Toxic Heavy Metal Ions Activate the Heme-regulated Eukaryotic Initiation Factor-2α Kinase by Inhibiting the Capacity of Hemin-supplemented Reticulocyte Lysates to Reduce Disulfide Bonds*

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Addition of toxic heavy metal ions (Cd²⁺, Hg²⁺, and Pb²⁺) to hemin-supplemented rabbit reticulocyte lysate brings about the activation of the heme-regulated eukaryotic initiation factor 2α kinase (HRI) and the inhibition of protein chain initiation. In this report we examined the effects of monothiol and dithiol compounds, metal ion-chelating agents, and metallothioneins (MT) on metal ion-induced inhibition of protein synthesis. The dithiol compounds dithiothreitol and 2,3-dimercaptopropane sulfonic acid prevented and relieved the inhibition of protein synthesis caused by Cd²⁺ and Hg²⁺ in hemin-supplemented lysates, but the monothiol compounds 2-mercaptoethanol, cysteamine, D-(−)penicillamine, and glutathione had no effect. The inhibition of protein synthesis caused by Cd²⁺ was reversed by the addition of excess EDTA but not by the addition of excess nitrilotriacetic acid. Toxic heavy metal ions inhibited the capacity of hemin-supplemented lysate to reduce disulfide bonds. Addition of excess EDTA to Cd²⁺-inhibited lysates restored the capacity of the lysate to reduce disulfide bonds and inhibited the phosphorylation of eukaryotic initiation factor eIF-2. MTs and their apoproteins (apoMTs) inhibited the activation of HRI and protected protein synthesis from inhibition by Cd²⁺, Hg²⁺, and Pb²⁺. Addition of apoMTs to heavy metal ion-inhibited lysates restored the capacity of lysates to reduce disulfide bonds. The restoration of the lysate's thioredoxin/thioredoxin reductase activity was accompanied by the inactivation of HRI and the resumption of protein synthesis, indicating that apoMTs can “detoxify” metal ions already bound to proteins. Several observations presented in this report suggest that the binding of metal ions to the α-domain of MT is responsible for the ability of MT to sequester bound metal in a non-toxic form. Addition of glucose 6-phosphate or NADPH had no effect on protein synthesis in metal ion-inhibited lysates, and NADPH concentrations in Cd²⁺-inhibited and hemin-supplemented control lysates were equivalent. The data suggest that the metal ions cause the inhibition of protein synthesis by binding to vicinal sulfhydryl groups present in some critical protein(s), possibly the dithiols present in the active site of thioredoxin and (or) thioredoxin reductase, which leads to the activation of HRI.

Toxic heavy metal ions (i.e. Cd²⁺, Hg²⁺, and Pb²⁺) have been found to inhibit protein synthesis in hemin-supplemented reticulocyte lysates with biphasic kinetics (1). The shut off of protein synthesis was due to an inhibition of protein chain initiation and occurred in conjunction with the phosphorylation of the α-subunit of eukaryotic initiation factor (eIF)², the loss of eIF-2B activity (also currently designated RF), reversing factor (2), and GEF, guanine nucleotide exchange factor (3)), and the disaggregation of polyribosomes. Inhibition of protein synthesis initiation in hemin-supplemented lysates also occurs in response to a variety of other conditions (reviewed in Refs. 4–6), which include addition of oxidants or sulfhydryl reactive agents, and heat stress (7–20). The data indicate that the inhibition of protein synthesis, which is observed under these conditions, apparently occurs due to the activation of the heme-regulated eIF-2α kinase (HRI) or a protein kinase with similar antigenic and physical properties (1, 8, 11, 21). The mechanism by which HRI becomes activated in the presence of hemin is not yet well understood, but maintenance of protein synthesis in hemin-supplemented reticulocyte lysates has been shown to require the presence of certain sugar phosphates (e.g. Glc-6-P), a NADPH-regenerating system, and a functional thioredoxin/thioredoxin reductase system (7–11).

Metallothioneins (MT) are low molecular weight, cysteine-rich, high affinity metal-binding proteins (reviewed in Refs. 22 and 23). The observations that MT have the ability to bind to heavy metal ions, and that their synthesis is induced by the ions, suggest that MTs play a role in cellular metal metabolism, homeostasis, or detoxification. MT synthesis is also induced in certain tissues and cell types in response to a variety of other chemical and physical stresses, suggesting that MT may have additional biological roles. Regulation of intracellular oxidation-reduction potential or activated oxygen detoxification are some of the other possible functions which have been proposed for MT (22, 23). Gene transfer studies have directly demonstrated the ability of MT to protect cells from the toxic effect of heavy metal ions. However,

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the observation that the accumulation of excessive amounts of Cd²⁺-containing MT appears to be the cause of renal damage in chronic Cd²⁺ poisoning has cast some doubt on the importance of MT as a specific and effective defense mechanism against Cd²⁺ exposure in animals (24).

