# Metallothionein Detoxification Function Is Impaired by Replacement of Both Conserved Lysines with Glutamines in the Hinge between the Two Domains†

Chris W. Cody and P. C. Huang*

*Department of Biochemistry, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Maryland 21205-2103*

*Received December 3, 1992; Revised Manuscript Received March 2, 1993*

ABSTRACT: Mammalian metallothioneins (MTs) possess eight highly conserved lysine residues, two of which constitute the hinge between two metal binding domains. By site-directed mutagenesis and recombinant DNA techniques, we replaced the interdomain lysines in Chinese hamster ovary MT2 with all possible combinations of glutamic acid and/or glutamine. The resultant MTs were expressed and assayed for detoxification feunction in a transformed yeast system. Results showed that these mutant MTs, like the native protein, bound seven atoms of divalent metal per molecule and conferred cadmium resistance to a metal-sensitive yeast host. Replacement of one or both of the lysines in the interdomain region was inconsequential to the structure and function of MT, unless both substituted residues were uncharged. When both lysines were replaced by glutamine (K30,31Q), a reduction in the ability of MT to protect yeast transformants against otherwise toxic levels of cadmium was observed. This diminished metal detoxification capacity was due to a decrease in the steady-state level of MT. These results suggest that at least one charged amino acid must be present in the hinge for the proper expression of MT.

Conserved amino acids in a protein are often critical to maintain protein structure and function (Bowie et al., 1990). Mammalian metallothioneins (MTs) are small proteins of 61 amino acids which contain 20 invariant cysteines and 8 highly conserved lysines [see review in Kagi and Kojima (1987)]. The conserved cysteines bind a series of transition IB and IIB metals. Because of this metal sequestering capability, MT is able to render cells or organisms resistant to otherwise toxic levels of metals such as cadmium (Kagi & Schaffer, 1988). MT consists of two metal–thiolate clusters, the $\beta$ and $\alpha$ domains, which bind three and four divalent metals, respectively. This has been demonstrated by $^{113}$Cd NMR (Otvos & Armitage, 1980; Vasak et al., 1987), homo- and heteronuclear 2D NMR (Arseniev et al., 1988; Schultze et al., 1988; Messerle et al., 1990), and X-ray crystallography (Robbins et al., 1991). Recombinant DNA studies have revealed that individual cysteines differ in their contribution to the metal chelation and detoxification capacity of MT in a domain-specific manner (Chernaik & Huang, 1991; Cismowski & Huang, 1991a; Cismowski et al., 1992).

The reason that lysine residues are also conserved is less well understood. They might be essential for proper metal chelation by neutralizing the excess negative charge of the cysteine thiolates that are tetrahedrally coordinated to metals (Kojima et al., 1976), thus stabilizing the overall structure of MT (Rakshit & Vasak, 1992). This idea has been supported by proton NMR chemical titration experiments which show that MT lysines have an elevated p$K_a$ (Pande et al., 1985; Vasak et al., 1985) and by studies which show that chemical modification of MT lysines can reduce metal binding capacity (Pande et al., 1985). However, chemical modification of MT lysines does not always affect metal chelation (Templeton & Cherian, 1984; Pande et al., 1985). Previous studies have been unable to distinguish specific lysines (Templeton & Cherian, 1984; Pande et al., 1985; Vasak et al., 1985) and imply that individual lysines, like cysteines, may contribute differentially to the structure and function of MT.

The eight lysines in MT are distributed with three in each of the two domains. The remaining two, K30 and K31, are completely conserved in mammals and constitute a hinge which separates the domains. This dipeptide is much shorter than the hinges found in most multidomain proteins (Agros, 1990). X-ray diffraction studies show that the $\epsilon$-amino group of K31 is close enough to C19 and C21 to form hydrogen bonds (Robbins et al., 1991).

In this study, we used site-directed mutagenesis to specifically substitute either or both of the interdomain lysines, and examined the contribution of these residues to the structure and function of mammalian MT.

