Re-evaluation of the Binding of ATP to Metallothionein*

Klaus Zangger‡, Gülün Öz§, and Ian M. Armitage¶

From the Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455

In a recent paper Jiang et al. (Jiang, L. J., Maret, W. & Vallee, B. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9146–9149) reported that metallothionein interacts with adenosine triphosphate (ATP) to form a 1:1 complex with a dissociation constant of \( K_d = 176 \pm 33 \mu M \) at pH 7.4. In an effort to characterize further this interaction using nuclear magnetic resonance spectroscopy, titration calorimetry, gel-filtration chromatography, affinity chromatography, and ultrafiltration, we were unable to find any evidence for the binding of ATP to metallothionein.

Metallothioneins (MTs)\(^1\) are a class of small (<7 kDa) proteins with a high cysteine content (~30%) and the highest known metal content after ferritins, which bind both essential (Cu\(^{+2}\) and Zn\(^{+2}\)) and nonessential (Cd\(^{+2}\) and Hg\(^{+2}\)) metals. MTs are ubiquitous proteins, found in animals, higher plants, eukaryotic organisms, and some prokaryotes. Metal coordination in metallothionein has a high thermodynamic but low kinetic stability. This means that metal binding is very tight, but very facile metal exchange occurs with other proteins. For this reason, MTs are thought to function biologically as intracellular distributors and mediators of the metals they bind (2, 3).

Despite the fact that MTs have been investigated for over 40 years, no clear physiological role can be unambiguously assigned to this protein. A recent series of articles by Vallee and co-workers (4–7) on the redox state dependence of the amount of zinc bound to MT has attracted much attention (7). They have shown that an oxidoreductive mechanism modulates the affinity of zinc for the cysteine thiolate ligands, and key players have shown that an oxidoreductive mechanism modulates the binding of zinc to MT. They used the Hummel-Dreyer method (13) to extract the ATP dissociation constant, and they found that the release of zinc from MT and the behavior of MT on a gel-filtration column was modified in the presence of ATP. In our experimental effort to characterize the structural details of the binding of ATP to metallothionein and the identity of the binding site, we could not find any evidence of an interaction between ATP and MT. In these experiments, several independent methods were used that included NMR spectroscopy, titration calorimetry, gel-filtration chromatography, affinity chromatography, and ultrafiltration.

**EXPERIMENTAL PROCEDURES**

Materials—A recombinant mouse [Cd\(_7\)]-MT1 was expressed from a pET3d vector (Novagen) in the Escherichia coli BLR(DE3) strain. 5 ml of LB starter cultures were inoculated with 5 \( \mu \)l of glycerol stock and grown for 4–6 h before two of these were used to inoculate 200-ml LB cultures. These were pelleted after 3 h, resuspended in brain heart infusion media, and used for inoculation of fresh circle grow medium with 1.5% (v/v) pre-culture. All cultures were grown at 37 °C and had 150 mg/liter ampicillin. Expression of MT1 was induced by 1 \( \mu \)M isopropyl-\( \beta \)-thiogalactopyranoside at an \( A_{600} \) of 1.2, followed by a 30-min growth before addition of 0.4 \( \mu \)M CdSO\(_4\). Cells were harvested 12 h post-induction, pelleted, washed with 20 \( \mu \)l Tris-HCl, 0.25 \( \mu \)l sucrose, pH 8.0, and resuspended in the same buffer with 0.04% \( \beta \)-mercaptoethanol. They were lysed with a French pressure cell. After centrifugation the supernatant was loaded onto a POROS HQ 20 anion exchange column (2 × 25 cm) run with an A\textsubscript{280} fast performance liquid chromatography system (Amersham Pharmacia Biotech) and equilibrated with 20 \( \mu \)l Tris-HCl, pH 8.6. MT1 was eluted by washing the column with 3.5 column volumes of the same buffer. Fractions were monitored at 280, 254, and 220 nm, and their cadmium content was assessed by atomic absorption spectroscopy (Varian SpectrAA-100). Pooled fractions that contained MT1 were concentrated by ultrafiltration (Amicon, YM3 membrane) in the presence of 1.2 \( \mu \)M dithiothreitol before loading onto a Sephadex G-75 gel filtration column (2.6 × 80 cm) equilibrated with 20 \( \mu \)l Tris-HCl, pH 8.6. The final peak was pooled and concentrated for further studies.