The observation that the relative order of potency of the metal ions in bringing about the inhibition of protein synthesis (Hg²⁺ > Pb²⁺ > Cd²⁺ > Zn²⁺) (1) is similar to the established association constants for the metals with cysteine and with sulfides (Hg²⁺ > Pb > Cd²⁺ > Zn²⁺) (25-27), suggests that the metals are acting through binding to sulfhydryls. Furthermore, the inhibition of protein synthesis by arsenite and the inhibition of protein synthesis by Cd²⁺ at concentrations 30-50 times lower than those required for Zn²⁺ (1), imply that this inhibition is brought about through the interaction of the metal ions with spatially adjacent (vicinal) sulfhydryls (28, 29). In this report we have compared the ability of monothiol compounds, dithiol compounds, other metal-chelating agents, and MTs to prevent or relieve the inhibition of protein synthesis by heavy metal ions in hemin-supplemented reticulocyte lysates. The data presented here fulfill all the additional basic criteria suggested for demonstrating the presence of functional dithiols in the active sites of proteins (30-32).

The observations that toxic heavy metal ions have no effect on NADPH levels, yet inhibit the capacity of hemin-supplemented reticulocyte lysates to reduce disulfide bonds, suggests that the site of action of the metal ions may be the vicinal sulfhydryl groups present in the active site of thioredoxin and (or) thioredoxin reductase (33, 34). Addition of MT and their apoproteins (apoMT) to hemin-supplemented lysates protected protein synthesis from inhibition by toxic heavy metals. Furthermore, apoMTs were observed to have the ability to "detoxify" the metal ions, restoring the capacity to reduce disulfide bonds, and protein synthesis in previously inhibited lysates.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cyclic AMP, diethyl dithiocarbamic acid, 2,3-dimercaptopropanoic acid, dithiotreitol, EDTA, nitritriacetic acid, diethylenetriamine pentaacetic acid, 2-mercaptopoethylamine, HC₁ (cysteamine), D(-)-penicillamine, glutathione (GSH), 5' adenosine monophosphate, diethylenetriamine pentaacetic acid, 2-mercaptoethylamine. Furthermore, apoMTs were observed to have the ability to "detoxify" the metal ions, restoring the capacity to reduce disulfide bonds, and protein synthesis in previously inhibited lysates.

**Protein Synthesis in the Reticulocyte Lysate**—Reticulocyte lysates were prepared from anemic rabbits as described (35), using buffered saline containing 5 mM glucose to wash the reticulocytes prior to their lysis. The inclusion of glucose in the wash buffer yields reticulocytes replete in glucose. Reticulocyte lysates prepared in this manner are capable of maintaining high constant levels of NADPH and NADP⁺ in hemin-supplemented lysates. These MTI preparations were  more effective in protein synthesis than when added alone to incubation mixtures than was the MTI preparation with the lower Cd content. Removal of the metal ion from these preparations, as described above, yielded an active apoMTI preparation.

**Phosphoprotein Profiles**—Protein synthesizing lysates were pulsed with [γ³²P]ATP (0.5 mM/µl protein synthesis mix final concentration) at 10-14 or 25-29 min (during and after shut off). Aliquots, containing an equivalent of 2.5 µl of protein synthesis mix, were denatured in SDS sample buffer and analyzed by SDS-PAGE on 8% gels (37.5:1 acrylamide/bis) followed by autoradiography as described (38).

**Assay for the Capacity of Lysates to Reduce Disulfide Bonds**—The ability of lysates to reduce disulfide bonds present in insulin was measured by a modification of the method described by Jackson et al. (11). Lysates were incubated under protein synthesis conditions at 30 °C. At the times indicated in the figure legends, 10-µl samples were added to 7 µl of a 1.2 mg/ml insulin solution. After incubation for 15 min at 30 °C, reaction mixtures were diluted with 32 µl of buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM Mg(OAc)₂, and 50 mM NaCl, and 1 µl of a 50 mM thioredoxin MB solution (prepared in acetonitrile) was then added. Assay mixtures were incubated for another 5 min at 30 °C, and the reactions were then terminated by the addition of 20 µl of 4 times concentrated SDS sample buffer. Aliquots (25 µl) of each sample were analyzed by SDS-PAGE on 20% gels. After electrophoresis, gels were fixed for soaking for 30 min in a 0.008 N HCl solution containing 40% methanol and 10% acetic acid. Gels were then illuminated with a long wavelength ultraviolet light box (Chromato-vue, Ultra-Violet Products, Inc.) and fluorescent bands were photographed with Kodak Panatomic X film through a yellow wratten gelatin filter No. 8 (cut off at 460 nm) using an exposure time of 2 min (39).