## MATERIALS AND METHODS

*Mutagenesis and Construction of pYCHE-MT.* Site-directed mutagenesis was performed as previously described (Kunkel et al., 1987; Chernaik & Huang, 1991; Cismowski & Huang, 1991a), using wild-type or mutant CHO MT2 cDNAs (Griffith et al., 1983) cloned into M13mp19 as templates. Complementary oligonucleotides (18mers) containing a single mismatch to the template were used as primers. Codons 30 (AAG) and 31 (AAA) code for lysine in wild-type MT; changing the first base of either codon to C or G changes lysine to glutamine or glutamic acid, respectively. The single lysine replacement mutants (K30Q, K31Q, K30E, and K31E) were generated using single-stranded antisense M13mp19MT as template DNA. Two of the resultant sequences, M13mp19K30Q MT and M13mp19K30E MT, were then utilized as templates for the subsequent construction of the four doubly substituted MTs (K30Q,K31E; K30,31Q; K30,-31E; and K30E,K31Q). Mutants were confirmed by dideoxynucleotide sequencing (Sanger et al., 1977).

Native and mutant MT cDNA sequences were subcloned into the *Nco*I site of a high-copy yeast episomal plasmid,

† This work was supported in part by NIH Grant GM R01 32606 to P.C.H.

* Correspondence should be addressed to this author at the Department of Biochemistry, Johns Hopkins University, School of Hygiene and Public Health, 615 N. Wolfe St., Baltimore, MD 21205. Telephone: 410-955-3844. Telefax: 410-955-2926.

0006-2960/93/0432-5127$04.00/0 

pYCHE, which contains the constitutive TDH3 promoter and the full-length *trp 1* gene (Ecker et al., 1986). Constructs with the proper orientation, as verified by restriction analysis with *Bam*HI and *Cla*I, were transformed into the *cup 1*$^{\Delta}$, *trp 1-289* yeast strain AB-DE 1, in which the endogenous thionein gene had been previously deleted (Ecker et al., 1986).

*Media.* Yeast minimal medium was prepared by autoclaving 1.7 g of yeast nitrogen base/L, 5 g of ammonium sulfate/L, and 5 g of casamino acids/L for 30 min to destroy tryptophan before adding sterilized glucose (20 g/L), adenine (30 mg/L), histidine (20 mg/L), arginine (30 mg/L), leucine (40 mg/mL), and lysine (40 mg/mL). YTD medium contained bactoyeast (10 g/L), bactotryptone (20 g/L), and glucose (20 g/L).

*Metal Resistance Assays.* Plate assays were carried out by streaking transformants onto minimal plate with or without cadmium and allowing the cells to grow for 3 days at 30 °C before scoring. Liquid assays were carried out by diluting a dense overnight culture of the transformants grown in minimal medium 1:50 into YTD medium with or without 20 μM cadmium. Cultures were grown shaking at 250 rpm, 30 °C, and turbidity was measured periodically, with a Klett Summerson colorimeter.

*DNA and RNA Analyses.* Total RNA or DNA was isolated from yeast (Rose et al., 1990). Concentration was determined by the absorbance at 260 nm, assuming an OD of 1 corresponds to 50 μg/mL DNA and 40 μg/mL RNA, respectively. For Southern analysis (Southern, 1975), 20-μg samples of DNA were digested with *Eco*RI and electrophoresed on a 1.2% agarose gel. For Northern analysis (Morris & Huang, 1987), 20-μg samples of RNA were electrophoresed onto a 1.2% formaldehyde gel, followed by blotting onto Gelman Biotrace RP membrane filters and hybridization to a nick-translated $^{32}$P-labeled *Eco*RI fragment of CHO MT2 or a *Sma*I fragment of yeast Ura3, the latter to serve as a control. After hybridization, the filters were washed in 0.2 × SSC/0.1% SDS at 65 °C for 40 min, dried, and exposed to Kodak X-Omat AR film. RNA bands on the autoradiogram were quantitated using an LKB Ultrascan XL laser densitometer.

