The ATP/Metallothionein Interaction: NMR and STM†

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ABSTRACT: We have previously established that ATP binds to mammalian metallothionein-2 (MT). The interaction between ATP and MT and the associated conformational change of the protein affect the sulfhydryl reactivity and zinc transfer potential of MT [Jiang, L.-J., Maret, W., and Vallee, B. L. (1998) The ATP−metallothionein complex. *Proc. Natl. Acad. Sci. U.S.A. 95*, 9146−9149]. NMR spectroscopic investigations have now provided further evidence for the interaction. ^{35}Cl NMR spectroscopy has further identified chloride as an additional biological MT ligand, which can interfere with the interaction of ATP with MT. ^{1}H NMR/TOCSY spectra demonstrate that ATP binding affects the N- and C-terminal amino acids of the MT molecule. Scanning tunneling microscopy recorded images of single MT molecules in buffered solutions. Moreover, this technique demonstrates that the otherwise nearly linear MT molecule bends by about 20° at its central hinge region between the domains in the presence of ATP. These results may bear on the development of mild obesity in MT null mice and the role of MT in the regulation of energy balance. The interaction suggests a mechanism for the cellular translocation, retention, and reactivity of the ATP·MT complex in the mitochondrial intermembrane space. Both MT and ATP are localized there, and MT and thionein alternately bind and release zinc, thereby affecting mitochondrial respiration.

Metallothionein (MT)[1] has been known to bind zinc for more than 40 years (*1*). The zinc coordination of MT is unusual with 2 cluster networks, in which 20 cysteines bind 7 zinc atoms very tightly. The clusters differ from all other zinc-binding motifs of proteins, and they make zinc available to other cellular proteins. Our work has continued to focus on the mechanisms by which this is achieved, how MT binds and releases metals, and which biological ligands trigger zinc release and binding. A report that GTP binds to MT (*2*) prompted investigations of other nucleotides as potential MT ligands (*3*). We and others found that ATP also binds to MT and that this interaction affects the reactivity of this protein (*3*, *4*). ATP enhances both zinc transfer from MT to zinc-depleted sorbitol dehydrogenase and the reactivity of the cysteine sulfhydryl groups of MT with disulfides. Moreover, the interaction with ATP brings about a conformational change of MT which leads to a transition from a molecule that has the shape of a dumbbell to one that is more globular. Thus, in addition to glutathione and glutathione disulfide (*5*, *6*), nucleotides constitute a second class of biological ligands that interact with MT and modulate its zinc transfer potential and redox behavior. These effects suggest a metabolic link between MT and energy metabolism. Indeed, several lines of investigation now provide evidence for a function of MT and zinc in energy metabolism and regulation of energy balance. First, MT is imported into the intermembrane space of liver mitochondria, releases zinc, and inhibits ADP-stimulated respiration (*7*). The intermembrane space is particularly rich in adenine nucleotides. Second, mice with a genetic knock-out of MT-1 and MT-2 develop a mild form of obesity, which is characterized by significantly increased hepatic lipids and plasma leptin levels (*8*). Third, MT is cold-induced in brown adipose tissue (*9*). Fourth, both in vitro and in vivo studies suggest that MT and its apoprotein thionein control the availability of cellular zinc, thereby affecting regulatory zinc functions in carbohydrate metabolism (*10*, *11*).

With the goal of further characterizing the interactions between MT and ATP, we have extended structural aspects of this work by employing ^{35}Cl and ^{1}H NMR spectroscopy. As a consequence, we have identified chloride as an MT ligand and shown that binding of chloride competes with that of ATP. Moreover, we have now found direct evidence of a conformational change of the MT molecule in the presence of ATP by scanning tunneling microscopy. Images of single MT molecules in buffered solutions reveal submolecular details of its structure. ATP pronouncedly effects bending the domains of MT around a central interdomain region that acts as a hinge. We believe that these ligand-induced changes of the structure and reactivity of MT signify a link of MT with energy metabolism and that they are

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[1] Abbreviations: MT, metallothionein; STM, scanning tunneling microscopy.