**Characterization of NADPH and NADP⁺**—The levels of NADPH and NADP⁺ in hemin-supplemented and Cd²⁺-inhibited lysates were measured by the method described by Ernst et al. (7). Fluorescence of samples was measured in a 1 × 1-cm quartz cuvette using a Perkin-Elmer Cetus 650-40 Fluorescence Spectrophotometer with an excitation wavelength of 340 nm and an emission wavelength of 460 nm. The slit width was 5 nm, respectively. Determinations were done in triplicate, and the experiments were repeated at least twice.
Effects of Monothiol and Dithiol Compounds and Metal Chelating Agents on Protein Synthesis in Heavy Metal Ion-inhibited Reticulocyte Lysates—DTT was found to protect protein synthesis in hemin-supplemented lysates from inhibition by Cd** or Hg** (Fig. 1, A and B). Furthermore, addition of DTT to lysates containing Cd** or Hg**, after the shutoff of protein synthesis, was found to restore protein synthesis to near control rates. We subsequently examined the ability of a variety of monothiol and dithiol compounds to protect protein synthesis from inhibition by Cd** or Hg**. The monothiol compounds cysteamine, 2-mercaptoethanol, D(-)-penicillamine and glutathione at concentrations of 1 mM did not protect protein synthesis in hemin-supplemented lysates from inhibition by Cd** or Hg** (data not shown). However, protein synthesis was partially or fully protected from inhibition by Cd** when 50 μM DTT, DMPS, or diethyl dithiocarbamic acid was added to the lysate (Table I). Furthermore, the delayed addition of these dithiol compounds to Cd**-inhibited lysates at 10 min was observed to stimulate the rate of protein synthesis, indicating that these compounds have the ability to partially or fully reverse Cd**-induced inhibition of protein synthesis (Table I). DTT and DMPS, but not diethyl dithiocarbamic acid, were found to have similar effects on protein synthesis when added to hemin-supplemented lysates containing Hg** (Table I). Monothiol compounds similarly added at 10 min to Cd**- or Hg**-inhibited lysates had no stimulatory effect on protein synthesis (data not shown). Concentrations of DMPS and DCCA higher than 50 μM were found to be inhibitory to protein synthesis, such that the ability of higher concentrations of these compounds to fully restore protein synthesis in Cd**- or Hg**-inhibited lysates could not be accessed. The reversal of Cd** inhibition by dithiols, but not by monothiols, has been traditionally accepted as a criterion for the presence of an active-site dithiol within a protein (30, 31). However, since thiols can readily reduce protein disulfides and exert independent effects on proteins, Gaber and Fluharty (32) have suggested an additional criterion (the reversal of Cd** inhibition by a 10-fold excess of EDTA but not NTA) be met to establish the presence of an active-site dithiol. The binding affinity of EDTA for Cd** is close to the estimated binding affinity of dithiols to Cd**, while the binding affinity of NTA for Cd** is approximately the same as the binding affinity of monothiols to Cd** (25, 27, 40). EDTA and DTPA, but not NTA, effectively protected hemin-supplemented lysates from inhibition of protein synthesis by Cd** (Table II). In addition, EDTA and DTPA were able to reverse the inhibition of protein synthesis upon their addition to Cd**-inhibited lysates at 10 min (Table II). Little effect of EDTA, DTPA, or NTA was observed upon their addition to lysates in which protein synthesis was inhibited by the addition of Hg** (Fig. 1, other data not shown). At very low Hg** concentrations (0.5 pM) slight protection by DTPA and EDTA was observed (data not shown). Addition of EDTA or DTPA (but not NTA) to Pb**-inhibited lysates was also observed to protect and restore protein synthesis (data not shown). DTPA, which has a 100 times higher binding affinity than EDTA for Cd**, was also more effective than EDTA in protecting protein synthesis from inhibition by Cd**. Only a 2-fold excess of DPTA over Cd** was required to protect hemin-supplemented lysates from inhibition of protein synthesis; 50 μM DTPA was observed to be capable of fully protecting protein synthesis from inhibition by 25 μM Cd** (data not shown). Effects of MTI and II, and ApoMTI and II on Heavy Metal Ion-induced Inhibition of Protein Synthesis in Hemin-supple-
mented Lysates—Titrations of metal ions into hemin-supplemented reticulocyte lysate were carried out to determine the concentrations of $Hg^{2+}$, $Pb^{2+}$, and $Cd^{2+}$, which inhibit protein synthesis by 50% of the hemin-supplemented control ($IC_{50}$) in a 30-min incubation (Fig. 2). Rabbit liver MTI (20 µM) and MTII (30 µM) added at the beginning of the incubation were found to protect hemin-supplemented lysates from inhibition by lower levels of $Hg^{2+}$ and $Pb^{2+}$, and somewhat higher levels of $Cd^{2+}$ (Fig. 2, A-C). Based on their metal content, 20 µM MTI and 30 µM MTII were estimated to contain equivalent amounts of unoccupied metal-binding sites (~56 µM). The change in the $IC_{50}$ for the inhibition of protein synthesis by $Hg^{2+}$, $Pb^{2+}$, and $Cd^{2+}$ in the presence of 20 µM MTI were calculated to be approximately 7, 8, and 15 µM, respectively. In the presence of 30 µM MTII, the $IC_{50}$ for $Hg^{2+}$, $Pb^{2+}$, and $Cd^{2+}$ inhibition were approximately 3, 6, and 13 µM, respectively. Two other MTI preparations obtained from Sigma, containing approximately 20% more bound $Cd^{2+}$ (77.7 and 76.4 µg of $Cd$/$mg$ of protein) were found to have essentially the same capacity to protect protein synthesis from inhibition by $Pb^{2+}$ and had a greatly reduced capacity to protect protein synthesis from inhibition in the presence of $Hg^{2+}$ ($IC_{50}$ ~1 µM) and $Cd^{2+}$ ($IC_{50}$ ~3 µM) (data not shown).

