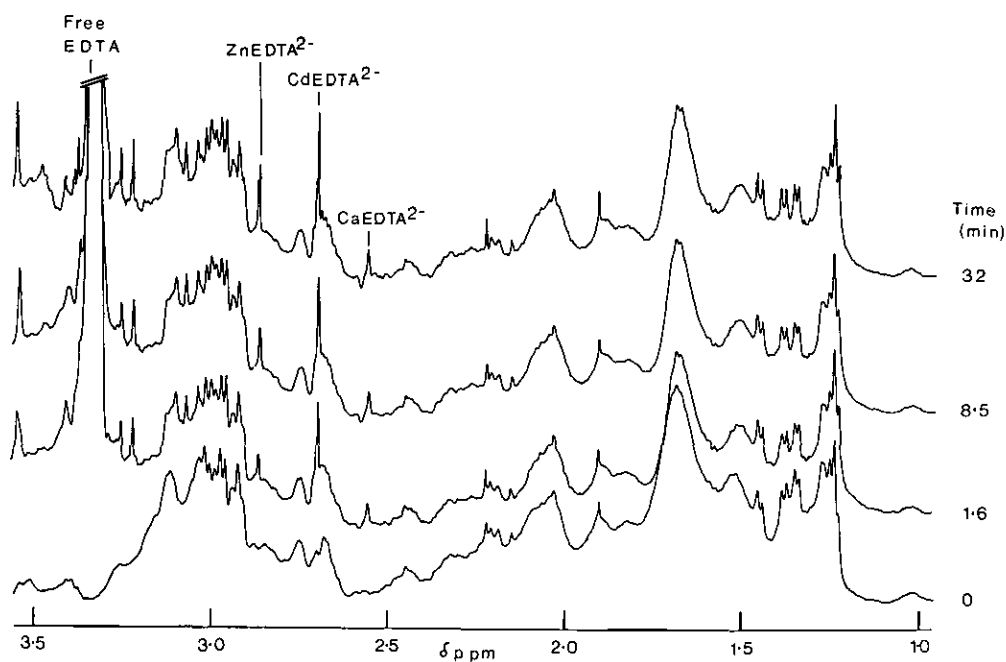


FIGURE 4. 500 MHz proton NMR spectrum of crab metallothionein before, and at various time intervals after the addition of a 20-fold excess of EDTA. Note the increase in intensity of the resonances due to [ZnEDTA]²⁻ and [CdEDTA]²⁻ complexes, with time.

Each satellite is one-eighth the height of the central component. Thus the height, and subsequently the concentration of $[\text{CdEDTA}]^{2-}$ may be calculated.

Kinetics of Metal Removal from Crab Metallothionein

Figure 4 shows proton NMR spectra of crab metallothionein at pH 7.0 in the presence of a 20-fold excess of EDTA (over protein) at various time intervals after mixing. The intensities of the singlets at 2.87 ppm and 2.76 ppm due to $[\text{ZnEDTA}]^{2-}$ and $[\text{CdEDTA}]^{2-}$, respectively, increase with time. A small peak was also observed at 2.56 ppm which was assigned to $[\text{CaEDTA}]^{2-}$. The time course of metal removal is plotted in Figure 5.

Since EDTA was present in large excess, the data were analyzed in terms of pseudo-first-order kinetics. A typical plot for crab metallothionein (Fig. 5b) shows that there were several distinct phases of metal removal. For both cadmium and zinc an initial very fast step (with corresponding rate constant k_1 , which was too fast to measure accurately), lasting less than 2 min was followed by two slower steps (with corresponding rate constants k_2 and k_3) (Table 1). In some cases an apparent fourth step was observed for cadmium removal. This step, however, coincided with removal of the sample from the NMR probe and was possibly due to the slight difference between the probe temperature and room temperature. A further slow removal of cadmium, however, proceeded over several days. The reaction was therefore brought more rapidly to equilibrium by heating (see "Methods"). Removal of further metal from the protein, either by incubating for several days or heating, resulted in precipitation of the protein if oxygen was present. Protein precipitation did not occur if the solution was purged with nitrogen, or in

control solutions of the protein that did not contain EDTA.