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critical for the specific manner in which MT distributes zinc in the cell.

MATERIALS AND METHODS

Materials. ATP (disodium salt, SigmaUltra) and rabbit liver Cd,Zn-metallothionein II (Cd,Zn-MT-2) were obtained from Sigma; sodium chloride (Suprapur) was from Merck, Darmstadt, Germany. Cd_7MT-2 was prepared by in vitro substitution of the metals using cadmium chloride (Puratronic) from Johnson Matthey, Royston, Hertfordshire, Great Britain (*12*).

^{1}H NMR Spectroscopy. TOCSY spectra (Varian UnityPlus 400 MHz spectrometer equipped with a 5 mm triple resonance probe) were acquired at 25 °C on samples of 2 mM Cd,ZnMT-2 in 20 mM deuterated Tris-HCl, pH 7.2, containing 20 mM KCl and 10% deuterium oxide in the presence and absence of ATP (SigmaUltra). Samples were kept under argon (*13*). Each spectrum was acquired with 4096 points and 32 scans per FID in t2 and 256 complex FIDs in t1. The spectral width was 5000 Hz in both dimensions. The mixing time was 80 ms, using a DIPSI-3 clean TOCSY mixing sequence with an RF field of 10 kHz.

^{35}Cl NMR Spectroscopy. Chloride NMR was performed on a Bruker 500 MHz spectrometer at 25 °C. Cd_7MT was concentrated with Centricon 3 (Amicon) centrifugal filters in 20 mM Tris-HCl, pH 7.4, and added to 100 mM sodium chloride in 10% (v/v) D_2O in the same buffer, which had been treated with Chelex-100 (Bio-Rad, Hercules, CA). For titrations, small aliquots of a stock solution of ATP, which had been adjusted to pH 7.4 with Tris base, were added to the sample. The pH of the samples was monitored with a microelectrode from Orion (Beverly, MA) to ascertain that there are no changes of pH after additions of ligands. All other methods were as described by Jiang et al. (*3*).

Scanning Tunneling Microscopy. The STM experiments were performed by use of a Molecular Imaging Pico STM (model MS300). The substrates (platforms) were made by evaporating gold (Goodfellow Ltd.) on freshly cleaved muscovite mica (Agar Scientific) at 250 °C under a pressure of 0.1 N m^{-2} and deposited at a rate of 0.2 nm s^{-1}. Mr. Chris Goodwin from Thin Film Facility, Nuclear Physics Department at University of Oxford, prepared the platforms. Additional gold samples were bought from Metallhandel Schroer GmbH. These were vapor-deposited on chromium (1−4 nm thick) on borosilicate glass with a final gold thickness of 200−300 nm. Flame annealing of gold/mica substrate was required to generate Au(111) crystal surfaces. These are atomically flat and suitable for studying adsorption of proteins by STM. Flame-annealing was carried out in a butane flame; the sample was then quenched in air and immersed in pure deionized water (Millipore, 18 MΩ) or 15 mM AnalaR potassium phosphate buffer (pH 7, prepared with 18 MΩ Millipore water) within the Molecular Imaging fluid cell. The latter was soaked overnight in a mixture of 30% H_2O_2 and 70% concentrated H_2SO_4 (caution!), and then rinsed in boiling Millipore water for at least 1 h prior to use. Tips were prepared by electrochemical etching of tungsten wire (0.25 mm, Agar Scientific) in 2 M NaOH and coated in Apiezon wax.

For ambient imaging, a freshly annealed gold substrate was immersed in a solution of MT (1−1.5 μM) and was removed from the solution after 5−15 min and then immediately immersed in buffer (15 mM potassium phosphate, pH 7.4) within the fluid cell.

All buffer components, solvents, and other reagents were of biotechnology grade, or the highest purity available. All buffer solutions used in protein preparation were filtered through sterile 0.22 μm filters prior to use.

