# SYNTHESIS OF METALLOTHIONEIN IN A POLYSOMAL CELL-FREE SYSTEM

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SUMMARY

A new covalent chromatography system utilizing Activated Thiol Sepharose 4B was employed to quantitate the content of thionein chains synthesized in a polysomal cell-free system. Liver polysomes from zinc injected rats directed the translation of more thionein-like polypeptide chains than polysomes from control rats. The increase was similar to the stimulation in MT synthesis in vivo following a zinc injection. This evidence supports the concept that metallothionein synthesis is regulated by changes in the pool of translatable thionein mRNA.

## INTRODUCTION

Zinc plays a role in the regulation of liver metallothionein (MT) synthesis (1-3), however the mechanism of the control system is not clear. Pulse label experiments with $^{35}$S-cystine indicate that the rate of MT synthesis is markedly increased following a zinc injection (3), in a manner similar to the effect of iron on ferritin synthesis (4). Control at the translational level has been suggested for iron in the ferritin system (5). In contrast, however, inhibitors of mRNA synthesis and processing, i.e. actinomycin D and cordycepin, block MT synthesis in response to zinc, which suggests that control of the synthesis of this protein is more analogous to the induction of specific gene products in response to certain steroid hormones (6,7).

In vivo experiments to date have not differentiated between the two most likely sites of action of zinc in the regulation of MT synthesis. Zinc may act as an inducer of the synthesis of mRNA for MT. Alternatively zinc might increase the hepatic MT content by decreasing the degradation rate of rapidly turning over thionein (apo-metallothionein) polypeptide chains translated from a short-lived mRNA. In this study we report data from

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a cell-free protein synthesizing system, utilizing liver polysomes, which show that polysomes isolated from zinc-injected rats are able to direct the synthesis of increased amounts of thionein-like chains compared to polysomes isolated from control, non-zinc injected rats. Identification of thionein in the *in vitro* reaction mixture was accomplished using Activated Thiol Sepharose 4B covalent chromatography.

## MATERIALS AND METHODS

(2-$^{3}$H) glycine (3.4 Ci/mmol), (L-3-$^{3}$H) serine (17 Ci/mmol), (L-4,5(n)-$^{3}$H) lysine monohydrochloride (18 Ci/mmol) and (L-$^{35}$S) cystine (63.5 mCi/mmol) were obtained from Amersham/Searle Corp. Activated Thiol Sepharose 4B was purchased from Pharmacia Fine Chemicals. Glassware and deionized water were sterilized prior to use. Male rats (150-200 g), of the Sprague-Dawley strain and fed a standard natural diet *ad-libitum*, were used in all experiments.

MT-containing liver cytosol samples and standard $^{35}$S-labeled MT were obtained by injecting rats with 25 umoles $Zn^{2+}$ as $ZnSO_4$ and 5 uCi $^{35}$S-cystine (ip) 18 hr prior to sacrifice. Control rats were injected with 0.9% (w/v) NaCl in place of the $Zn^{2+}$. Liver homogenates were centrifuged at 166,000 x g for 60 min to yield a clear cytosol fraction. MT was isolated by Sephadex G-75 chromatography of the liver soluble proteins with a purity of 85-90% (8).

Polysomes were isolated from rats injected with 25 umoles $Zn^{2+}$ 5 hr prior to sacrifice; control rats received an injection of 0.9% (w/v) NaCl. Livers were homogenized in 2 vol of 375 mM sucrose in TKM buffer (50 mM Tris-HCl, pH 7.6; 25 mM KCl; 5 mM $MgCl_2$) with polyvinyl sulfate (20 mg/l) and 1 mM mercaptoethanol (9). The homogenate was centrifuged at 19,000 x g for 15 min to yield a post-mitochondrial supernatant fraction (PMS) which was made 1% in deoxycholate. The PMS was layered on a discontinuous sucrose gradient (3 ml of 2.0 M sucrose and 4 ml of 0.5 M sucrose, both in TKM buffer) and centrifuged at 166,000 x g in a Beckman 50-Ti rotor for 15.5 hr (10). The polysomal pellet, containing both free and membrane bound ribosomes, was resuspended in water and stored in liquid $N_2$.