Bound $Cd^{2+}$ and $Zn^{2+}$ were removed from the MTIs by dialysis of the proteins against water at pH 2 followed by gel filtration. Five µM apoMTI increased the $IC_{50}$ for the inhibition of protein synthesis by $Hg^{2+}$, $Pb^{2+}$, and $Cd^{2+}$ by 22, 17, and 18 µM, respectively, and apoMTII (5 µM) increased the $IC_{50}$ for $Hg^{2+}$, $Pb^{2+}$, and $Cd^{2+}$ inhibition by 17, 24, and 22 µM, respectively (Fig. 1, A-C). Protein synthesis was completely protected from the inhibitory effects of 5 µM $Hg^{2+}$, 10 µM $Pb^{2+}$, and 12.5 µM $Cd^{2+}$ in the presence of 2 µM apoMTI or II, while protein synthesis was inhibited by 25–50% by these concentrations of metal ions in the presence of 1 µM apoMT (data not shown). ApoMTI and II (5 µM) alone were found to inhibit protein synthesis by approximately 5–15%. This inhibition was observed to be reversed in the presence of low concentrations of each metal ion. The capacity of metal ions to stimulate protein synthesis to levels above those present in the apoMT controls (Fig. 2) probably reflects the ability of bound metal ions to protect cysteines present in apoMT from oxidation. Concentrations of apoMT higher than 5 µM were found to be more inhibitory to protein synthesis in the absence of added metal ions (data not shown).

The Capacity of ApoMTI and II to Restore Protein Synthesis in Heavy Metal Ion-inhibited Lysates—The addition at 10 min of either 5 µM apoMTI or apoMTII to lysates incubated in the presence of 10 µM $Hg^{2+}$ restored protein synthesis to near the rate of proteins synthesis in lysates to which the apoMT was added at the beginning of the incubation (data not shown). Restoration was not immediate and occurred after a lag period of 5–10 min. Similarly, the addition of apoMTI or II to lysates after the shut off of protein synthesis had occurred in the presence of 10 µM $Pb^{2+}$ or 12.5 µM $Cd^{2+}$ stimulated protein synthesis to rates near those present in hemin-supplemented control lysates (data not shown). MTIs were in general ineffective in restoring protein synthesis in metal ion-inhibited lysates, giving only marginal stimulation in protein synthesis rates, at very low concentration of the inhibitory ions.

The Effects of Heavy Metal Ions on the Capacity of Hemin-

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**TABLE II**

**Effect of EDTA, DPTA, or NTA on $Cd^{2+}$-induced inhibition of protein synthesis**

Protein synthesis mixtures (40 µl) containing 20 µM hemin·HCl were incubated for 30 min at 30 °C as follows: with no additions (control); with 12.5 µM Cd(OAc)$_2$ at 0 min ($Cd^{2+}$); with 250 µM of the chelating agent (EDTA, DPTA, or NTA) added at 0 min; or with 12.5 µM Cd(OAc)$_2$, added at 0 min and 250 µM EDTA ($Cd^{2+}$ + EDTA), DPTA ($Cd^{2+}$ + DPTA), or NTA ($Cd^{2+}$ + NTA) added at 0 or 10 min. Aliquots (5 µl) of the incubation mixtures were taken at 5, 10, 15, 20, and 30 min. The data are reported as counts/min (cpm × 10$^3$) of [14C]leucine incorporated into acid-precipitable protein in 30 min from a 0.1 µl aliquot of the incubation mixture (0 min addition of chelating agent) or as the rate of [14C]leucine incorporated into acid precipitable protein (cpm/min) between 20 and 30 min of incubation (10 min addition of chelating agent).