RESULTS

We have previously established the binding of ATP to MT by using an equilibrium method (*3*, *14*) and now employed other physical methods to determine structural details of the interaction between ATP and MT. We investigated whether ^{35}Cl NMR spectroscopy could be used to monitor the interaction between MT and ATP. Enhancement of chloride quadrupole relaxation by macromolecules has been employed as a probe for anion-binding sites of proteins (*15*), either on metal centers (*16*) or on protein side chains (*17*). Addition of Cd_7MT to a solution of 100 mM sodium chloride in buffer to yield a final concentration of 0.11 mM broadens the ^{35}Cl resonance from 15.1 Hz (Figure 1A) to 42.6 Hz (Figure 1B). These results identify chloride as an MT ligand. When ATP is then added to a final concentration of 10 mM, the resonance sharpens to a line width of 25.0 Hz (Figure 1C). Thus, ATP perturbs the interaction between MT and chloride, possibly by competing for the same binding site(s). Successive additions of ATP to a solution of Cd_7MT increasingly sharpen the ^{35}Cl NMR line. An apparent binding constant of 933 ± 96 μM is estimated from fitting the titration data with a hyperbolic decay function (Figure 2). In the same buffer, we determine a line broadening by cadmium chloride of about 6 Hz per 100 μM Cd^{2+}, i.e., of the same order of magnitude as that observed earlier at higher chloride concentrations (*18*). The tight binding of cadmium in Cd_7-MT [stability constant of 5×10^{-17} M at pH 7 (*19*)] would not allow for a sufficiently large free cadmium concentration to be present in equilibrium with MT and to cause a broadening such as the one observed here. Furthermore, we have removed any free cadmium ions from the sample by gel filtration after reconstitution with cadmium. We have not observed any changes in line width when keeping the sample at 4 °C for 3 days, further attesting to the stability of Cd_7-MT under the experimental conditions.

We then examined the effect of the ATP/MT interaction on the structure of MT by two-dimensional ^{1}H NMR spectroscopy. TOCSY spectra in particular provide comprehensive fingerprints of resonance positions and chemical shifts induced by conformational changes. In the presence of 20 mM chloride, i.e., under conditions for which sequence-specific assignments were made for rabbit MT-2 (*13*), chemical shift changes are localized to the N-terminal Met, Asp and C-terminal Ala, Cys residues at ATP/MT ratios of 5 (Figure 3). Magnesium chloride (5 mM) has no effect on the TOCSY spectra of MT in the presence of ATP.

Scanning tunneling microscopy (STM) has now been applied successfully to image single biomolecules under physiological conditions. Submolecular details of metalloproteins have been resolved. The image obtained from STM of Cd_7MT adsorbed on a gold surface [Au(111)] is similar to that previously reported for Zn_7MT (*20*). MT is not adsorbed selectively onto the gold by introducing a geneti-