Polysomal RNA was translated in an *in vitro* polypeptide-synthesizing system modified from that described by Falvey and Staehelin (11). Reaction mixtures contained 1 umole ATP, 0.4 umole GTP, 10 umole creatine phosphate, 50 ug creatine phosphokinase, 0.2 ml of S-100 gel filtered liver cell sap (11), 50 nmoles of all amino acids, except lysine, serine and glycine, 5 uCi each of $^{3}$H lysine, glycine and serine and a uniform amount of 30-50 $A_{260nm}$ units of polysomal RNA. The final ionic conditions were 150 mM $NH_4Cl$, 4 mM Mg acetate, 20 mM Tris-HCl (pH 7.5) and 1 mM dithrothreitol. The final volume of the reaction mixture was 1 ml. After incubation for 1 hr at 37°C the released polypeptide chains were separated from the polysomes by centrifuging at 166,000 x g for 90 min. The amount of radioactivity incorporated into released polypeptides was measured by the method of Mans and Novelli (12) on a 10 ul aliquot.

MT synthesized in the cell-free protein synthesizing system was measured by chromatography of the reaction mixture on Activated-Thiol Sepharose 4B. The gel was prepared as outlined by Brocklehurst *et al.* (13). A sample containing the released polypeptide chains was mixed with an equal volume of 100 mM Na acetate buffer (pH 4) containing 300 mM NaCl and 1 mM EDTA (Buffer

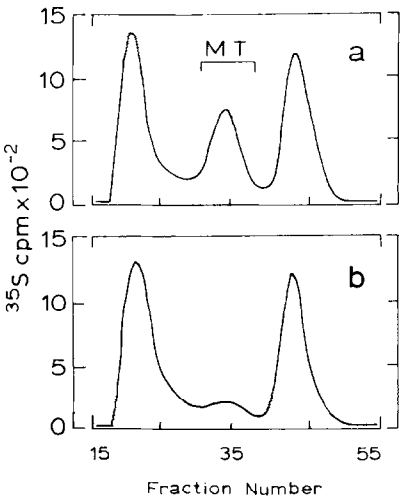


Figure 1. Gel-filtration chromatography of rat liver cytosol on Sephadex G-75 showing effect of zinc on zinc-thionein (metallothionein) synthesis.

Profile a: Rats were injected (ip) with 25 umoles of $Zn^{2+}$ as $ZnSO_4$ (in 0.9% NaCl) and 5 uCi $^{35}$S-cystine 18 hrs before sacrifice. MT designates the metallothionein (zinc-thionein) fraction. Profile b: Rats were injected (ip) with 0.9% NaCl and 5 uCi $^{35}$S-cystine 18 hrs before sacrifice. Fractions of 1.5 ml were collected.

A). The sample was centrifuged at 15,000 x g for 15 min to remove the precipitated proteins and 8 ug of carrier MT was added. The supernatant fraction was then applied to a column of Sephadex G-25 (1.6 x 40 cm) and the eluent was collected and void volume pooled. This sample was then applied to a column of Activated Thiol Sepharose 4B (0.9 x 5 cm) equilibrated with Buffer A. The column was washed with 30 ml of Buffer A, then with 30 ml of 100 mM Tris-HCl (pH 8), 300 mM NaCl, 1 mM EDTA (Buffer B) and finally with 30 ml of 50 mM Cysteine in Buffer B. Two ml fractions were collected and dissolved in 15 ml of Scintiverse (Fisher Scientific Co.) and the $^{3}$H content measured by liquid scintillation counting.