*Cell Labeling and Gel Analysis.* Transformed yeast cells were labeled with [$^{35}$S]cysteine in minimal medium containing 50 μM $ZnCl_2$, collected by centrifugation, washed with $H_2O$, and resuspended in lysis buffer (10 mM Tris, pH 8, 10 mM DTT, and 2 μM PMSF). Cell lysates were analyzed by 15% SDS–PAGE (Laemmli, 1970) after sulfhydryl residues were blocked with iodoacetamide (Sone et al., 1987). Alternatively, cells were lysed by vortexing with glass beads and run on 18% polyacrylamide nondenaturing gels in running buffer (25 mM Tris, pH 8.8, and 19 mM glycine) containing 50 mM sodium thioglycolate without further purification.

*Protein Purification.* Wild-type and mutant MTs were purified essentially as described previously (Cismowski & Huang, 1991b). The steps included (A) cell lysis with glass beads, (B) chromatography on G75 Sephadex, (C) chromatography on DEAE-Sephadex A25, (D) concentration with an Amicon YM-2 membrane, (E) HPLC on a Bio-Rad Hi-Pore RP-C18 column, (F) chromatography on an analytical G75 Sephadex column, and (G) ultrafiltration as in step D. For K30,31E MT, a linear gradient of 250 mL from 10 to 300 mM Tris, pH 7.4, was used in step C, while a 10–200 mM gradient was used for wild-type MT.

*Metal Stoichiometry.* Purified MTs were diluted to a concentration of approximately 15 μM in 20 μM HCl, and the absorbance at 220 nm was measured on a Hitachi U-2000 spectrophotometer. MT concentration was determined using the extinction coefficient at pH 1.7 and 220 nm of 47 300 $M^{-1}$ $cm^{-1}$ (Buhler & Kagi, 1979). Samples were then neutralized by dilution with 50 mM Tris-HCl, pH 7.5, in order to measure cadmium, zinc, and copper concentrations in the linear response range of 0.1–2.0 ppm with a Varian Techtron flame spectrophotometer.

*Circular Dichroism.* Samples were adjusted to a concentration of 8 μM in 10 mM Tris-HCl, pH 7.5, and scanned at an ambient temperature of 20.5 °C, using a JASCO 500C spectrophotometer under conditions as previously described (Cismowski & Huang, 1991a). Molecular ellipticity is expressed in terms of protein concentration as degrees per centimeter per decimole.

Table I: Metal Resistance Rendered by Wild-Type and Mutant MTs to AB-DE 1[a]

| MT | supplemented with | | |
|---|---|---|---|
| | none | 30 μM Cd | 300 μM Cd |
| K30Q | + | + | + |
| K31Q | + | + | + |
| K30E | + | + | + |
| K31E | + | + | + |
| K30,31Q | + | + | – |
| K30Q,K31E | + | + | + |
| K30E,K31Q | + | + | + |
| K30,31E | + | + | + |
| wild type | + | + | + |
| none | + | – | – |

[a] Yeast transformed with pYCHE-MT containing the indicated MT was streaked onto minimal medium plates supplemented with or without metals. Plates were scored for growth after incubation for 3 days at 30 °C; (+) = uninhibited growth, (–) = no growth.

## RESULTS

Eight mutants of wild-type Chinese hamster MT2 were constructed so that the two adjacent lysines in the hinge region between the domains were singly (K30Q, K31Q, K30E, and K31E) or doubly (K30,31Q; K30,31E; K30E,K31Q; and K30E,K31Q) substituted. We expressed each of these mutants in the metal-sensitive yeast strain AB-DE 1.

All eight mutant MTs could function in metal detoxification, as they, like wild-type MT, conferred cadmium resistance to AB-DE 1 (Table I). All transformants, except K30,31Q, grew on minimal plates containing 300 μM cadmium. Growth of transformants was also followed in liquid media supplemented with 20 μM cadmium, a concentration which is effective in identifying cysteine mutations that lower the detoxification capacity of MT (Chernaik & Huang, 1991). K30,31Q transformants grew more slowly than the other transformants (Figure 1).