| Additions | Metal ion | Chelating agent | $[^{14}C]$Leucine incorporated in 30 min | Rate of $[^{14}C]$Leucine incorporation |
|-----------|-----------|-----------------|----------------------------------------|----------------------------------------|
|           |           |                 | cpm × 10$^3$                            | cpm/min                                |
| Control   |           |                 | 29                                     | 870                                    |
| $Cd^{2+}$ |           | +EDTA           | 29                                     | 920                                    |
| $Cd^{2+}$ |           | +DPTA           | 28                                     | 670                                    |
| $Cd^{2+}$ |           | +NTA            | 26                                     | 900                                    |
| $Cd^{2+}$ |           | +NTA            | 8.8                                    | 28                                     |

**Fig. 2.** Effect of MTI and II, and apoMTI and II on $Hg^{2+}$, $Pb^{2+}$, and $Cd^{2+}$-induced inhibition of protein synthesis in hemin-supplemented lysates. Protein synthesis mixtures containing 20 µM hemin·HCl were incubated for 30 min at 30 °C in the presence of increasing concentrations of metal ions: $Hg^{2+}$ (panel A); $Pb^{2+}$ (panel B); and $Cd^{2+}$ (panel C). Protein synthesis mixtures contained no further additions (+——+), 20 µM MTI (O——O), 30 µM MTII (□——□), 5 µM apoMTI (●——●), or 5 µM apoMTII (○——○). $[^{14}C]$Leucine incorporated into acid-precipitable protein contained in a 5 µl aliquot was determined as described under "Experimental Procedures." Values are reported as percent incorporation relative to the control incubation containing no metal ion.
supplemented Lysates to Reduce Disulfide Bonds—The capacity of lysates to reduce the disulfide bonds present in insulin was used as an assay to determine the overall ability of lysates to maintain sulfhydryl groups in proteins in a reduced state. The assay presumably reflects the activity of the thioredoxin/thioredoxin reductase system present in the lysate (9–11). Toxic heavy metal ions (Cd²⁺, Hg²⁺, and Pb²⁺), which were all previously found to inhibit protein synthesis in hemin-supplemented lysates by bringing about the activation of HRI (1), were all found to inhibit the capacity of lysates to reduce disulfide bonds (Fig. 3). A low level of Zn²⁺ (50 μM), which is only slightly inhibitory to protein synthesis, was observed to inhibit the lysate’s capacity to reduce disulfides by 80–90%, while a higher, inhibitory level of Zn²⁺ (1 mM) completely inhibited the lysate’s capacity to reduce disulfides. The capacity to reduce disulfides was unaffected (if not stimulated) in lysates where protein synthesis was inhibited under non-oxidative conditions due to the activation of HRI by heme-deficiency, or due to the activation of the double-stranded RNA-activated eIF-2α kinase by the addition of double-stranded RNA (poly(I)·poly(C)) (Fig. 3).

The ability of Thiolyte to derivatize insulin (reduced by prior incubation with a hemin-supplemented lysate) in the presence of added Hg²⁺, demonstrated that the metal ion itself is not directly affecting the ability of Thiolyte to react with the sulfhydryls in reduced insulin. In addition, the heavy metal ion-induced inhibition of the lysates capacity to reduce disulfide bonds was found to be very rapid, occurring within the first 15-min incubation step of the lysate with insulin, even without any prior preincubation of the lysate in the presence of the metal ion (data not shown). On 10% SDS-polyacrylamide gels, the metal ions were found to have little effect on the overall derivatization of lysate proteins by the Thiolyte, demonstrating that these metal ions are not present at a concentration sufficient to undergo a generalized reaction with free thiol groups present in proteins within the lysate (data not shown).

Effects of EDTA and ApoMTs on Disulfide Bond Reduction and eIF-2α Phosphorylation in Metal Ion-containing Lysates—The ability of lysate to reduce disulfide bonds or phosphorylate eIF-2 was determined under conditions where the addition of EDTA, apoMT, or MT was previously found to preserve (addition at 0 min) or restore (addition at 10 min) protein synthesis in metal ion-containing lysates (Figs. 1 and 2). Addition of EDTA to Cd²⁺-containing lysates or apoMT (I or II) to Hg²⁺-containing lysates was observed to maintain and restore the capacity of these lysates to reduce disulfide bonds, in conjunction with the inhibition of HRI activation and/or activity (Fig. 4, apoMTII data not shown). ApoMTs were found to have similar effects on insulin reduction and eIF-2 phosphorylation in lysates incubated in the presence of Cd²⁺ (data not shown). MTII and II, also inhibited HRI activation and prevented the phosphorylation of eIF-2 in Cd²⁺-containing lysates (data not shown). Cyclic AMP inhibited eIF-2α phosphorylation (Fig. 5B) in the absence of any restorative effects on the capacity to reduce disulfide bonds (Fig. 5A), under conditions where cAMP was previously found to maintain protein synthesis in Hg²⁺-containing lysates (1).