## RESULTS AND DISCUSSION

Normally MT is separated from total liver soluble proteins by gel filtration chromatography using Sephadex G-75 in which advantage is taken of the protein's low molecular weight (6,000-10,000 daltons) (Figure 1). Purification of MT to this point results in a purity of 85-90% (8). However this routine purification procedure cannot be used to separate thionein, the demetalized polypeptide chain of MT, from a mixture of proteins synthesized in vitro because such systems generate a large number of small polypeptide chains, primarily subunits of normally larger proteins and incompleted

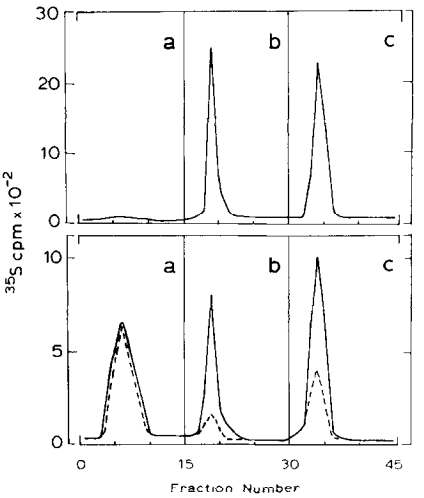


Figure 2. Covalent chromatography of $^{35}$S-thionein and $^{35}$S-liver cytosol using Activated-Thiol Sepharose 4B.

Top profile: Covalent chromatography of purified $^{35}$S-labeled liver thionein (derived from zinc-thionein by demetalization at pH 4.0). ——— $^{35}$S cpm/2 ml fraction.
Bottom profile: Covalent chromatography of pH 4.0 soluble liver cytosol proteins. Cytosol from rats injected with 25 umoles $Zn^{2+}$ (_____) or 0.9% NaCl (---) and 5 uCi $^{35}$S cystine. $^{35}$S cpm/2 ml fractions.

Columns were eluted with a) 30 ml of 100 mM Na acetate buffer (pH 4.0), b) 30 ml of 100 mM Tris-HCl (pH 8.0) and c) 30 ml of 50 mM cysteine in 100 mM Tris-HCl (pH 8.0). All buffer solutions contained 300 mM NaCl and 1 mM EDTA.

chains. For this reason a new method of detecting thionein was developed based on this protein's high cysteine content (25-30% of the total amino acid residues) (14). Brockhurst and coworkers(13) have prepared active papain by selective attachment of the active site thiol group of the enzyme to the thiol groups of Activated Thiol Sepharose. In a similar manner, thionein will bind to this gel matrix at a pH of 4, presumably through the formation of disulfide bonds between cysteine sulfhydryl groups and the thiol groups of the gel (Figure 2a). At a pH of 4 $Zn^{2+}$ is removed from the binding sites of MT. Thionein can be released from the column first by changing the eluent pH to 8, which probably increases the formation of intramolecular disulfide bonds within the thionein moiety, and then by elution with 50 mM

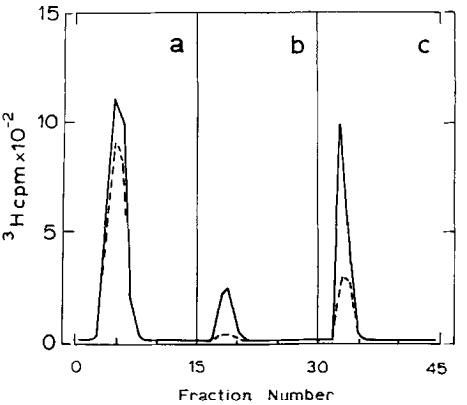


Figure 3. Covalent chromatography using Activated-Thiol Sepharose 4B of $^3$H-polypeptides produced by a polysomal cell-free protein synthesizing system.

Polysomal RNA was translated in a cell-free system containing 5 uCi each of $^3$H-glycine, $^3$H-serine and $^3$H-lysine. Free and membrane bound polysomes were prepared from livers of rats that were injected (ip) with either 25 umoles of $Zn^{2+}$ (———) or 0.9% NaCl (---) 5 hrs before sacrifice. The $^3$H-labeled products were adjusted to pH 4.0 and were eluted from the column with a) 30 ml of 100 mM Na acetate buffer (pH 4.0), b) 30 ml of 100 mM Tris-HCl (pH 8.0) and c) 30 ml of 50 mM cysteine in 100 mM Tris-HCl (pH 8.0). All buffer solutions contained 300 mM NaCl and 1 mM EDTA. Fractions of 2 ml were collected.

cysteine at pH 8 which competes with thionein for the Sepharose thiol groups. Both the A and B forms of thionein (8) exhibit this dual elution behavior.

