Metal Binding in Metallothioneins: Competition for Cadmium and Zinc between Chelex-100 and Metal Binding Sites in Metallothionein

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Abstract

A study of the use of the metal chelation properties of Chelex-100 in metal binding reactions of metallothionein (MT), is described. The stoichiometric ratios of bound metals in MT were determined at several stages during a titration in which the Zn(II) in Zn_7–MT was displaced by Cd(II), by using Chelex-100 to sequester the free zinc. The stoichiometric ratios provide convincing supporting evidence that the complicated circular dichroism spectral properties observed during the titration arise because the incoming cadmium is distributed across both domains in the protein. It is shown that Chelex-100 does not sequester zinc or cadmium directly from the metallothionein binding sites. Use of Chelex-100 over the temperature range −20 to 65 °C is demonstrated. The chelation capacity of Chelex-100 (in terms of μg metal ion/mg resin) has been determined for a range of elements important in metal toxicology, including: cadmium (33 μg), zinc (22 μg), copper (19 μg), silver (38 μg), lead (40 μg) and mercury (40 μg).

Introduction

Metallothionein, MT, is a metalloprotein commonly isolated from the livers and kidneys of mammals, but generally found in many species [1, 2]. The protein is characterized by a molar mass of about 7000 daltons, with 20 cysteines out of a total of 61 amino acid [1, 2]. No aromatic amino acids are present [1]. MT is unique in binding a remarkably wide range of metals both *in vivo* and *in vitro*. *In vitro* studies of the metal binding properties of the protein have shown that up to 7 atoms of metal bind in two cluster domains [3, 4]. While the binding site stoichiometry and geometry is now well known for protein containing cadmium and zinc [5, 6], from optical, NMR and X-ray studies, far less information is available about the actual metal binding reaction and the selectivity of the metal for any one of the 7 binding sites. There is even less information available for metals such as copper, mercury, silver and gold, each of which bind tightly to MT [7–10], yet the spectroscopic probes for these metals are generally less helpful than for cadmium. Measurement of accurate stoichiometries during reactions of metal salts with both Zn_7–MT and apo-MT is essential before the binding site geometry can be determined.

Circular dichroism (CD) and magnetic CD (MCD) spectra measured during titrations of dilute solutions of the protein with solutions of metal salts can provide considerable detail about the mechanism of the metal binding reaction [5, 10]. The distinctive CD signal from ligand to metal charge transfer transitions can be used to follow changes at the metal binding site as a new metal is added during a titration [5, 10–13]. In this manner, metal ions that were bound initially to the protein, are replaced by new metals. The CD spectrum has recently been characterized as arising from exciton splitting of degenerate states of a clustered Cd_4–α MT domain [5].

The major requirement for a successful analysis of spectral data obtained when the metals bound *in vivo* are displaced *in vitro*, is that quantitative exchange of the metal occurs. This does not mean that the stoichiometric ratio is 1 Zn for 1 incoming metal, as metals such as copper and silver are expected to adopt a geometry other than tetrahedral. For these metals it becomes even more important to establish the ratio of metals bound: metals added, to determine that all the incoming metal ions bind to the protein and to determine whether all the initially-bound metals are actually displaced and become free ions.

Traditional methods used to remove free metal ions from solutions of proteins, involve the chromatographic separation of the protein from the free metal ions on Sephadex G-25 columns, followed by atomic absorption spectroscopy (AAS) analysis of the protein-containing fraction. While this technique can provide a good estimate of the stoichiometry of metals binding to metallothionein, there are several problems. (i) There may be redistribution of metals during passage down the column (a significant problem for a metalloprotein like MT that binds several

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metals in different sites). (ii) The concentration of the solution will change. (iii) The protein may degrade on the column. Finally, (iv) the use of columns is relatively slow. An alternative is to use Chelex-100 to remove free metals directly from the solution. Chelex-100, a chelating resin with iminodiacetic acid functional groups anchored to a styrene divinylbenzene copolymer support [14], is frequently used for trace metal preconcentration because it is capable of quantitatively sequestering trace metal ions [15]. Although several groups working with metallothionein have applied Chelex-100 in their studies [6, 16–18], detailed working conditions have not been published.

In this paper, we have measured the concentration of zinc and cadmium at several stages during a titration of Zn_7–MT with cadmium in order to relate the Zn:Cd ratio to the unusual CD spectral data observed as the titration proceeds. We also describe the use of Chelex-100 with a wide range of metals (cadmium, zinc, copper, lead, silver and mercury), and over a temperature range of −20 to 65 °C.