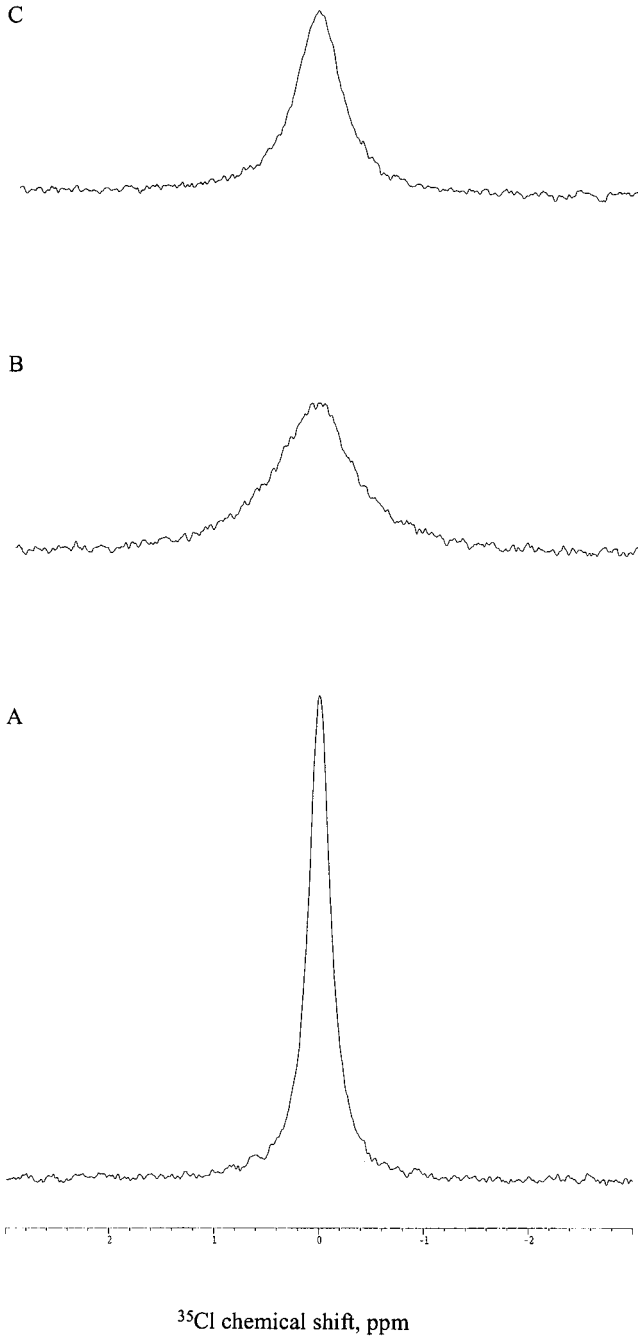

FIGURE 1: ^{35}Cl NMR spectra. (A) 100 mM sodium chloride, (B) addition of Cd_7MT to a final concentration of 0.11 mM, and (C) addition of ATP to a final concentration of 10 mM.

cally engineered surface cysteine residue (*21*). Yet, MT molecules are spread on the gold surface with remarkable uniformity that indicates specific interactions. The side view of a single MT molecule (Figure 4A) shows peaks, consistent with enhancement of the tunneling current, presumably by four cadmium ions in the C-terminal domain (left), separated by the interdomain cleft from the remaining three cadmium ions in the N-terminal domain (right). These same features are present in the top view of a single MT molecule (Figure 4B). Work in progress seeks to offer an explanation of the origin of the differential enhancement of the tunneling current.

When Cd_7MT (1.2 μM) is examined after the addition of ATP/Mg to give a solution containing a ratio of 1 between

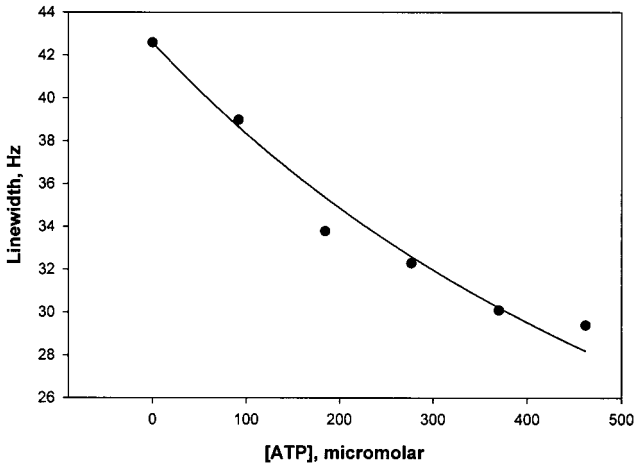

FIGURE 2: ^{35}Cl NMR line width titration. Cd_7MT-2 (110 μM) in 100 mM sodium chloride, 20 mM Tris-HCl, pH 7.4 (10% v/v D_2O), was titrated with a stock solution of ATP in the same buffer. The titration data were fitted with a 2-parameter hyperbolic decay function (SigmaPlot, SPSS Inc., Chicago, IL).