Cd,Zn-metallothionein II (Cd,Zn-MT2) and ATP were purchased from Sigma, and NHS-activated Hi-Trap affinity columns were obtained from Amersham Pharmacia Biotech.

**NMR Spectroscopy**—The \(^{31}P\) NMR experiments were carried out on a Varian Unity INOVA 500 MHz NMR spectrometer and all other NMR experiments on a Varian Unity INOVA 800 MHz NMR spectrometer. Watergate solvent suppression (14) was used for the experiments involving proton detection. All measurements were carried out at 25 °C, and the pH was kept constant at 7.4. The proton spectra were referenced using the chemical shift of water, which is 4.76 ppm at 25 °C (15). For the titrations of metallothionein with ATP, the concentration of mouse-MT1 was kept at 0.29 mM, and depending on the sensitivity of the experiment various ranges of ATP concentrations were used (between 0.1 and 6.0 mM). NOESY spectra (16) with mixing times between 100 and 400 ms were acquired on samples containing 0.6 mM mouse-MT1 and concentrations of ATP varying between 0.2 and 20 mM in a search for transfer NOE peaks. Longitudinal (\( T_1 \)) \(^{31}P\) NMR relaxation times were acquired with the inversion recovery method (17), whereas the proton \( T_1 \) relaxation times were obtained with a diffusion-edited inversion recovery experiment (18) in order to suppress the signals of the protein.

* This work was supported in part by National Institutes of Health Grant DK18778 (to I. M. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by Erwin Schrödinger Fellowship Project Number J-1618CHE from the Austrian Fonds zur Förderung der Wissenschaftlichen Forschung. Current address: Institute of Pharmaceutical Chemistry, University of Graz, Universitätsplatz, A-8010 Graz, Austria.

§ Supported by a Louise T. Dosdall fellowship from the Graduate School at the University of Minnesota.

¶ To whom correspondence should be addressed: Dept. of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, 6-155 Jackson Hall, 321 Church St. S.E., Minneapolis, MN 55455. Tel.: 612-624-5977; Fax.: 612-625-2163; E-mail: armitage@bscl.msi.umn.edu.

\(^1\) The abbreviations used are: MT, metallothionein; NOESY, nuclear Overhauser effect spectroscopy; NOE, nuclear Overhauser effect; NHS, N-hydroxy succinimide.

(Received for publication, December 1, 1999, and in revised form, December 29, 1999)
Prepacked NHS-activated 1-ml Hi-Trap columns from Amersham Pharmacia Biotech were used for all affinity chromatography experiments. The gel is based on highly cross-linked agarose beads with 6-atom spacer arms attached to the matrix by epichlorhydrine and activated by N-hydroxysuccinimide, which binds to primary amine groups. The substitution level of this gel is −10 μm NHS groups/ml gel. A coupling buffer of 0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3, was used, and unreacted NHS groups were deactivated by repeated washing with 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3, and 0.1 M acetate, 0.5 M NaCl, pH 5, solutions. Sample volumes of 50 μl were put onto the column and operated at a flow rate of 0.44 ml/min.

### Results and Discussion

**NMR Spectroscopy**—Mouse metallothionein-1 was titrated with ATP, and the ¹H NMR chemical shifts of both mouse-MT1 and ATP and the ³¹P NMR chemical shifts of ATP were monitored. Binding phenomena whether or not they involve structural changes should be easily detected by this method and manifested in changes in either or both the respective chemical shifts and line widths. However, no changes in any of the chemical shifts or line widths were observed (Fig. 1). More dramatically perhaps are the superimposable NOESY spectra of mouse MT1 and mouse MT1 + ATP as shown in Fig. 2 for the fingerprint region. The indiffERENCE of even the labeled side chain lysine protons, which according to Jiang et al. (1) should be the closest to the binding, is clearly manifested. It is worth mentioning that care needs to be exercised when dissolving ATP in the 20 mM Tris buffer at pH 7.4 since the acidity of ATP disodium salt is high enough to change the pH in this buffer even with minute amounts of ATP. The chemical shifts of the ³¹P resonances are particularly susceptible to pH changes, and thus careful matching of the pH of the ATP solution used for the titration to the pH of the MT sample is necessary.