Experimental

Chelex-100 resin (Bio-Rad Laboratories) was washed in 1 M HCl, followed by 1 M NaOH, then rinsed with distilled water; the final pH was between 9 and 10. Portions of Chelex-100 resin were added to solutions of the metal ions or protein, mixed, then the supernatant was poured off leaving the resin; metals remaining in the supernatant were determined by AAS analysis. The contact time was recorded. The metallothionein used in this study was Zn–MT 2 which was isolated from the liver of a rabbit following a method previously described [19]. The metal ion solutions were prepared from $ZnAc_2$ (Fisher), $CdCl_2$ (Fisher), $AgNO_3$ (Fisher), $HgCl_2$ (Fisher) and $Pb(NO_3)_2$ (Fisher). All of the solutions used in the experiments (except the Pb(II) and Cu(I) solutions) were prepared in a 0.05 M Tris-HCl, pH 7.5 buffer. $Pb(NO_3)_2$ solutions were prepared in 0.01 M HNO_3 and Cu(I) solutions were prepared by dissolving $[Cu(CH_3CN)_4]ClO_4$ in 30% acetonitrile (*v*/*v*). The zinc solutions used for the low temperature (−20 °C) experiments were prepared in 40% ethylene glycol (*v*/*v*). Metal concentrations were estimated with a Varian 875 atomic absorption spectrometer. The concentrations of Zn, Cd, Cu and Ag were estimated in the flame mode; the concentration of Pb was estimated in the furnace mode and Hg was estimated by cold vapour generation (Varian VGA-76). Circular dichoroism (CD) spectra were recorded on a Jasco J-500 spectrometer that was controlled by an IBM-9001 computer with the program CDSCAN5 (R. Kitchenham and M. J. Stillman unpublished program). The spectral data were processed with the data base program Spectra Manager [20].

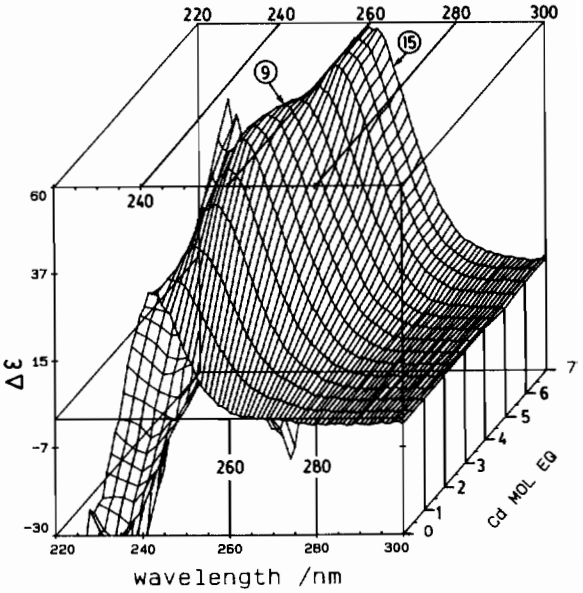

Fig. 1. A 3-dimensional perspective view of CD spectra recorded during a titration of Zn_7–MT with Cd(II). Cadmium was added in aliquots to give total concentrations (as mol eq) for each trace as indicated on the 'z' axis.

Results and Discussion

Figure 1 shows a set of CD spectra recorded as Cd was added to a solution of Zn_7–MT. In this 3-dimensional perspective view, the positive CD band at 250 nm due to the Zn_7-MT would be expected to diminish as a new band due to Cd_7–MT intensified. The cross lines indicate constant wavelengths; so that the red shift of the band that grows in during the titration, is shown by the rise in the cross line that marks 260 nm. Clearly the band shown as line 9 is not part of an isodichroic set. Although at this point in the titration only 3.5 mol eq Cd had been added, it cannot be assumed that the Zn has been replaced stoichiometrically so assignment of the spectral changes is difficult. Chelex-100 strongly chelates many divalent ions, and previous studies have suggested that Chelex-100 can compete successfully with MT for metals, so that it might be expected that if Chelex-100 was mixed with Cd, Zn–MT, at least the Zn would be pulled out of the protein by the Chelex-100. This would appear to limit the use of Chelex-100 in metal binding studies. However, as we demonstrate below, under the conditions required for optical measurements, Chelex-100 does not remove Cd or Zn from MT.