Approximately 12% of the total cadmium was removed from crab metallothionein by EDTA and, of this, ca. 60% was removed during the first 2 min (step 1). Zinc was extracted more slowly, but almost all the zinc was removed from the protein (100% from preparation 1 and 92% from preparation 2). The second stage of cadmium removal had a half-life of only 1 min and a k_2 of $1.8 \times 10^{-2} \text{ sec}^{-1}$. In contrast, the second stage of zinc removal was slower ($k_2 = 8 \times 10^{-3} \text{ sec}^{-1}$, with an associated half-life of 1.4 min; step 3 proceeded at a similar rate. The crab metallothionein from the second preparation contained substantially more zinc, and the rate of zinc removal was slightly faster. The rate of cadmium removal from this protein was unaffected.

Almost twice as much cadmium (22%) was removed from the cadmium-reconstituted protein, and most of the additional cadmium was released during step 3.

Traces of calcium appeared to be present in all three crab metallothionein preparations at similar levels, approximately 0.3 mole/mole protein. With a 20-fold excess of EDTA, all the calcium (as determined by atomic absorption spectroscopy) was removed during the first 2 min of the experiment.

Metal removal from crab metallothionein (preparation 2, Cd:Zn ratio 4.5:1) by an equimolar amount of EDTA (to protein) was also investigated. As EDTA was no longer in excess over metal, a kinetic analysis was not attempted. Under these conditions approximately half the zinc but no cadmium was removed from the protein. Removal of calcium proceeded over a period of approximately 20 min, suggesting perhaps that calcium may be weakly bound to the protein. Addition of one equivalent of EDTA did, however, lead to the removal of cadmium from the reconstituted crab metallothionein, but only 4.5% of the cadmium initially present. It is possible that reconstitution under the conditions de-