Another sensitive NMR parameter to detect binding of a small ligand to a larger protein is by observing the line widths...
or more accurately the relaxation times of the ligand. The longitudinal ($T_1$) relaxation times of $^1$H and $^{31}$P resonances of ATP in Fig. 1 do not indicate a change in the motional behavior of ATP, which can only be explained if ATP is not bound to or interacting with metallothionein to any significant extent.

The binding of a small ligand to a protein can be effectively studied by transfer NOE experiments (18, 21, 22) in the case of fast exchange between the bound and free ligand. These transfer NOE peaks result from the change in rotational correlation time and thus relaxation times of a small ligand upon the binding to a relatively large protein. The free ligand has small and positive NOEs, but when it is bound to a macromolecule, the effective correlation time is increased which results in large and negative NOEs. We acquired NOESY spectra on samples with ATP:mouse-MT1 ratios of 2:1, 5:1, 10:1, and 20:1 but could not detect any transfer NOE peaks. In fact, the small and positive NOE peaks that were also found for free ATP did not change their appearance at any ATP:MT ratio.

**Tritration Calorimetry**—The binding isotherm corresponding to a plot of integrated heats of titrating a sample of 0.34 mM mouse MT1 or 0.2 mM Cd,Zn-MT2 in 20 mM Tris-HCl, pH 7.4, with solutions of 10 mM ATP in the same buffer and at the same pH did not show any release of heat upon mixing, which is consistent with a zero change in enthalpy. Due to restricted mobility and flexibility of both the protein and the water-soluble ligand, a binding interaction would lead to an entropy change that would be negative for this reaction, and therefore any binding must be accompanied by a larger negative enthalpy change to give a negative free energy $\Delta G = \Delta H - T\Delta S$.

**Gel-filtration Chromatography**—Jiang et al. (1) reported that the apparent molecular size of metallothionein as measured by a gel-filtration column was reduced in the presence of ATP. We used a Sephadex G-75 column (1.5 x 80 cm) to investigate this behavior and found no changes in the retention times of Cd7-MT1 in the chromatograms (Fig. 3). The trough at the position of ATP in the chromatogram of mouse-MT-1 on a column equilibrated with a buffer containing 0.5 mM ATP (Fig. 3D) stems from the fact that the sample applied to that column contained just 0.36 mM ATP.

A reduction in the apparent molecular size of MT would very likely be related to a change in the tertiary structure of MT as mentioned by Jiang et al. (1). The dumbbell-shaped metallothionein molecules are known to elute at an apparent molecular mass about twice the actual molecular mass of about 7 kDa. Elution at a lower molecular weight indicates a more compact structure, which could arise from enhanced interdomain interactions that would result in a more compact metallothionein structure and a loss of the dumbbell shape. Another example of a related behavior in MTs was described by Palumaa et al. (10) when MT2 was treated with excess cadmium in the presence of phosphate ions. This resulted in a form of MT that contained 13 cadmium atoms and 2 phosphate groups per monomer which was termed Cd13-(Pi)2-MT. What is even more concerning in the report by Jiang et al. (1) is that phosphate competes with ATP for the binding to MT, since phosphate has already been shown to bind just to dimerized MT, to MT crystals, and to the above-mentioned Cd13-(Pi)2-MT. Is it possible, therefore, that Jiang et al. (1) may have somehow produced a form of MT resembling Cd13-(ATP)x-MT? In this regard it is perhaps noteworthy that our attempts to produce such a species according to the procedure used to produce Cd13-(Pi)2-MT were unsuccessful.