(1) Optimal Working Conditions

(A) Time dependence

2 ml portions of a 1.5 ppm solution of Zn(II) or Cd(II) were mixed with Chelex-100 for varying

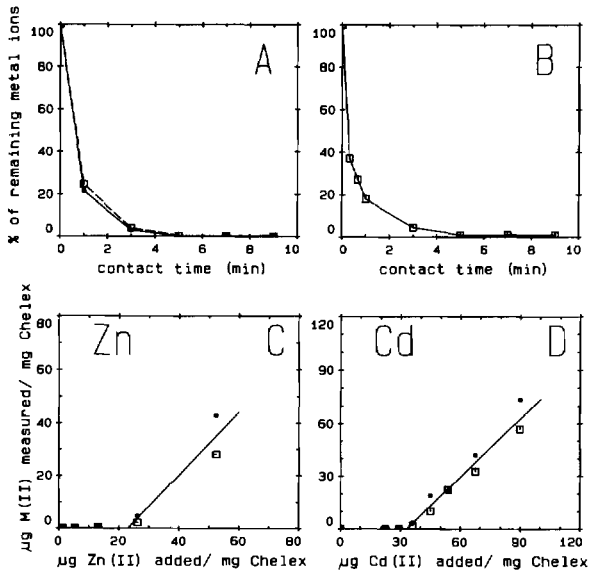

Fig. 2. (A) and (B) Dependence of the free metal ion concentration (expressed as a % of the initial concentration) on the contact time with Chelex-100 resin. (A) Using dilute solutions: 1.5 ppm Cd(II) (solid line) and 1.5 ppm Zn(II) (dotted line). (B) Using a concentrated solution: 1300 ppm Zn(II). (C) and (D) Chelation capacity of Chelex-100 for Zn (C) and Cd (D). (Units are µg metal added/mg Chelex-100 used, and µg metal measured/mg Chelex-100 used).

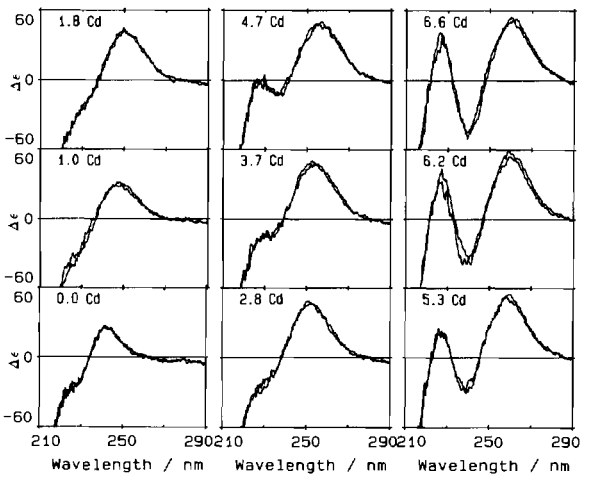

Fig. 3. CD spectra recorded for individual solutions of Zn_7–MT 2 with increasing concentrations of Cd(II) added as single aliquots; the spectra were recorded before and after Chelex-100 was added and the series represent the separate stages of the reaction shown in Fig. 1.

times. Figure 2A shows the dependence of the concentration of metal ions remaining in the solution on the contact time with Chelex-100. To remove 97% of the free Zn(II) and Cd(II) from the solution required a minimum of 5 minutes. 2 ml aliquots of a 1300 ppm $ZnAc_2$ solution were mixed with 200 mg Chelex-100 for varying times, Fig. 1B. After 5 min, under these high concentration conditions, only about 2 ppm (or 0.15%) Zn(II) still remained in solution. The time taken to remove more than 99% of the Zn(II) from the solutions used for Fig. 2B, where the metal ion concentrations are 600 times and the amount of Chelex/ml is 4 times greater than that used in Fig. 2A, was almost the same as for the dilute solution used for Fig. 2A. We suggest that, because the reaction occurs at the resin, the observed reaction rate is probably controlled by the efficiency of mixing.

(B) Chelation capacity of Chelex-100

50 mg portions of Chelex-100 were mixed with 2 ml aliquots of varying concentrations of metal ion (Zn, Cd, Cu(I), Ag(I), Pb(II), Hg(II)) solutions for 5 min. It is necessary to take special precautions with Pb(II) solutions. Chelex-100 works well only at pH 3 and above, but at high pH values (near 6) Pb(II) forms a $Pb(OH)_2$ precipitate. Using diluted NaOH, the pH of a $Pb(NO_3)_2$ and Chelex-100 solution was adjusted from less than 2 to just above pH 3. The results indicate that under these conditions, $Pb(OH)_2$ does not precipitate and Chelex-100 will chelate Pb(II). The data in Figs. 2C and 2D indicate that Chelex-100 has a maximum chelation capacity of 22 µg Zn(II)/mg Chelex, and 33 µg Cd(II)/mg Chelex. Similar experiments carried out for the other metal ions, indicated chelation capacities (as µg/mg Chelex-100) of 19 µg Cu(I)/mg, 38 µg Ag(I)/mg, 40 µg Pb(II)/mg and 40 µg Hg(II)/mg.