To confirm that all transformants expressed MT, cultures were labeled with [$^{35}$S]cysteine, and cell lysates were run on nondenaturing polyacrylamide gels (Figure 2). Using this system, MTs can be visualized as single bands that are well resolved from other cellular proteins (Chernaik & Huang, 1991). Two points are readily apparent from Figure 2. First, the expressed level of K30,31Q MT was much lower than the level of wild-type MT; other mutant MTs appeared to be expressed at about the same level as wild type. Second, mutant MT mobility was dependent on the net interdomain charge of the molecule. In general, the more negatively charged the interdomain residues were, the faster the mutant MT migrated.

Reduced expression of K30,31Q MT protein could be due to a decrease at the DNA or RNA level. We determined that the plasmid copy number of pYCHE-MT was identical in the K30,31Q and wild-type transformants (Figure 3). Furthermore, the MT mRNA level was similar in both transformants as determined by Northern analysis (Figure 3C), implying

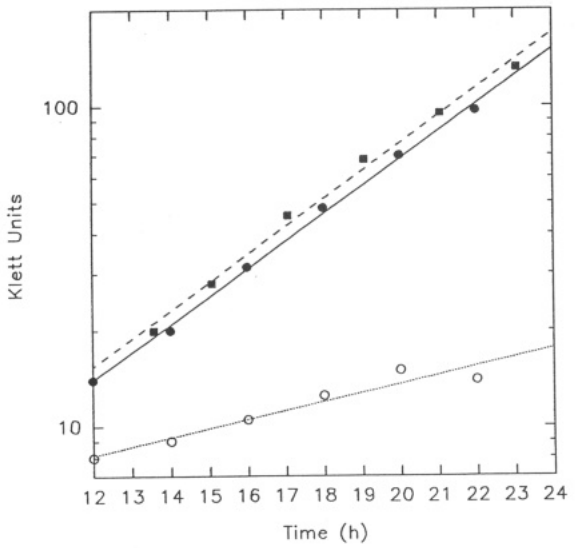


FIGURE 1: Comparative growth of yeast AB-DE 1 transformed with pYCHE-MT wild type and mutants. Turbidity of transformants in log-phase growth in YTD medium containing 20 $\mu$M $CdCl_2$ was measured as described under Materials and Methods, beginning 12 h after inoculation. Representative data for yeast transformed with wild-type pYCHE-MT (●), mutant pYCHE-K30,31E-MT (■), or vector pYCHE (○) are shown as plots of Klett units vs time. Regression coefficients obtained with IBM Sigma Plot 4.0 were used to calculate doubling times for AB-DE 1 transformed with pYHE vector alone (10.5 ± 0.2 h), wild-type MT (3.6 ± 0.2 h), K30Q (3.5 ± 0.1 h), K31Q (3.6 ± 0.3 h), K30,31Q (6.7 ± 1.4 h), K30E (3.0 ± 0.1 h), K31E (3.4 ± 0.2 h), K30Q,K31E (3.4 ± 0.0 h), K30E,-K31Q (3.3 ± 0.2 h), and K30,31E (3.5 ± 0.1 h). Doubling times shown were the average of 2–5 measurements with the indicated standard deviation.