Cd_7MT and ATP/Mg, many of the MT molecules appear to be 'bent' (Figure 5A). The additional features in Figure 5A are presumably due to molecules that did not survive the tip scanning across the surface. The change of molecular shape is exemplified by the image of a single MT molecule (Figure 5B) that shows a distinct bending of the two domains of the protein. Rather than being arranged in a linear fashion as in Figure 4B, the domains have now changed their position with respect to one another through pivoting on the linker region that serves as a hinge of the molecule. It is a fair assumption that this change is due to the addition of ATP/Mg, since magnesium by itself did not elicit any changes. Vectors applied to 40 molecules of MT have a bending angle of $8 \pm 1°$, while in the presence of ATP the bending angle of the same number of molecules is $26 \pm 3°$.

DISCUSSION

Chlorine and proton NMR spectroscopic data reveal the interaction between ATP and MT. These data are completely consistent with our findings (*3*). In both ATP- and GTP-binding sites of other proteins, the β-phosphate interacts with a lysine side chain (*22*). Protein lysines are involved in the Walker motif of ATP-binding sites (*23*) and in ATP-binding sites of purinergic receptors (*24*). We have shown that modification of the lysines in MT by carbamoylation abolishes the interaction with ATP (*3*), thus clearly implicating the lysines as part of the ATP-binding site. Among the eight lysines of the protein, Lys-31 binds phosphate in crystals of MT (*25*). The side chains of lysines are also typical chloride-binding sites in many proteins. Therefore, it is no surprise that ATP affects chloride binding to MT.

In our earlier work, the Hummel−Dreyer method (*14*) was the only one we used to measure the MT/ATP interaction (*3*). A subsequent report questioned the validity of this method for the determination of the binding of ATP to MT (*26*). The authors indeed report peaks and troughs in the chromatograms, thus confirming our observations; their interpretation differs from ours, however. They do not attribute these chromatographic features to an interaction between MT and ATP. Two reasons were given for this conclusion. First, similar peaks and troughs were noticed with