In some experiments, we noted that the sulfhydryl groups of a 24-kDa protein became oxidized in hemin-supplemented lysates in the presence of toxic heavy metals. These sulfhydryls were observed to be restored to the reduced state upon restoration of the lysates capacity to reduce disulfide bonds (Fig. 4). The oxidation of the 24-kDa protein occurred at or after the time of protein synthesis shut off, suggesting that its oxidation is not involved in bringing about the activation of HRI. Jackson and co-workers (11) have similarly noted the oxidation of a 24-kDa protein in gel-filtered (glucose deprived) lysates, and have previously reported that cAMP stimulated protein synthesis, but did not promote disulfide bond reduction in these lysates.

Fig. 3. Effect of heavy metal ions on the ability of hemin-supplemented lysates to reduce disulfide bonds. Protein synthesis mixtures were assayed for their ability to reduce the disulfide bonds present in insulin after incubation at 30 °C for 15 min as described under “Experimental Procedures.” Hemin-supplemented lysates were incubated with: no additions (lane 1); 50 μM Zn²⁺ (lane 2); 1 mM Zn²⁺ (lane 3); 25 μM Hg²⁺ (lane 4); 50 μM Cd²⁺ (lane 5); 50 μM Pb²⁺ (lane 6); 100 ng/ml poly(I)·poly(C) (lane 7); and, the absence of added insulin to the reduction assay (lane 8). Hemin-deficient lysate (lane 9). Hb, indicates the florescence of derivatized hemoglobin in the sample, while I indicates fluorescence due to the labeling of reduced insulin.

Fig. 4. Changes in the capacity of metal ion-inhibited lysates to reduce disulfide bonds and phosphorylate eIF-2α in the presence of EDTA or apoMT. Hemin-supplemented lysates were incubated under conditions for protein synthesis for 25 min at 30 °C and were then assayed for their ability to reduce insulin (A) or phosphorylate the α-subunit of eIF-2 (B) as described under “Experimental Procedures.” Hemin-supplemented lysate incubated with no additions (lanes 1 and 6); with 12.5 μM Cd²⁺ (lane 2); with 250 μM EDTA (lane 3); with 12.5 μM Cd²⁺, plus 250 μM EDTA added at 0 min (lane 4); with 12.5 μM Cd²⁺, plus EDTA added after 10 min (lane 5); with 10 μM Hg²⁺ (lane 7); and with 10 μM Hg²⁺ plus 5 μM apoMT added at 10 min (lane 8). Similar results were obtained in protection experiments where EDTA or apoMT were added to metal containing lysates at 0 min, and the ability of the lysates to reduce disulfide bonds or phosphorylate eIF-2 were measured at 10 min. The addition of 5 μM apoMTII gave the same results as the addition of apoMT (data not shown). In control experiments, addition of apoMTI or II alone was found to have no effect on the capacity of lysate to reduce disulfide bonds or the phosphorylation of eIF-2 (data not shown). Data show photograph of fluorescently labeled proteins (Hb, hemoglobin; I, insulin; *, 24-kDa protein) (A), and an autoradiogram of ³²P-labeled proteins (B), separated by SDS-PAGE.
in hemin-supplemented lysates than similar concentrations of Cd$^{2+}$ is more consistent with the observed interaction of Pb$^{2+}$ with enzymes containing active-site diethiol than with its interaction with enzymes containing single functional sulfhydryls (41). Arsenite was also observed previously to be a potent inhibitor of protein synthesis (1). Both arsenite and Cd$^{2+}$ are known to have a high affinity and selectivity for vicinal sulfhydryl groups (28, 42–47). Cd$^{2+}$ was also found to be a much more potent inhibitor of protein synthesis than Zn$^{2+}$ (30–50 times). These observations support the hypothesis that toxic heavy metal ions are binding to some protein(s) containing a vicinal sulfhydryl group, whose function is critical for maintaining protein synthesis in hemin-supplemented reticulocyte lysates. The data presented in this study further support this hypothesis. The ready reversal of Cd$^{2+}$-inhibition by diethiols, but not by monothiols, is a commonly accepted criterion for demonstrating the presence of active site diethiols in enzymes (30, 31). The reversal of Cd$^{2+}$-induced inhibition of protein synthesis by EDTA, but not by NTA, meets an additional criterion for establishing the presence of a functional diethiol in a protein (32) that is not compromised by the problem attendant with the ability of thiols to readily reduce protein disulfides and thus exert independent effects on proteins. Comparable results in experiments examining the effects of mono or diethiol compounds, and metal-chelating agents on Hg$^{2+}$- and Pb$^{2+}$-induced inhibition of protein synthesis suggest that these ions are also acting through a similar mechanism. The ability of EDTA and DTPA to reverse the inhibition of protein synthesis induced by Cd$^{2+}$ or Pb$^{2+}$, but not Hg$^{2+}$, is consistent with the fact that only Hg$^{2+}$ has a stability constant for the binding to cysteine (Hg(cys)2) that is much greater than the stability constant of its binding to EDTA and DTPA (28, 40, 41).

