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# The Effect of pH on $Cd^{2+}$ Binding to Rat Liver Metallothionein

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SUMMARY: Acidification of a solution of Cd, Zn-thionein results in a decrease in the UV absorbance at 250 nm and CD intensity at 260 nm. When the pH of the solution is raised to about 7, both the absorption and CD spectra are found to resemble the spectra of the original solution quite closely. Addition of $Cd^{2+}$ to Cd, Zn-thionein at pH 7 increases the loading of $Cd^{2+}$ in the protein, apparently in the same type of sites as the originally-bound $Cd^{2+}$. $Cd^{2+}$ added to thionein at pH 1.0 (and the pH then raised to 7) is found to bind at sites that exhibit the expected strong absorbance at 250 nm, but unlike the pH 7 loading experiment no parallel increase in CD intensity is observed.

## INTRODUCTION

Following the injection of aqueous $CdCl_2$ into rats, a low molecular weight (ca. 6800) metalloprotein that has a high sulphur content and contains a large amount of cadmium can be isolated from the liver. Kagi and Vallee named this protein metallothionein (1). Their early spectroscopic studies demonstrated that $Cd^{2+}$ and $Zn^{2+}$ were bound to the protein (1-3). The induction, isolation and biochemical properties of many metallothioneins have been extensively studied (4-6). More recently, a wide range of techniques have been used to study the coordination properties of the Cu, Zn and Cd in this protein (7-15). In order to understand better the biological sequence of events that result in the formation of Cd, Zn-thionein following the increase in $Cd^{2+}$ concentration in the liver, it is important that the conditions for binding and release of $Cd^{2+}$ from the metallothionein be determined. We report here the pH dependence of the rebinding of $Cd^{2+}$ to thionein formed at low pH, the stability of the thionein at pH 2.5, and the subsequent efficiency of the metal-cysteine complex formation.

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## MATERIALS AND METHODS

Male Sprague-Dawley rats were injected with 1 mg Cd/kg body wt over a two week period. The metallothionein was purified and freeze dried as previously reported (4). Protein samples were dialysed against triply distilled water for 20 hrs. The pH at this stage was 6.9. The pH of a solution in a cuvette was reduced by adding μL aliquots of concentrated HCl. Spectra were recorded immediately after addition of the acid and once again after 2 hours before the addition of base. The pH was raised by addition of μL aliquots of concentrated NaOH. Absorption spectra were recorded on a Cary 219 spectrophotometer. CD measurements were made with a JASCO CD/ORD-5 which had been modified to Sproul SS-20 specifications.

## RESULTS AND DISCUSSION

In Fig. 1a, we show the absorption spectra recorded for samples of rat liver Cd, Zn-thionein† at pH 6.9, 4.1, 3.1, 1.9, 0.9 and 0.52. The pH was adjusted in the cuvette by the addition of μL aliquots of concentrated HCl to different samples of the same pH 6.9 stock solution. The extinction coefficient, $\varepsilon_{250nm} = 1.45 \times 10^4$ litre $mol^{-1}$ $cm^{-1}$ (2,3), was used to calculate the concentration of the initial pH 6.9 solution. Fig. 1b shows the CD spectra for the same solutions. The 250 nm shoulder in the absorption has been associated with a Cd-S charge transfer transition (2). The CD intensity, which has a maximum at 260 nm, can either arise from a chiral coordination of the $Cd^{2+}$ in the protein, or from the chirality of the coordinating cysteines, as both the $Cd^{2+}$ and the cysteines are involved in the charge transfer transition. The loss of intensity at 250 nm as the pH is dropped indicates that the Cd-S bonds have been broken, although in an alternative scheme it is possible that the sulphurs could be protonated, which would reduce any charge transfer ($RS^- \rightarrow Cd^{2+}$) intensity, while leaving the Cd-bonds intact. This would require the $K_{EQ}$ of the $Cd\text{-}(SR)_n$ complex to be comparable to that for the competition for the sulphur sites by the protons alone.

The pH of the solutions were adjusted to ca. 7 with NaOH and Fig. 2a shows the reappearance of absorption at 250 nm. In each of these spectra, there is an increase in the absorbance at 280 nm compared with the initial pH 6.9 trace. This same trend is also observed at 250 nm for all but the pH 3.1

†metal-free metallothionein will be referred to as thionein throughout this paper.