(C) Temperature dependence of the rate of chelation

We examined the conditions for using Chelex-100 at a range of different temperatures. Increasing the temperature leads to an increase in the rate of the chelation reaction. The minimum time required to remove 99% of free Cd(II) from the solution was: 30 min at −20 °C, 6 min at 4 °C, 5 min at room temperature (23 °C), 4 min at 37 °C and 1 min at 65 °C. The temperature dependent experiments were carried out in a refrigerated circulating water bath with a magnetic stirrer located below the reaction flask.

(2) Application of Chelex-100 to the Study of Metal Binding to MT

The spectra in Fig. 1 were obtained during a titration of a single sample of Zn_7–MT (2 ml of 10 µM) with multiple aliquots of Cd(II). In a separate experiment, separate solutions of Zn–MT were prepared and single aliquots of Cd were added so that a matching set of spectral data were obtained, Fig. 3. Chelex-100 was added to each solution to sequester the free ions. Table I summarizes these metal concentration values. Four sets of solutions were used to obtain these analytical results; the experimental errors for the numbers in Table I are within 6%.

TABLE I. Zn(II) and Cd(II) Concentrations in the Solutions of Zn_7–MT 2 Used in Fig. 3

Zn EST[a] (mol eq)	Zn AAS[b] (mol eq)	Cd EST (mol eq)	Cd AAS (mol eq)	Σ(Zn + Cd) AAS (mol eq)
7.0	7.0			
6.1	6.0	0.9	1.0	7.0
5.2	5.2	1.8	1.8	7.0
4.3	4.1	2.7	2.8	6.9
3.5	3.2	3.5	3.7	6.9
2.3	2.1	4.7	4.7	6.8
1.4	1.3	5.6	5.3	6.6
0.6	0.5	6.4	6.2	6.7
0	0.3	7.0	6.6	6.9

[a]'EST': these concentrations were estimated from the concentration of the cadmium added to the Zn–MT, for Zn(II), it was assumed that 1 mol eq Cd(II) displaced 1 mol eq Zn(II). [b]'AAS': these concentrations were measured by AAS following addition of Chelex-100.

From Table I, and Figs. 1 and 3, we can determine the distribution of Cd and Zn bound to the protein at various stages during the titration. The AAS values for the solutions used in Fig. 3, give the following stoichiometric ratios: for the native Zn–MT, Zn = 7.0 and Cd = 0.0; for the CD spectrum with the maximum peak intensity mid-way between Zn_7–MT and Cd_7–MT, Cd = 3.7 and Zn = 3.2, and at the end of the titration, Cd = 6.6 and Zn = 0.3. The metal concentration values for the intermediate species confirm that a total of 7 metal ions are bound to each MT molecule at all times during the titration. This suggests that the species which forms at trace 9 (Fig. 1) is different from the species finally observed as trace 15 (Fig. 1). We interpret this behaviour in terms of a distributed model in which Cd binds across both domains. These results provide essential supporting data missing in the previous studies of cadmium binding to Zn–MT which involved detailed analyses of CD spectral data [5] and ^{113}Cd NMR data [6].

Measurement of the concentrations of the metals remaining in solution after addition of an agent that can be considered to be a competitive chelator for the metals bound to MT, assumes that the chelator does not pull out the protein-bound metals. In this work we tested that addition of Chelex-100 did not change the spectral properties of the protein that arise from thiolate to metal charge transfer. Individual solutions used to construct Fig. 3, were measured before and after the addition of Chelex-100. In the absence of Chelex-100 there will be a significant concentration of free zinc in the solution. 50 mg Chelex-100 was added and mixed for 5 min, then the CD spectrum was recorded. The two superimposed lines show the CD spectra recorded in the presence and absence of Chelex-100. It is clear from Fig. 3 that the addition of Chelex-100 does not affect the CD spectra of Cd, Zn–MT species; this means, that under our conditions, Chelex-100 does not compete for either the Zn or Cd bound to the protein. This contrasts other reports using metal free apometallothionein reconstituted with Zn, where the Chelex-100 resin probably did remove bound Zn [16].

Conclusions

Chelex-100 is a valuable reagent in the study of metal binding reactions of proteins. It operates over a wide range of temperatures with many different metal ions. Several minutes are required to sequester greater than 90% of free ions. Cadmium binds to metallothionein in a distributed manner which results in a unique CD spectrum being observed for the protein with the stoichiometric ratio Cd_4, Zn_3–MT. It is demonstrated that Chelex-100 does not compete for either the Cd or Zn that is bound to native metallothionein.

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