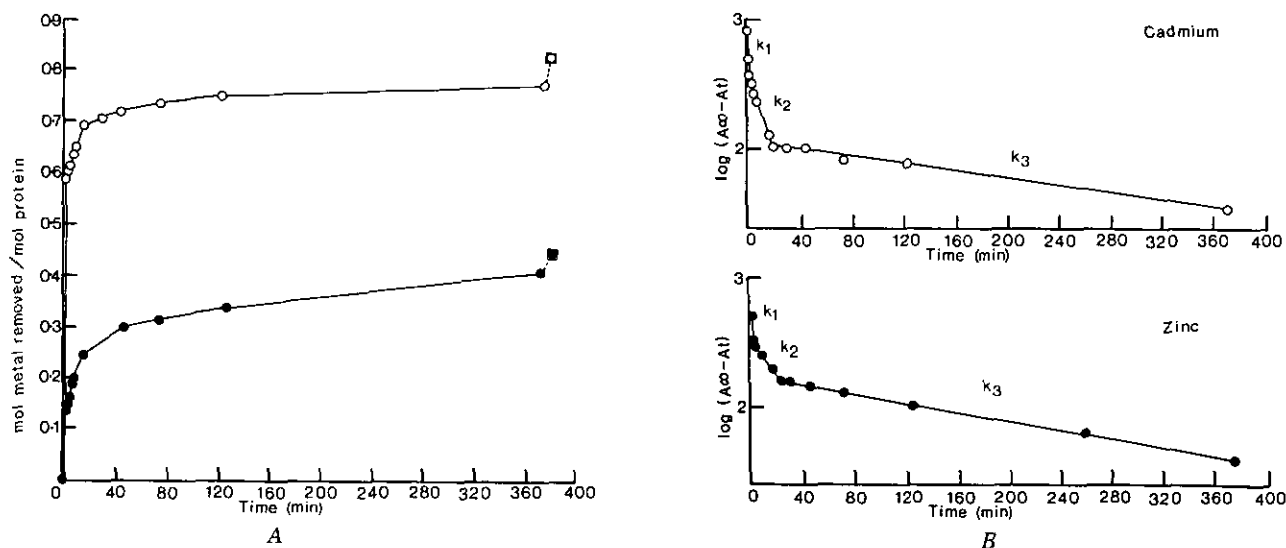


FIGURE 5. Plots of (A) time dependence and (B) pseudo-first-order kinetics of metal removal from crab metallothionein (preparation 1) in the presence of 20-fold excess EDTA: (●) zinc; (○) cadmium; (□) metal removed after heating.

Table 1. Metal removal from crab metallothionein by EDTA.

Preparation	Metal	Pseudo-first-order rate constant, sec^{-1}	$t_{1/2}$, min	Metal removed ^a mole/mole protein %	
Preparation 1, Cd:Zn = 16:1	Cd	k_1 fast	—	0.50	61
		k_2 1.8×10^{-2}	0.7	0.19	23
		k_3 2.2×10^{-4}	53	0.07	9
		Heat	—	0.06	7
		Total Cd removed		0.82	
	Zn	k_1 fast	—	0.13	31
		k_2 8.1×10^{-3}	1.4	0.16	40
		k_3 4.0×10^{-4}	29	0.10	24
		Heat	—	0.02	5
		Total Zn removed		0.41	
		Total metal removed		1.23	
Preparation 2, Cd:Zn = 4.5:1	Cd	k_1 fast	—	0.50	59
		k_2 1.8×10^{-3}	0.7	0.20	23
		k_3 4.2×10^{-4}	28	0.12	14
		Heat	—	0.03	4
		Total Cd removed		0.85	
	Zn	k_1 fast	—	0.36	38
		k_2 9.0×10^{-3}	1.3	0.48	50
		k_3 7.0×10^{-4}	17	0.04	4
		Heat	—	0.07	7
		Total Zn removed		0.95	
		Total metal removed		1.8	
Preparation 3, reconstituted ¹¹³ Cd only	Cd	k_1 fast	—	0.85	56
		k_2 1.2×10^{-2}	1	0.20	13
		k_3 9.8×10^{-4}	12	0.38	26
		Heat	—	0.07	5
		Total Cd removed		1.52	
	Zn	k_1 fast	—	0.58	43
		k_2 1.8×10^{-4}	7	0.49	36
		k_3 1.3×10^{-4}	89	0.26	19
		Heat	—	0.04	3
		Total Zn removed		1.37	
		Total metal removed		1.77	

^aAs a percentage of the total metal removed.

scribed leads to the binding of cadmium to a lower affinity site than is present in the native protein.

Table 2. Metal removal from plaice metallothionein by EDTA (Cd:Zn 4:1).

Metal	Pseudo-first-order rate constants, sec^{-1}	$t_{1/2}$, min	Metal removed ^a mole/mole protein %	
Cd	k_1 fast	—	0.35	86
	k_2 very slow	—	0.05	
Total Cd removed			0.40	
Zn	k_1 fast	—	0.58	43
	k_2 1.8×10^{-4}	7	0.49	36
	k_3 1.3×10^{-4}	89	0.26	19
	Heat	—	0.04	3
Total Zn removed			1.37	
Total metal removed			1.77	

^aAs a percentage of the total cadmium removed.