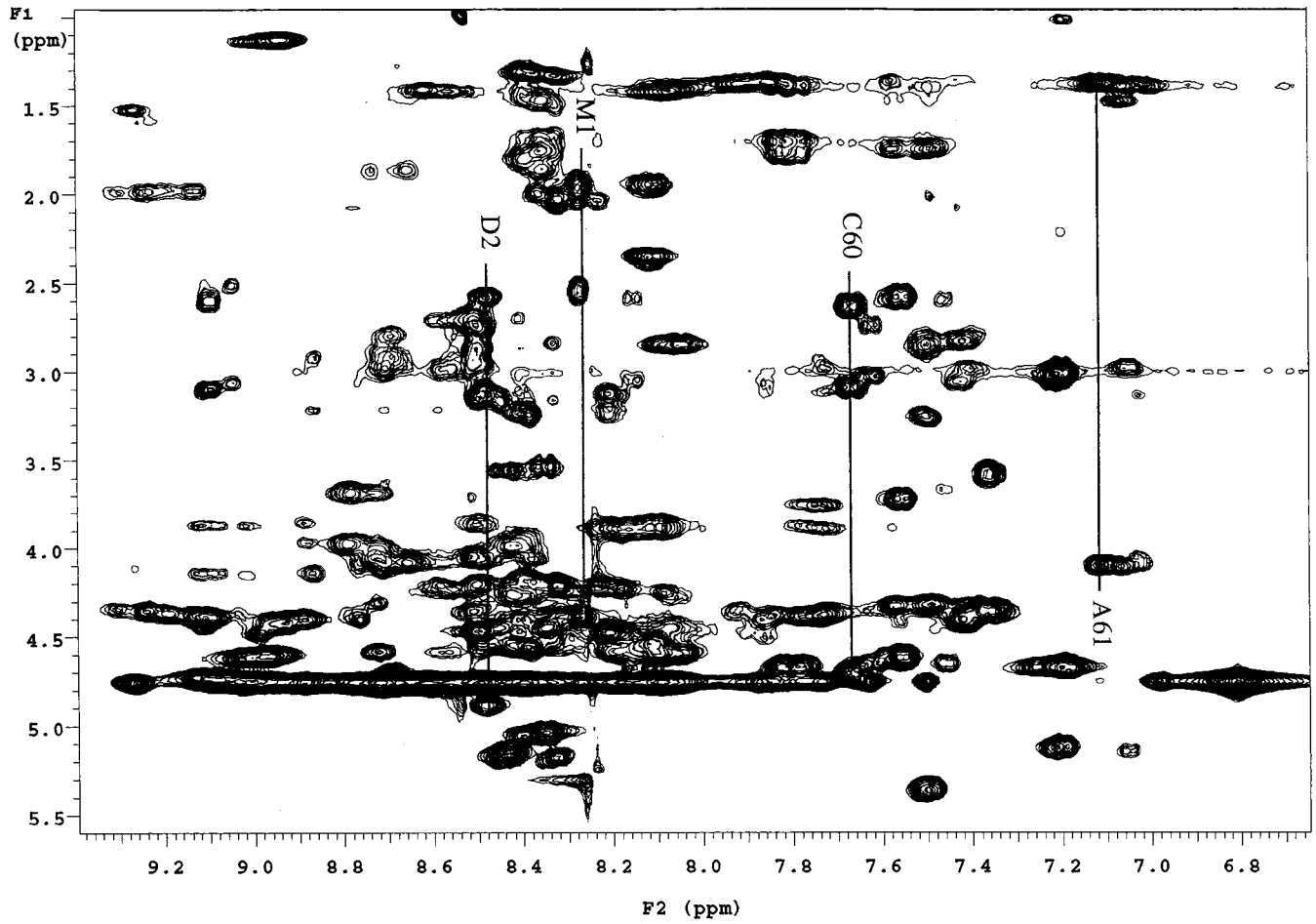

FIGURE 3: Superimposed TOCSY spectra of Cd,ZnMT in the presence and absence of ATP. Amide proton chemical shift changes are given in Hertz relative to the spectrum of MT in the absence of ATP and are referenced to DSS at 0.00 ppm: A61, −7.6; C60, −6.0; M1, +5.4; and D2, +5.1.

other proteins. Second, the peaks and troughs disappeared at higher ionic strengths of sodium chloride. These results do not seem to invalidate a method that has stood the test of time. At least two of the other five proteins used, i.e., ribonuclease A (*27*) and albumin (*28*), clearly bind ATP as has been confirmed by other methods. Also, changes of the ionic strength that were interpreted as affecting the interaction of ATP with the column matrix actually affect the interaction of ATP with MT as demonstrated by our ^{35}Cl NMR experiments. In fact, our Hummel−Dreyer experiments demonstrated saturable binding, which was abolished when employing lysine-modified MT or AMP as controls. Thus, there is no reason to suspect that this method fails in this instance, in particular since it has been employed to determine ATP binding to other proteins (*29*, *30*). Last, but not least, the interaction between MT and ATP has now been measured with a new method (*4*). ATP enhances the reaction of MT with Ellman's reagent (*3*, *4*). From a hyperbolic dose−response curve, a binding constant of 310 μM for ATP was calculated (*4*). It is noteworthy that this determination was made in the presence of 0.15 M potassium chloride (pH 7.6). Thus, in that investigation as well as in the present study, the apparent binding constant for ATP to MT in the presence of chloride is significantly higher than that of 176 μM determined in the absence of chloride (*3*).