Another way to analyze the binding of a ligand to a protein by gel-filtration chromatography is the Hummel-Dreyer method.
For this procedure, the column is equilibrated with a buffer containing the ligand. The protein is dissolved in the buffer containing the same concentration of ligand as the elution buffer. The ligand that needs to be detectable by the monitor gives rise to a constant but elevated base line. Binding of the ligand to the protein leads to a reduced concentration of the ligand in the buffer where the sample is applied onto the column. The deficit of ligand travels with the speed of the ligand through the column.
and gives a negative peak or trough at the position where free ligand would elute. By repeating the Hummel-Dreyer method as reported by Jiang et al. (1), we too found a trough at the position of ATP if MT was applied onto the column. However, we also found troughs for every other protein tested in this way (lysozyme, bovine serum albumin, α-chymotrypsinogen, RNase, and insulin). Critical evaluation of this experiment revealed that by increasing the ionic strength, the trough disappeared. The Sephadex gel (G-25) used for this experiment is known to bind positively charged and aromatic molecules to some extent (23–25), and the partial exclusion of negatively charged ATP from this column material has been reported (24). It is also known that increasing the ionic strength reduces or eliminates the repulsion of ATP on Sephadex columns (24). We therefore postulate that by loading a protein onto the column, the exclusion of ATP is decreased by either slight binding of the protein to the column or increasing the local ionic strength and thereby decreasing the effective charge of ATP. As soon as the protein moves down the column, ATP molecules can occupy more space due to the now partially shielded charges, leading to a higher local concentration of ATP in the moving protein zone and thereby reducing the concentration of ATP behind the protein, leading to the observed trough. In other words, the free energy concentration of ATP behind the protein, leading to the observed trough. In other words, the free energy of ATP in the moving protein zone and thereby reducing the partial shielding of charges, leading to a higher local concentration of ATP on Sephadex columns through 6-atom-spacer arms. Samples of 50 μl, 50 μM ATP were applied onto the MT affinity columns and eluted with a flow rate of 0.275 ml/min. The elution volume in the absence of binding was obtained with solutions of MT and other proteins, like lysozyme, bovine serum albumin, or α-chymotrypsinogen. Another column was prepared with hexokinase bound to serve as a reference. The elution times of various ligands on the protein binding columns are shown in Fig. 4, along with the elution times of various proteins on the ATP column. Although the binding of ATP to hexokinase and glutathione to metallothionein was clearly demonstrated by this technique (Fig. 4), no indication of an interaction between either MT isomorph and ATP was found (Fig. 4, B–D). The proposed sites on MT responsible for ATP binding are lysine NH₂ groups, and these groups are also responsible for the binding of MT to the affinity columns. However, MT contains MT2 or MT1, Cd,Zn-MT2, or ATP was immobilized to NHS-activated HiTrap affinity columns via their primary NH₂ groups through 6-atom-spacer arms. Samples of 50 μl, 0.4 mM Cd,Zn-MT2 no increase in this amount was observed. One can calculate that binding with a Kd = 176 μM, as reported (1), would give a decrease of ATP concentration in the filtrate of 0.62 mM (mouse-MT1) or 0.36 mM (Cd,Zn-MT2).

The fact that each and every one of these very different experiments to probe molecular interactions did not give any indication of an interaction between MT and ATP should perhaps not be too surprising considering that MT does not carry any ATP binding consensus sequence (26), and simple electrostatic interaction between a lysine side chain and the phosphate groups on ATP is not sufficient to give a Kd = 176 μM. Jiang et al. (1) also reported an increased metal release and metal transfer between metallothionein and sorbitol dehydrogenase upon the addition of ATP to MT. We can only hypothesize that either the transfer or release of metal from metallothionein is enhanced by the presence of weak electrostatic interactions with ATP or that by dissolving ATP at the concentration (1 mM) used, the pH of the sample was lowered, which is well known to result in increased metal release in MTs (27–29).

In conclusion, with the use of five independent methods to probe molecular interaction, we could not find any indication of the binding of ATP to metallothionein or a structural change of MT in the presence of ATP.

Acknowledgment—NMR instrumentation was provided by National Science Foundation Grant BIR-961477 and the University of Minnesota Medical School.