Kinetics of Metal Removal from Plaice Metallothionein

Plots showing the time dependence of the removal of cadmium and zinc from plaice metallothionein in the presence of excess EDTA at pH 7 are shown in Figure 6. As for crab metallothionein, EDTA removed almost all the zinc (> 85%) from the protein. The kinetics of zinc removal are also similar to those for crab metallothionein; an initial fast step was followed by a slower phase, with a half-life of approximately 7 min. The rates of zinc removal during this second phase are faster for crab than for plaice ($k_2 = 8 \times 10^{-3} \text{ sec}^{-1}$ for crab, $k_2 = 1.8 \times 10^{-3} \text{ sec}^{-1}$ for plaice). A third phase of zinc removal coincided with removal of the sample from the

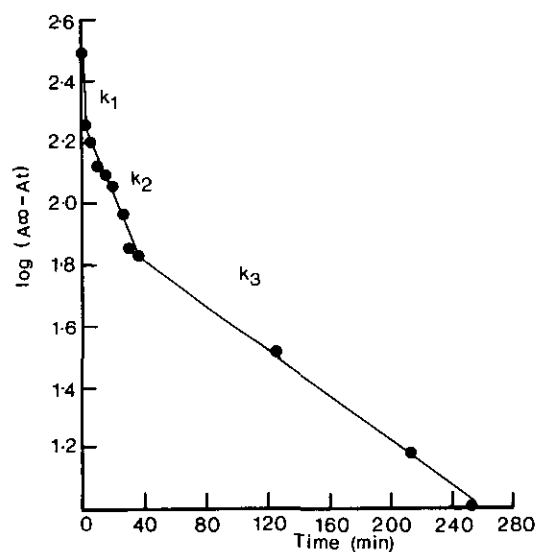
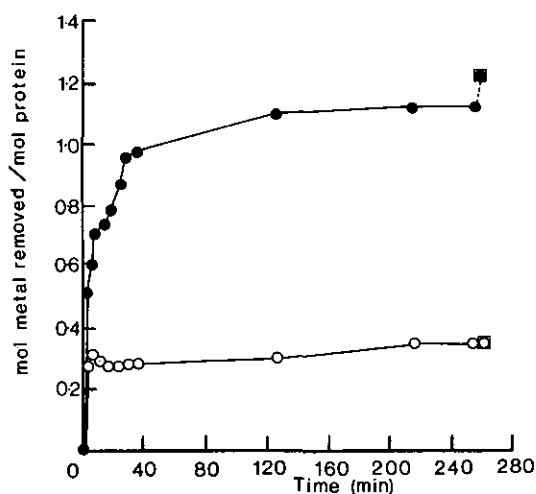


FIGURE 6. Plots of (A) time dependence and (B) pseudo-first-order kinetics of metal removal from plaice metallothionein in the presence of 20-fold excess EDTA: (●) zinc; (○) cadmium; (□) metal removed after heating.

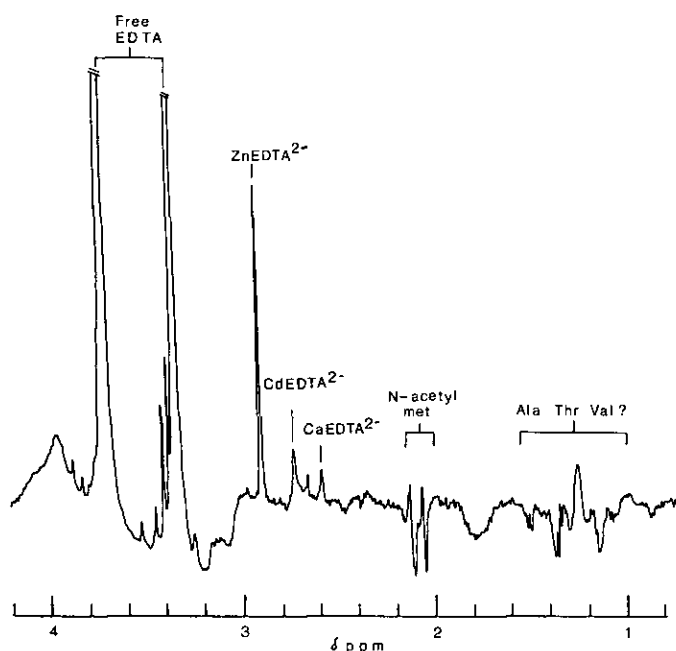


FIGURE 7. A 400 MHz proton NMR difference spectrum of plaice metallothionein before and after the removal of 1.77 g-atoms metal/mole protein. Changes in several resonances, particularly those due to *N*-acetylmethionine and threonine, were observed during metal removal.