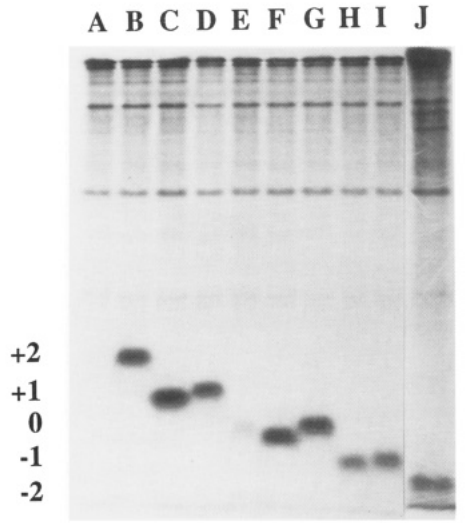


FIGURE 2: Nondenaturing gel analysis of wild-type and mutant MTs. $^{35}$S-Labeled MTs were prepared by diluting saturated cultures of AB-DE 1 transformants 1:10 into 1 mL of minimal medium containing 50 $\mu$M $ZnCl_2$. After 2 h of growth at 30 °C, 250 rpm, [$^{35}$S]cysteine (specific activity >600 Ci/mmol) was added to 20 $\mu$Ci/mL, and the cultures were incubated for 6 more h and processed as described under Materials and Methods. $2 \times 10^5$ cpm of each sample was electrophoresed on an 18% polyacrylamide gel. MTs in each lane are (A) pYCHE vector alone (no MT), (B) native, (C) K30Q, (D) K31Q, (E) K30,31Q, (F) K30E, (G) K31E, (H) K30Q,K31E, (I) K30E,K31Q, and (J) K30,31E. Net interdomain charge is shown on the vertical label. Samples A–I were run on the same gel, and sample J was run on a separate gel with native MT (not shown) as a control.

that a posttranscriptional mechanism accounted for the reduced expression of K30,31Q.

A pulse–chase experiment was carried out with a K30,31Q MT transformant to determine if the mutant MT was stable

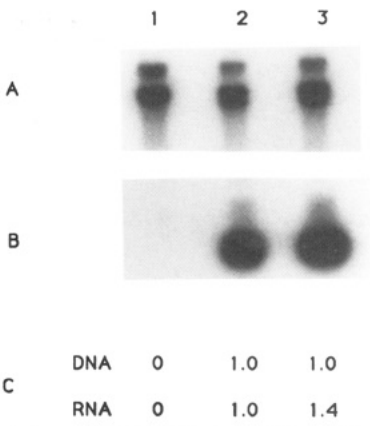


FIGURE 3: Relative pYCHE-MT plasmid levels in K30,31Q and wild-type transformants. DNA was isolated, digested with *Eco*RI, and analyzed as described under Materials and Methods. (A) Hybridization to $^{32}$P-labeled *Ura* 3 probe. (B) Hybridization to $^{32}$P-labeled MT probe. (1) AB-DE 1 (untransformed); (2) AB-DE 1/pYCHE MT (wild type); (3) AB-DE 1/pYCHE K30,31Q MT. (C) Relative ratio of $^{32}$P counts hybridized to MT versus *Ura* 3 probe, as determined by scanning densitometry and normalized to 1.0 for wild-type MT. DNA counts were determined from the autoradiograms in panels A and B. RNA counts were determined from similar autoradiograms obtained by Northern analysis as described under Materials and Methods (data not shown).

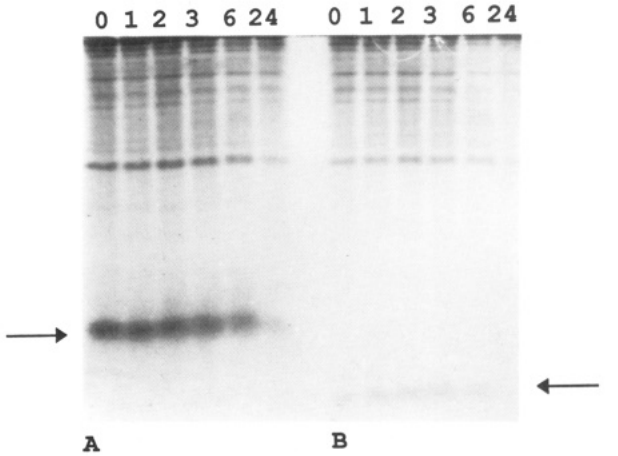


FIGURE 4: Pulse–chase experiment with K30,31Q and wild-type MTs. Transformed cells were labeled for 1 h with 100 $\mu$Ci/mL [$^{35}$S]cysteine (specific activity) >600 Ci/mmol) and chased for various periods of time up to 24 h in minimal medium with 60 $\mu$M nonradioactive cysteine. At the end of each indicated time period (0, 1, 2, 3, 6, or 24 h), 500-$\mu$L aliquots of cells were harvested by centrifugation and subjected to electrophoresis as described under Materials and Methods. (A) Wild-type MT (left arrow); (B) K30,-31Q MT (right arrow).

once it was synthesized. It can be seen in Figure 4 that the level of K30,31Q MT, albeit low, remained steady for at least 6 h. The decrease in intensity seen at 24 h can be accounted for by dilution due to the increased cell volume. In order to determine the kinetics of MT accumulation, cells were labeled with [$^{35}$S]cysteine for different periods of time and analyzed by SDS–PAGE. Results showed that for labeling times between 1 and 24 h, the level of K30,31Q protein was lower than that of wild type (Figure 5). The rate of increase, however, was similar. Thus, the reduced expression of K30,-31Q MT seen after 6 h of labeling (Figure 2) could not have been due to a shorter half-life of the mutant relative to wild-type MT.