Through the use of the thiol covalent chromatography system it is possible to separate thionein from the majority of the soluble $^{35}$S-cysteine labeled liver proteins to a sufficient degree to detect changes in the amount of thionein in hepatic cytosol (Figure 2b). The two MT-containing peaks are increased in samples from zinc-injected rats compared to controls. However there is no difference in the size of the first peak (pH 4 buffer) containing the non-bound proteins, which is compatable with evidence which suggests that zinc selectively increases the synthesis of MT compared to other liver proteins (Figure 1). Incorporation of $^{35}$S-cystine into high molecular weight proteins ($\geq$ 75,000 daltons) was equivalent when the cytosol was fractionated by gel filtration (Figure 1). Moreover zinc injections were found not to significantly alter TCA precipitable radioactivity

in the cytosol fraction (3). Therefore it appears that the increases in radioactivity measured in the second two peaks from Activated Thiol Sepharose chromatography are related to the increase in the MT present in the hepatic cytosol.

Chromatography of the products of the cell-free protein synthesizing reaction mixture on Activated Thiol Sepharose is shown in Figure 3. Radioactivity in the second two peaks was increased in samples derived from polysomes isolated from zinc-injected rats compared to control rats, while the first peak was increased only slightly (about 0.25 fold). However, the second (pH 8 buffer) and third (50 mM cysteine) peaks were increased by 2.6 and 1.9 fold, respectively. The increase in radioactivity in the second peak is comparable to the increase in the rate of MT synthesis observed when measured, *in vivo*, at 5 hours after a zinc injection (3).

The increase in the amount of thionein-like polypeptides synthesized from polysomal RNA from zinc-injected animals suggests that zinc does, indeed, increase the translation of MT mRNA. This evidence is consistent with the concept that increased MT synthesis following elevation of zinc status involves the transcription of more translatable thionein mRNA.

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REFERENCES

1. Richards, M. P. and Cousins, R. J. (1975) Biochem. Biophys. Res. Comm. 64, 1215-1223.
2. Richards, M. P. and Cousins, R. J. (1976) J. Nutrition 106, 1591-1599.
3. Squibb, K. S., Cousins, R. J. and Feldman, S. L. (1977) Biochem. J. 164, In Press.
4. Drysdale, J. W. and Munro, H. N. (1966) J. Biol. Chem. 241, 3630-3636.
5. Zahringer, J., Baliga, B. S. and Munro, H. N. (1976) Proc. Natl. Acad. Sci. 73, 857-861.
6. O'Malley, B. W., Woo, S. L. C., Harris, S. E., Rosen, J. M. and Means, A. R. (1975) J. Cell. Physiol. 85, 343-356.
7. Chan, L. and O'Malley, B. W. (1976) N. Engl. J. Med. 294, 1372-1381.

8. Feldman, S. L. and Cousins, R. J. (1976) Biochem. J. 160, 583-588.
9. Baliga, B. S., A. W. Pronczuk and Munro, H. N. (1968) J. Mol. Biol. 34, 199-218.
10. Blobel, G. and Potter, V. R. (1967) J. Mol. Biol. 26, 279-292.
11. Falvey, A. K. and Staehelin, T. (1970) J. Mol. Biol. 53, 1-19.
12. Mans, R. J. and Novelli, G. D. (1961) Arch. Biochem. Biophys. 94, 48-53.
13. Brocklehurst, K., Carlsson, J., Kierstan, M. P. J. and Crook, E. M. (1973) Biochem. J. 133, 573-584.
14. Bremner, I. and Davies, N. T. (1975) Biochem. J. 149, 733-738.