Maintenance of protein synthesis initiation in rabbit reticulocyte lysates has been shown to require the presence of hemin, a sugar phosphate (i.e. Glc-6-P) and a reducing system capable of reducing disulfide bonds (7–11). Sugar phosphates are required as a stimulatory “cofactor” affecting the rate of protein chain initiation and for NADPH generation by way of their metabolism through the pentose phosphate shunt. The requirement for reducing power is thought to be met by this NADPH generation, together with an active thiorodoxin/thiorodoxin reductase system (10, 11). The data in this report indicate that toxic heavy metal ions inhibit the lysate’s capacity to reduce disulfide bonds. The ability of cAMP, a competitive inhibitor of HRI (6, 48), to stimulate protein synthesis in lysates whose reducing capacity remains inhibited, suggests that the capacity to reduce disulfide bonds is required for maintaining HRI in an inactive state, but it is not required for the maintenance of protein synthesis in general. The data indicate that heavy metal ions neither deplete lysates of Glc-6-P or NADPH, nor inhibit the lysate’s capacity to generate NADPH. Cd$^{2+}$ has been reported to be a potent inhibitor of partially purified thiorodoxin/thiorodoxin reductase activity (49) and of protein disulfide isomerase (50, 51). Heavy metal ions may then inhibit the lysate’s reducing capacity directly, through binding to the vicinal sulfhydryl groups present in the active sites of either thiorodoxin or thiorodoxin reductase (33, 34).

MTs and apoMTs were observed to maintain protein synthesis in hemin-supplemented lysates in the presence of Cd$^{2+}$, Pb$^{2+}$, or Hg$^{2+}$ by preventing the inhibition of the lysate’s thiorodoxin/thiorodoxin reductase activity, thus inhibiting the activation of HRI. Presumably this is the result of the apoMT’s capacity to bind and sequester these toxic heavy metal ions (22, 23). The ability of apoMTs to restore the

**Fig. 5. Effect of cAMP on the capacity of Hg$^{2+}$-inhibited lysates to reduce disulfide bonds or phosphorylate eIF-2α.** Panel A, hemin-supplemented lysates were incubated under conditions for protein synthesis for 25 min at 30 °C and then assayed for their ability to reduce insulin as described under "Experimental Procedures." Incubations contained: no additions (lane 1); no added insulin in reduction assay (lane 2); 10 μM Hg$^{2+}$ (lane 3); and 10 μM Hg$^{2+}$ plus 10 mM cAMP added at 10 min (lane 4). Data show photograph of fluorescently labeled proteins (Hb, hemoglobin; I, insulin) separated by SDS-PAGE. Panel B, hemin-supplemented lysates were incubated under conditions for protein synthesis for 25 min at 30 °C in the presence of: no additions (lane 1); 10 μM Hg$^{2+}$ (lane 2); and 10 μM Hg$^{2+}$ plus 10 mM cAMP added at 10 min (lane 3). Lysates were pulsed with [γ-32P]ATP as described under "Experimental Procedures." Data are an autoradiogram of 32P-labeled proteins.

**DISCUSSION**

Hg$^{2+}$, Pb$^{2+}$, and Cd$^{2+}$ bind avidly to thiols, forming mercaptides with the sulfhydryl group of cysteine, which are more stable than complexes that these metal ions form with other amino acid side chains (41). Their relative order of inhibitory potency for protein synthesis (Hg$^{2+}$>Pb$^{2+}$>Cd$^{2+}$>Zn$^{2+}$) is similar to the order of their binding affinities to sulfides and cysteine (25–27). The observation that Hg$^{2+}$ inhibits the activity of partially purified HRI in vitro,3 suggests that the metal ions are not activating HRI in situ by binding directly to the kinase. Because of its high binding affinity, Hg$^{2+}$ is generally thought to bind specifically to sulfhydryl groups at low concentrations. The observation that low concentrations Pb$^{2+}$ (5–10 μM) inhibit protein synthesis to a greater extent

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3 R. L. Matts, unpublished observations.
capacity of metal ion-inhibited lysates to reduce disulfide bonds, in conjunction with the inactivation of HRI and the resumption of protein synthesis, indicate that apoMTs also have the ability to detoxify metal ions already bound to proteins. The affinity constant for the binding of Hg²⁺ to cysteine (Hg(cys)_2) has been reported to be 10¹⁴ M⁻² (52).

Given the very slow off rates expected for Hg' from such a complex, the relatively rapid restoration of protein synthesis that is observed upon the addition of apoMTs to Hg²⁺-inhibited lysates suggests that a direct interaction of apoMT with the protein-bound metal may be occurring. MTs have been shown to be capable of donating bound Cu or Zn to metal-requiring enzymes, activating their catalytic activity (53-57). The kinetics of the metal ion-exchange between MT and these enzymes has led to the suggestion that a direct interaction between MT and other proteins may occur and that MT contains unusually reactive metal-binding sites which are capable of donating metal to apometalloproteins (57). The data presented in this report indicate that apoMT contains metal-binding sites that are also capable of extracting tightly bound metal ions from proteins.