A conformational change of MT in the presence of ATP was postulated earlier on the basis of different elution volumes of MT and MT/ATP on a gel filtration column (*3*). This conformational change of monomeric MT has now also been recognized by direct observation of bending of the MT molecule through scanning tunneling microscopy. This method appears to be particularly well suited for this purpose, attesting to its emerging role as a structural technique complementary to both NMR spectroscopy and X-ray diffraction. Because of the dynamics of the MT molecule in solution, NMR spectroscopy does not resolve the orientation of the two domains relative to each other (*31*). So far, only proton NMR (TOCSY) demonstrated changes in the MT molecule in the presence and absence of MT, but these changes are localized to the terminal amino acids of each domain. This is perhaps not surprising, because these residues undergo the largest movement when the molecule is bent around its central hinge region. While ensembles of molecules are analyzed with NMR and crystallography, STM can provide images of single molecules, in the case of MT at submolecular resolution.

Functional Implications. Various cellular translocations of MT have now been reported, in particular to mitochondria (*7*), the nucleus (*32*), and the extracellular space (*33*). MT does not have a known mitochondrial import sequence, a signal peptide for cellular export, or a nuclear localization signal. Hence, ligand interactions and associated conformational changes of MT might be necessary for any or all of these cellular translocations. Thus, MT has been found in

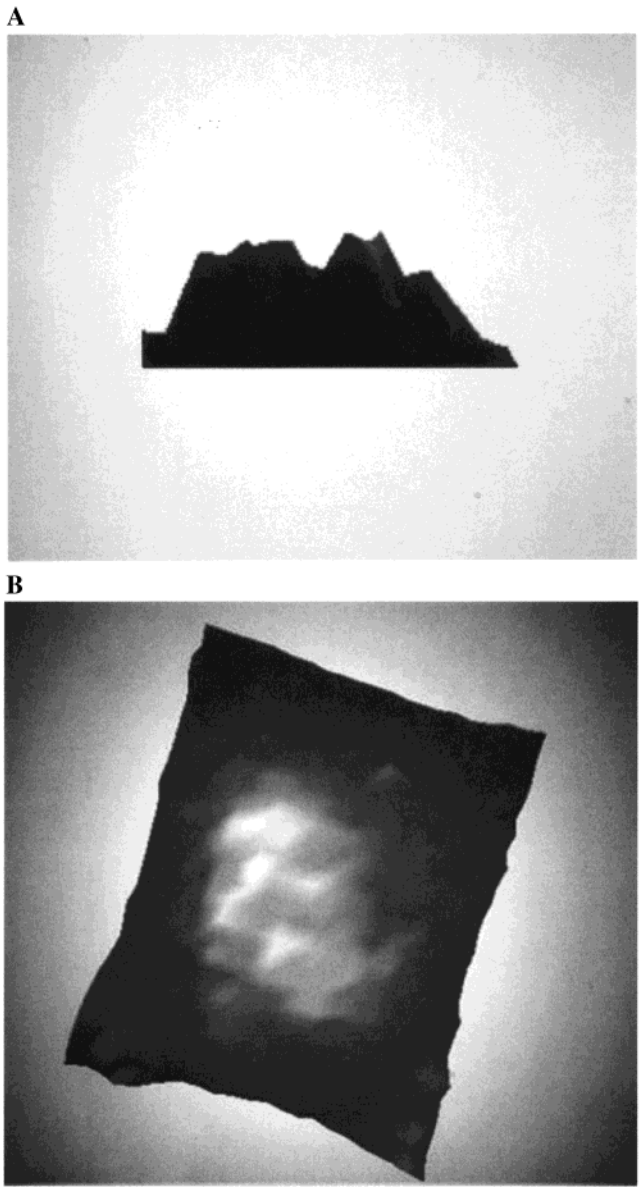

FIGURE 4: STM images of Cd_7-MT. (A) Profile of the MT molecule with the presumed Cd_4 cluster on the left, followed by the cleft and then the Cd_3 cluster. (B) Top view of a single MT molecule. *x*, 4.0 nm; *y*, 2.0 nm; *z*, 1.6 nm. The image is 6.2 (width) × 4.9 nm. Bias, −1.08 V; set point, 0.096 nA; scan speed, 2.9 L/s.