Wild-type MT and K30,31E MT from transformants grown in the presence of cadmium were purified and analyzed. Each bound 7 mol of metal/mol of MT (Table II); only a minor difference in the ratio of cadmium/zinc/copper was observed.

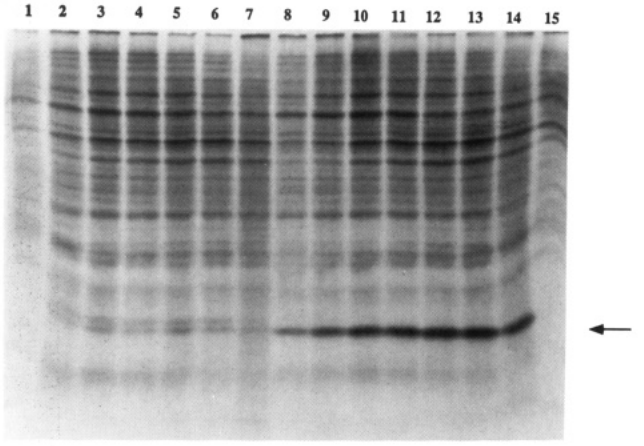


FIGURE 5: SDS–PAGE analysis of the accumulation of wild-type and K30,31Q MTs with time. AB-DE 1 transformants were labeled with [$^{35}$S]cysteine for the indicated number of hours: 1 (lanes 1, 8); 2 (lanes 2, 9); 4 (lanes 3, 10); 6 (lanes 4, 11); 9 (lanes 5, 12); 12 (lanes 6, 13); 24 (lanes 7, 14). $10^5$ cpm/lane were loaded on a 15% SDS–PAGE gel following iodoacetamide treatment as described under Materials and Methods. Samples were AB-DE 1 transformed with pYCHE K30,31Q MT (lanes 1–7), pYCHE MT (wild type) (lanes 8–14), or pYCHE vector alone (lane 15). K30,31Q MT migrated as a doublet on a 15% SDS gel. The arrow points to MT.

Table II: Metal Binding Stoichiometry[a]

| | wild-type MT | K30,31E MT |
|---|---|---|
| $A_{220}$ | 0.70 | 0.61 |
| μm MT | 15 | 13 |
| μM Cd | 95 | 71 |
| μM Zn | 5.0 | 12 |
| μM Cu | 4.5 | 6.6 |
| Cd/MT | 6.4 | 5.5 |
| Zn/MT | 0.34 | 0.92 |
| Cu/MT | 0.30 | 0.51 |
| metal/MT | 7.0 | 6.9 |

[a] Protein concentrations were determined from the absorbance at 220 nm of purified MTs at pH 1.7 as described under Materials and Methods. Then the samples were diluted 1:100 with 50 mM Tris-HCl (pH 7.4) for cadmium determination or 1:10 for zinc and copper determination by atomic absorption spectrophotometry. All values shown in the table refer to the undiluted sample at pH 1.7. Cd/MT, Zn/MT, Cu/MT, and metal/MT are expressed as moles of metal per mole of protein. Thus, wild-type and mutant MTs bind 7 mol of metal per mole of protein to the nearest integer.

Circular dichroism spectra of both proteins were characteristic of mammalian Cd–MTs (Rupp & Weser, 1978; Pande et al., 1986; Willner et al., 1987; Stillman & Zelazowski, 1989), having maxima at 225 and 258 nm, and a minimum at 237 nm (Figure 6). The ellipticity maximum at 258 nm, which is ascribed to cadmium thiolate complexes, was of similar intensity for both MTs.