The log of the average apparent binding constant of MT at neutral pH has been calculated to be 15.7 for Cd²⁺ binding and 11.7 for the binding of Zn²⁺ (22, 23). MTs are composed of two binding domains which vary in their metal binding specificity and affinity (22, 23, 58-63). The α-domain is capable of binding four divalent metal ions. The initial binding of Cd²⁺ to apometallothionein occurs preferentially to the α-domain (55-63), with an apparent affinity constant which is greater than the binding constant of Cd²⁺ to EDTA[log k > 16.6] (63, 64). The β-domain, which can bind three divalent metal ions, has a lower average metal ion binding affinity and a greater tendency to lose metal ions. The overall binding affinity of Cd²⁺ to the β-domain has been estimated to be lower than the binding affinity of Cd²⁺ to EDTA, and the lability of Cd²⁺ bound in the β-domain has been suggested to be similar to that of Zn²⁺ (63, 64). After its initial binding to MT, Cd²⁺ redistributes preferentially to the α-domain displacing bound Zn²⁺ (60, 63). This observation has led to the proposal that the α-domain of MT may function in the sequestration of toxic metal ions, while the β-domain may function in the storage of essential metals, such as Zn and Cu, and in the donation of these ions to apometalloproteins (60). The observation that only chelating agents with a binding affinity for Cd²⁺ equal to or greater than EDTA (e.g. DPTA, but not NTA) have the capacity to maintain or restore protein synthesis in the presence of Cd²⁺ suggests that only the four metal-binding sites present in the α-domain of MT would have high enough binding affinity to protect protein synthesis from Cd²⁺ inhibition. This proposal is supported by several other observations. The ΔG°₂₅ (13 μM) for the inhibition of protein synthesis by Cd²⁺ in the presence of MTI correlated best with the concentration of displacable Zn²⁺ calculated to be bound to the α-domain (16 μM). MT preparations, whose α-domains were estimated to be saturated with bound Cd²⁺, were observed to have little or no capacity to protect protein synthesis from inhibition by Pb²⁺, and only marginally changed the IC₅₀ values for the inhibition of protein synthesis by Hg²⁺ or Cd²⁺. Furthermore, the average ΔG°₂₅ for the inhibition of protein synthesis by Cd²⁺, Hg²⁺, or Pb²⁺ in the presence of 5 μM apoMTI or II was 21 μM. A ΔG°₂₅ of 35 μM would be expected if the chelation of metal ions by all seven binding sites played a role in protecting protein synthesis from inhibition.

Thioredoxin maintains the sulphydryl groups in a variety of cytoplasmic proteins in a reduced state, and it has been suggested that thioredoxin plays a direct role in the regulation of a variety of protein functions and enzymatic activities through reversible oxidation of selenodiglutathionyl dithiols to disulfides (33, 34). Previous studies with partially purified HRI in vitro have demonstrated that sulphydryl groups play an important role in the regulation of HRI activation and activity (5, 6, 65, 66), as does its phosphorylation state (6, 67, 68). In the absence of a functional thioredoxin/thioredoxin reductase system, a change in the redox state of certain critical sulphydryl groups in HRI could occur, either through direct oxidation or through thiol-disulfide exchange (5, 6, 11), leading to its activation. An alternate possibility, discussed by Jackson et al. (11), is that the real target of oxidation may not be HRI, but a protein which plays a role in regulating the activation or inactivation of HRI. HRI has recently been demonstrated to interact with the 90-kDa heat shock protein (hsp 90) in hemin-supplemented lysates in situ (69) and hsp 90 has been reported to affect the activity of HRI in vitro (70, 71).

Conditions or agents which bring about the activation of HRI in hemin-supplemented reticulocyte lysates are also known to bring about the heat shock or stress response in eukaryotic cells (72-75). Phosphorylation of eIF-2 occurs in cultured cells in response to a variety of stresses (76, 77), and activation of an eIF-2α kinase with properties similar to HRI has been reported to occur in HeLa cells in response of heat shock (78). It has been suggested that accumulation of improperly folded proteins within a cell may be the signal that actually initiates the heat shock response (79). Several of the major heat shock proteins (hsp's) are members of multigene families. These proteins are thought to play a role in the folding and/or unfolding of incompletely folded or improperly folded polypeptides (75). Thioredoxin has been shown to greatly accelerate the rate at which denatured proteins, which contain incorrect disulfide bonds, refold and recover activity (80). Inhibition of thioredoxin activity could lead to the accumulation of oxidized, improperly folded, or denatured proteins, which then might affect the association of hsp 90 with HRI, and therefore, the activity of HRI. We are currently investigating how toxic heavy metal ions affect the sulphydryl status of HRI and hsp 90, and the association of these proteins with one another in the reticulocyte lysate.

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