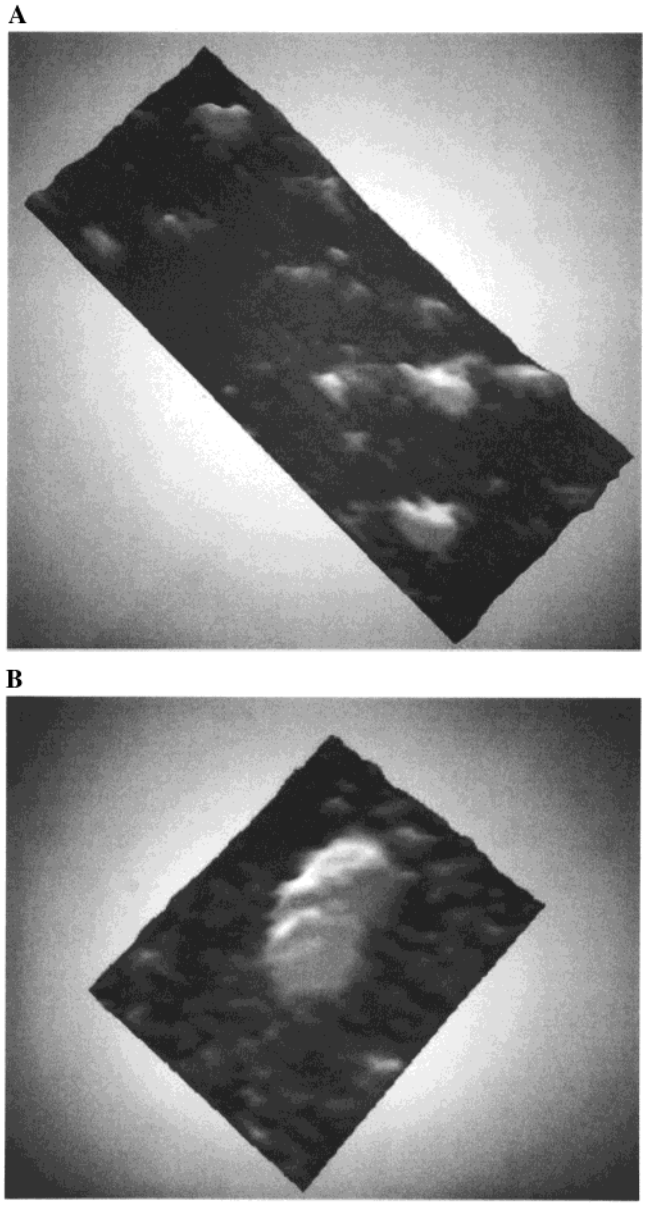

FIGURE 5: STM images of Cd_7-MT in the presence of ATP. (A) Ensemble of MT molecules. The image is 33.4 × 14 nm. Bias, −0.920 V; set point, 0.120 nA; scan speed, 1.1 L/s. (B) Top view of a single MT molecule. The image is 6.2 × 7.6 nm. Bias, −0.8 V; set point, 0.09 nA; scan speed, 1.3 L/s.

the intermembrane space of mitochondria where the concentration of ATP is high (*7*). The interaction between ATP and MT and the associated conformational change of the protein might be necessary for the retention of MT in the mitochondrial intermembrane space. Further, the interaction of MT with ATP affects the reactivity of its thiolate ligands of zinc and the zinc transfer potential (*3*). Therefore, ATP might be an effector for zinc release from MT in the intermembrane space and ensuing inhibition of ADP-stimulated mitochondrial respiration (*7*). The interaction between ATP and MT could also initiate the formation of a Zn−ATP complex as a specific cofactor for enzymes in energy metabolism. Thus, a Zn−ATP complex is the preferred substrate for both pyridoxal kinase and flavokinase (*34*, *35*). Moreover, MT activates pyridoxal kinase (*34*). Taken together, these data identify mitochondrial respiration and ATP metabolism as targets of the role of MT in the regulation of energy balance as evident from the mild obesity and hyperleptinemia in MT-I and -II knock-out mice (*8*).

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