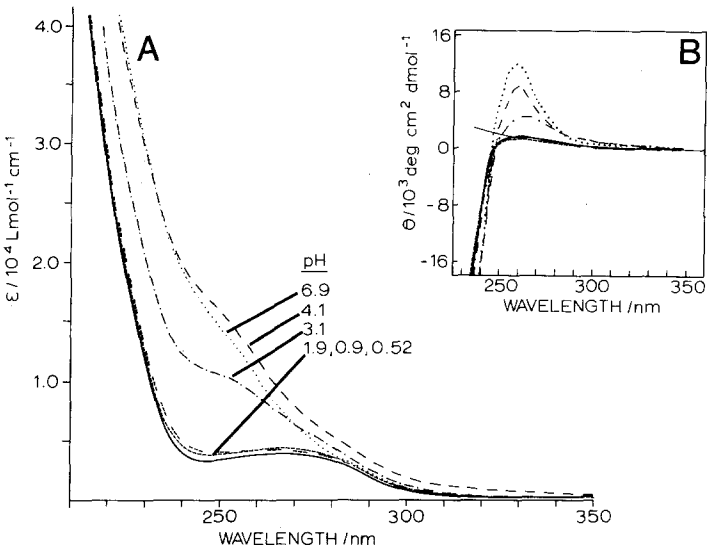


Figure 1. Conversion of Cd, Zn-thionein to thionein by addition of concentrated HCl, absorption (A) and CD (B). (.....) Cd,Zn-thionein, pH 6.9; (— — —) pH 4.1; (—·—·—) pH 3.1; (--- --- ---) pH 1.9; (-----) pH 0.9; (———) pH 0.52.

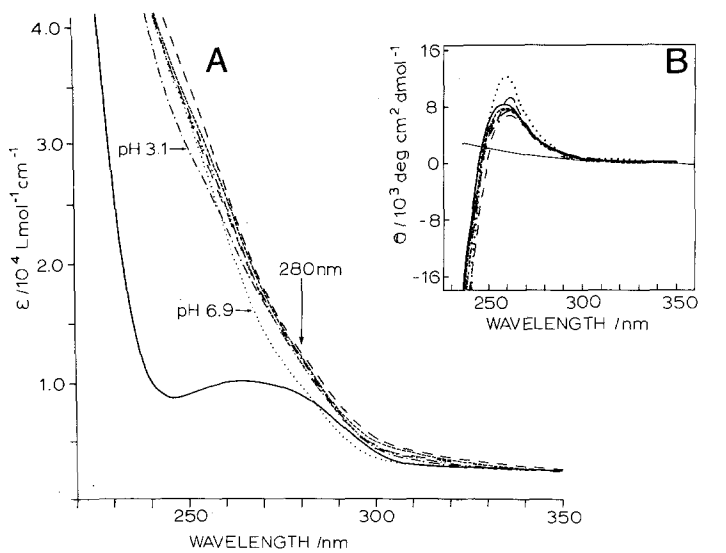


Figure 2. Absorption (A) and CD (B) spectra of the reconstituted protein at pH 7. The low pH solutions shown in Fig. 1 (4.1 - 0.52) were used, the final pH was close to 7.0. (.....) stock Cd,Zn-thionein at pH 6.9; (— — —) from pH 4.1; (—·—·—) from pH 3.1; (--- ---) from pH 1.90; (-----) from pH 0.90; (—··—··—) from pH 0.52. Thionein at pH 0.52 (———).

solution, which shows a slight drop in absorbance. Fig. 2b shows the corresponding CD spectra. The CD spectrum of each of the reconstituted proteins is slightly broadened and lower in intensity for solutions in which the pH went below 4. The band is relatively sharp for solutions where the pH was only dropped to 4. A return to the original CD intensity is not observed for any of these solutions. This decrease in intensity may be due to the fact that not all the free $Cd^{2+}$ or $Zn^{2+}$ can rebind to the protein in the original sites, this is perhaps especially true for the $Zn^{2+}$. We note that the CD spectra for all solutions where the pH was lowered to less than 4 were similar when the pH was returned to 7. Thus, the extent of rebinding seems to be constant for solutions that were brought up from a pH lower than 4.

In Fig. 3, we illustrate the effect on the spectrum of the protein of adding free $Cd^{2+}$ to the cuvette. The results of two experiments are shown.

(i) 1.55 and 3.62 molar equivalents of $Cd^{2+}$ were added to a pH 6.9 solution of Cd, Zn-thionein. We can see in Fig. 3a that the 250 nm absorption shoulder intensity increases considerably with the first 1.55 mol eq. At the same time the shoulder becomes more resolved and the band centre shifts towards 255 nm. Increasing the $[Cd^{2+}]_{added}$ to a total of 3.62 mol eq results in a smaller increase in the absorption intensity. At this point, we assume that the protein has reached the maximum binding limit for $Cd^{2+}$. Comparison between the intensities of the CD spectra, Fig. 3b, of the native pH 6.9 Cd, Zn-thionein and the $Cd^{2+}$-loaded protein at pH 6.9 (where $[Cd^{2+}]_{added}$ was 3.62 mol eq) indicates that a similar increase has taken place in the concentration of $Cd^{2+}$ that is bound to chiral sites, both the CD and absorption increase by ca. 30%.