NMR probe, and may be influenced by a difference between probe and ambient temperature (Table 2).

In contrast to crab metallothionein, only 5% of the total cadmium in plaice metallothionein was EDTA-chelatable. Almost all of this cadmium (86%) was removed rapidly during the first fast step. A further very slow step in the removal of metal from plaice metallothionein was also observed. This resulted in protein precipitation from solutions which were not purged with nitrogen.

Specific changes in the proton resonances of the protein were also observed during metal removal, indicating changes in the tertiary structure of the protein. For crab metallothionein, the methyl proton resonances (1–1.7 ppm) and resonances in the region around 3 ppm (CH_2 groups of Cys and Lys) became appreciably sharper. For plaice metallothionein, several resonances also sharpened, particularly those of Ala and Val. In addition a marked upfield shift of a methyl resonance (Thr?), and a downfield shift of the *N*-acetylmethionine S-CH_3 resonance were observed (Fig. 7).

^{113}Cd -NMR Studies

The proton decoupled ^{113}Cd -NMR spectra of native (Cd:Zn 4.5:1) and reconstituted (all ^{113}Cd) crab metallothioneins were very similar, with peaks at 619, 622, 639, 642, 650, and 670 ppm. However, in the spectrum of the native protein, the peaks at 650 and 670 ppm are relatively more intense, and there is an additional shoulder at 668 ppm. This may indicate a partial redistribution of metals during reconstitution. When a Gaussian resolution enhancement function was applied to the FID

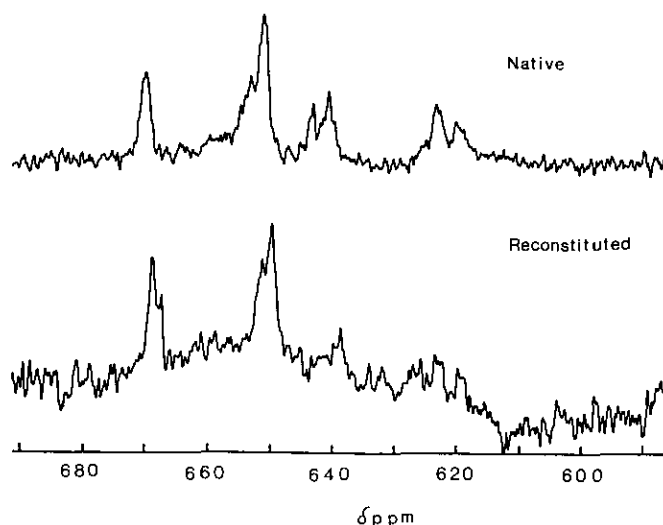


FIGURE 8. 44 MHz (proton decoupled) ^{113}Cd -NMR spectrum of crab metallothionein. The native protein was isolated from crabs injected with ^{113}Cd . The reconstituted protein was obtained by inserting ^{113}Cd into the apoprotein *in vitro* (see methods).

prior to Fourier transformation, splittings of approximately 33 Hz could be measured (although the signal-to-noise ratio was poor). These are the same order of magnitude as the ^{113}Cd - ^{113}Cd spin-spin couplings observed for mammalian metallothioneins (7,8) (Fig. 8).

The ^{113}Cd -NMR spectra of metallothioneins from *Cancer pagurus*, *Scylla serrata*, and rabbit liver metallothioneins are compared in Figure 9. The peak at 650 ppm is present in all three metallothioneins and is perhaps indicative of a highly conserved site.