REFERENCES
1. Jiang, L. J., Maret, W. & Valle, B. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 9146–9149
2. Kaj, J. H. & Rejima, Y. (1987) Exper. Suppl. (Basel) 32, 25–61
3. Kaj, J. H. & Schaffer, A. (1988) Biochemistry 27, 8509–8515.
4. Jiang, L. J., Maret, W. & Valle, B. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3438–3448
5. Jacob, C., Maret, W. & Valle, B. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3489–3494
6. Maret, W., Larsen, K. S. & Valle, B. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2233–2237
7. Valle, W. & Valle, B. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 3478–3482
8. Brouwer, M. & Brouwer-Hoexu, T. (1991) Arch. Biochem. Biophys. 290, 207–213
9. Brouwer, M., Hoeuex-Brouwer, T. & Cashon, R. I. (1993) Biochem. J. 294, 219–225
10. Palumaa, P., Zerbe, O. & Vasak, M. (1993) Biochemistry 32, 2874–2879
11. Furey, W. F., Robbins, A. H., Clancy, L. L., Winge, D. R., Wang, B. C. & Stout, C. D. (1986) Science 231, 704–710
12. Robbins, A. H., Mclner, D. E., Williamson, M., Collett, S. A., Xuong, N. H., Furey, W. F., Wang, B. C. & Stout, C. D. (1986) Science 231, 704–710
13. Hummel, J. P. & Dreyer, W. J. (1962) Biochim. Biophys. Acta 56, 530–532
14. Piotto, M., Saudek, V. & Sˇklenar, V. (1992) J. Biolmol. NMR 2, 661–665
15. Wishart, D. S., Bigam, C. G., Yao, J., Abildgaard, F., Dyson, H. J., Oldfield, E., Markley, J. L. & Sykes, B. D. (1993) J. Biomol. NMR 6, 135–140
16. Jerner, J., Meier, B. H., Bachmann, P. & Ernst, R. R. (1979) J. Chem. Phys. 71, 4546–4563
17. Vold, R. L., Waugh, J. S., Klein, M. P. & Phelps, D. E. (1968) J. Chem. Phys. 49, 3831–3832
18. Maret, W. & Anglistor, J. (1993) Biochemistry 32, 654–761
19. Wiseman, T., Williston, S., Brandts, J. F. & Lin, L.-N. (1989) Anal. Biochem. 179, 131–137
20. Sehile, B., Zini, E., Madjar, C. V., Thauz, N. & Tillment, J. P. (1990) J. Chromatogr. 517, 51–77
21. Ni, F. (1994) Macromol. Spectrosc. 36, 517–606
22. Sykes, B. (1993)Curr. Opin. Biotechnol. 4, 392–396
23. Jansen, J. G. (1997) J. Chromatogr. 728, 12–20
24. Gelotte, B. (1960) J. Chromatogr. 2, 330–342
25. Determann, H. & Walter, I. (1968) J. Chromatogr. 39, 131–137
26. Jeener, J., Meier, B. H., Bachmann, P. & Ernst, R. R. (1979) J. Chem. Phys. 71, 4546–4563
27. Vold, R. L., Waugh, J. S., Klein, M. P. & Phelps, D. E. (1968) J. Chem. Phys. 49, 3831–3832
28. Maret, W. & Anglistor, J. (1993) Biochemistry 32, 654–761
29. Wiseman, T., Williston, S., Brandts, J. F. & Lin, L.-N. (1989) Anal. Biochem. 179, 131–137
Re-evaluation of the Binding of ATP to Metallothionein
Klaus Zangger, Gülin Öz and Ian M. Armitage

J. Biol. Chem. 2000, 275:7534-7538.
doi: 10.1074/jbc.275.11.7534

Access the most updated version of this article at http://www.jbc.org/content/275/11/7534

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 29 references, 7 of which can be accessed free at http://www.jbc.org/content/275/11/7534.full.html#ref-list-1
Additions and Corrections

Vol. 275 (2000) 7534–7538

Re-evaluation of the binding of ATP to metallothionein.

Klaus Zangger, Gülin Öz, and Ian M. Armitage

Thanks to a reader, our attention has been drawn to the following errors in our manuscript:

Page 7535: Fig. 1B, the labeling of H-6 should be H1’; Fig. 1C, The uppercase Greek gamma should be γ; Fig. 1D, δ (ppm) should be δ (Hz). The corrected Fig. 1 appears below.

Page 7537: In the caption to Fig. 3, the column size should be 1.5 × 80 cm, not 1 × 80 cm.

Page 7538: The reported flow rate of 0.275 ml/min in the first paragraph under “Affinity Chromatography” (under “Results and Discussion”) should be 0.44 ml/min for the data shown, as reported under “Experimental Procedures,” although this experiment was done with a flow rate of 0.275 ml/min and gave exactly the same results.

These minor corrections do not change the conclusions drawn in the paper.

Fig. 1. Proton longitudinal relaxation time (A), proton chemical shifts (B), phosphorus longitudinal relaxation time (C), and phosphorus chemical shifts (D) of ATP as a function of the [ATP]:[MT] ratio.