(ii) In the second experiment, we investigate the effect of additional cadmium on the rebinding of $Cd^{2+}$ to thionein. The pH of a Cd, Zn-thionein solution was dropped to 0.95 (Fig. 3a), a 1.55 mol eq aliquot of $Cd^{2+}$ was added and the pH returned to 7. While the absorption at 250 nm is clearly similar to the trace recorded after 1.55 mol eq of $Cd^{2+}$ had been added to the pH 6.9 solution, the CD intensity, Fig. 3b, is identical with the solution that

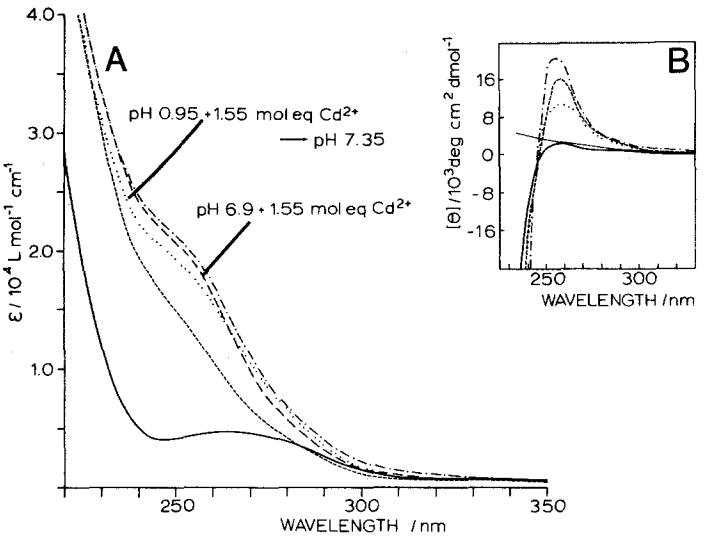


Figure 3. The absorption (A) and CD (B) spectra of $Cd^{2+}$-loaded metallothionein. (i) The effect of adding $Cd^{2+}$ on the Cd,Zn-thionein spectrum at pH 6.9. (-----) Cd,Zn-thionein stock at pH 6.9; (— — —) with 1.55 mol eq $Cd^{2+}$ added; (—.—.—) with a total of 3.62 mol eq $Cd^{2+}$ added. (ii) The effect of adding $Cd^{2+}$ at pH 0.95 on the thionein spectrum at pH 7.35. (-----) Cd,Zn-thionein stock at pH 6.9; (———) thionein at pH 0.95; (... ... ...) with 1.55 mol eq $Cd^{2+}$ added at pH 0.95, the pH was then brought up to 7.35 and the spectrum recorded.

did not have $Cd^{2+}$ added to it at pH 0.95 (Fig. 2). The data from the pH 7 loading experiment, Fig. 3, show that a considerable amount of additional $Cd^{2+}$ can be bound to the protein, both the absorption at 250 nm and the CD intensity rise by about 30%. This is well known (2) and it has been assumed that $Zn^{2+}$ is displaced by the $Cd^{2+}$. The results from the experiment where $Cd^{2+}$ is added at pH 0.95 (Fig. 3) are more difficult to explain. Apparently the number of Cd-S bonds does increase, as the absorbance at 250 nm rises to the 1.55 mol eq value found for the pH loading experiment. However, it is clear from the CD data that the concentration of $Cd^{2+}$ bound in chiral sites when the pH is returned to 7 is the same as in the absence of added $Cd^{2+}$. Because the CD intensity of the reconstituted Cd, Zn-thionein (Fig. 2) is less than that of the initial solution, and this intensity cannot be increased by adding $Cd^{2+}$ at the low pH, it seems reasonable to suggest that the sites used at pH 7 (probably $Zn^{2+}$ sites) are no longer available at pH 1.0.

It does not appear that the high proton concentration causes irreversible denaturation because the rebinding from a range of pH value (0.5 - 4.0) result in much the same CD signal (slightly higher with 4.0) which are all lower by at least 30% than the initial solution. In addition, a solution kept for 24 hours at pH 2.5 gave the same CD and absorption spectra upon returning to pH 7. We deduce from these data that thionein is chemically stable at low pH. Absorption and CD data measured for protein solutions where $Cd^{2+}$ has been added at pH 1.0 indicate that free sulphurs are available for bonding but that the resultant coordination geometry is not the same as for the original $Zd^{2+}$ or $Cd^{2+}$ We conclude that if $Zn^{2+}$ is lost through acidification, then $Cd^{2+}$ cannot rebind in the same manner at these sites. Finally, these data imply that if the $Cd^{2+}$ is released at low pH, it is able to bind again at the same or similar chiral sites that resemble the original pH sites, as most of the Cd intensity returns at pH 7 and the envelope shape in the 260 nm region is similar.

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