Discussion

The use of ^1H -NMR to monitor simultaneously the removal of Zn and Cd by EDTA from crab and plaice

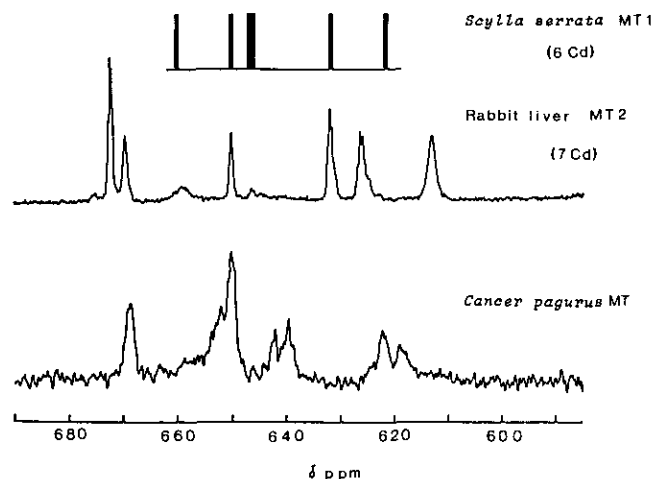


FIGURE 9. A comparison of the (proton-decoupled) ^{113}Cd -NMR spectra of *Cancer pagurus* metallothionein, *Scylla serrata* metallothionein (16), and rabbit liver metallothionein (8).

metallothioneins has several potential advantages over the use of electronic absorption in conjunction with atomic absorption spectroscopy. Zn removal from metallothionein is difficult to measure by UV spectroscopy. The Zn-S charge transfer absorption band at 220 nm is very close to the much larger absorption from the protein backbone and EDTA. Only gross changes in the protein structure can usually be measured by UV spectroscopy. However, proton NMR spectroscopy cannot easily be used to monitor the extraction of Cu(I) from metallothionein, as the formation of the paramagnetic $[\text{Cu(II)EDTA}]^{2-}$ complex would lead to very broad lines.

The accuracy and precision of the ^1H -NMR method has been assessed for the formation of calcium and magnesium EDTA complexes in plasma (9), and values were generally within 5% of those obtained by atomic absorption spectroscopy. Also kinetics of Cd and Zn removal from rabbit metallothionein, followed both by ^1H -NMR and electronic absorption spectroscopy were in good agreement (10).

The kinetic data suggest that there are one or possibly two bound zinc or cadmium ions per mole of protein that are reactive towards EDTA, but that these metals are extracted from several different environments (three-step kinetic plots). Triphasic kinetics were also observed by Li et al. (11). This could be interpreted in two ways. There may be several isoproteins in solution with slightly different metal contents (this would also explain nonintegral numbers of metals, i.e., plaice, 5.4 Cd, 1.6 Zn). Alternatively, proteins with the same metal composition may exist in several different conformations in solution. Evidence for such conformational substates has been provided by ^{113}Cd -NMR or reconstituted rabbit liver (7Cd) metallothioneins (8). The different conformations or isoproteins must then have similar sites that modify slightly the reactivities of the bound metals towards EDTA.

Zinc is normally found in the reactive site (or sites), but in a protein that contains predominantly cadmium the same sites often appear to be occupied by cadmium. We cannot rule out the possibility that removal of cadmium from one site allows EDTA to gain access to a second site.

The data suggest that the sample of plaice metallothionein may contain more than one isoprotein (i.e., proteins with different amino acid composition) as the ^1H -NMR suggests the presence of more than one type of *N*-acetylmethionine, the usual *N*-terminal amino acid of metallothionein. Only one of these acetyl methionines experienced a downfield shift when approximately one equivalent of metal was removed by EDTA (12). This suggests that the reactive site may be nearer the *N*-terminus of one isometallothionein compared to the other. Klauser et al. (13) have shown that purified plaice metallothionein contains two isoproteins with very different zinc to protein ratios.

Both the crab and plaice metallothioneins contained mainly cadmium. Yet almost all the zinc present in both

proteins was removed by EDTA, perhaps an indication that zinc is not distributed randomly among all the available metal-binding sites, but is only present in relatively accessible sites. It is important to note that the affinities of cadmium and zinc for EDTA at pH 7 (20°C) are very similar [$\log K_{\text{stab}}$ 16.46 and 16.5, respectively (14)], and therefore the rates of metal removal represent differences in the binding of the metals to the protein, i.e., reactivity and accessibility to EDTA. The apparent association constants between mammalian metallothionein and cadmium and zinc have been estimated to be $\log K_{\text{stab}}$ 12.3 and 16.3, respectively (15). However, these are average values for the bound metals. Although in most preparations of crab and plaice metallothioneins less cadmium was released than zinc, most of this cadmium (> 50% for crab, > 90% for plaice) was removed during an initial fast phase, suggesting that it is in a very reactive site. More cadmium was removed from crab metallothionein than from plaice metallothionein. Almost all the cadmium was removed from plaice metallothionein in one stage, whereas a complicated three-step removal of cadmium was observed from crab metallothionein. This suggests that cadmium in plaice metallothionein is present in only one reactive environment, whereas in crab metallothionein there are at least three possible, different, sites from which Cd can be easily removed.

In the reconstituted (all ^{113}Cd) crab metallothionein one (or both) reactive site must be occupied by cadmium. However, in this preparation (obtained by reconstituting the apo-crab metallothionein with 120% of its original cadmium content) it seems likely that additional cadmium may be bound. Cadmium was again removed in three distinct stages. Most of the additional cadmium appeared to be chelated by EDTA during the third step, suggesting the possibility that the crab metallothionein may have an additional metal-binding site.

In contrast to the situation for crab and plaice metallothioneins, Nicholson, Sadler, and Vasak (10) have found that no cadmium was removed from native Cd, Zn rabbit liver metallothionein by EDTA over a similar time period. Apparently, only zinc occupies the reactive site, unless the protein is reconstituted with 7 g-atoms cadmium per mole protein.

The presence of "reactive" or "exchangeable" zinc in a predominantly cadmium-containing protein may be of physiological importance, as these proteins may still have the ability to donate zinc to apoenzymes, a suggested function of metallothionein (4). EDTA has been used in this study as a probe of the reactivities of the cadmium and zinc ions bound to metallothioneins. The extent and rates of metal removal clearly depend on both the relative thermodynamic affinities of metallothionein and EDTA for the metal ions, and the mechanism of metal exchange. EDTA has only nitrogen and oxygen donor ligands available. *In vivo*, acceptors with stronger affinities may be present (e.g., apoenzymes) and the mechanisms of metal uptake and removal may involve sulfur-containing ligands such as glutathione as

intermediates. These may enhance or control the rates of metal transfer.

^{113}Cd -NMR also provides evidence of differences between cadmium-reconstituted crab metallothionein and the native protein. The shoulder at 670 ppm (an extra peak in the reconstituted metallothionein) may represent another binding site for cadmium in the crab metallothionein.

A comparison of the ^{113}Cd -NMR spectra of *Cancer pagurus* metallothionein with those of the *Scylla serrata* and rabbit metallothionein seems to suggest that the metal-binding sites in *Cancer pagurus* metallothionein are as closely related to rabbit metallothionein as to *Scylla serrata* metallothionein. This is surprising, considering that the amino acid compositions of the two crab metallothioneins are similar (6,15). Both have a lower Lys content than mammalian metallothioneins and contain no or little Val, no *N*-acetylmethionine, and a substantial amount of Pro.

To conclude, this work suggests that several conformations of metallothionein may exist in solution, each having one or two metal-binding sites that are reactive towards EDTA. These reactive sites are more often occupied by zinc than cadmium. The reconstituted (all ^{113}Cd) crab metallothionein appeared to have slightly different metal-binding sites to the native protein.

We thank the Science and Engineering Research Council, the Medical Research Council, Rio Tinto Zinc Services, and the University of London Intercollegiate Research Services for their support of this work.

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