## DISCUSSION

We have shown in this study that replacement of one or both of the interdomain lysines of MT with glutamine and/or glutamic acid did not affect metal detoxification function unless both lysines were replaced with glutamine. K30,31Q transformants were more sensitive to cadmium (Table I, Figure 1) and expressed less MT (as judged by the relative intensity of the bands in Figures 2, 4, and 5) than other transformants; hence, the reduction in cadmium resistance could be attributed to the lower steady-state level of MT.

The lower level of MT in the K30,31Q transformant was not caused by either a reduction in the copy number of vector per cell or a lower rate of transcription. Wild-type and K30,31Q transformants had equivalent plasmid MT DNA and

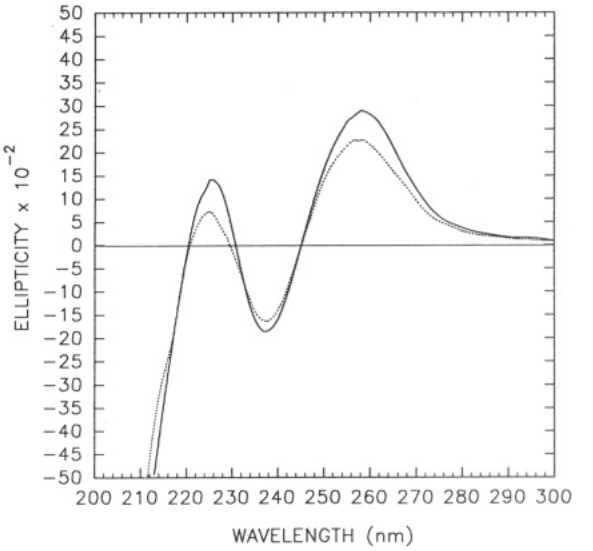


FIGURE 6: Circular dichroism spectra of purified wild-type and mutant MTs. Corrected spectra are of native MT (solid line) and K30,31E MT (dotted line).

MT mRNA levels (Figure 3). We can also rule out the explanation that K30,31Q MT has a shorter half-life in vivo; we observed by pulse–chase experiments that the level of K30,31Q or wild-type MT remained stable in vivo for at least 6 h (Figure 4). Both wild-type and mutant MT mRNAs obtained by transcription in vitro using T7 polymerase could be translated with rabbit reticulocyte lysates, but K30,31Q MT accumulated at a reduced level (Lin and Huang, unpublished results). These results suggest that K30,31Q MT expression is defective at a translational or posttranslational level. Since K30,31Q was the only MT in this study which did not contain at least one charged amino acid (lysine or glutamate) in the hinge, we infer that MT must have at least one charged residue between the $\alpha$ and $\beta$ domains for proper expression.

Proper expression of MT requires metal chelation by apoMT in addition to normal translation. We propose that if both hinge amino acids of MT are neutral, apoMT is less likely to properly fold into its fully metalated, two-domain structure. Instead, MT would be more likely to misfold or form stable, partially metalated intermediates. This would be consistent with the observed decrease in the steady-state level of MT (Figure 2). While we could not detect misfolded K30,31Q MT using nondenaturing electrophoresis (Figures 2 and 4) or by HPLC (data not shown), we observed that alkylated K30,-31Q MT could form two different SDS-complexed conformers (Figure 5). The ability of MT mutants to form two SDS-complexed conformers has been observed previously (Cismowski & Huang, 1991a).

Our results suggest that the positively charged interdomain lysines are not essential for charge neutralization of the negatively charged thiol groups of MT. Metal binding stoichiometry measurements of wild-type and K30,31E MTs showed that changing the net charge from +2 to −2 in the interdomain hinge did not reduce the net metal binding capacity (Table II). The similarity of their CD spectra suggested that the two proteins had virtually identical metal–sulfur interactions (Figure 6). The minor differences seen in the mutant CD spectrum were too small to correlate with reduced metal binding capacity or significant metal–thiolate rearrangement (Willner et al., 1987; Cismowski & Huang, 1991a). Loss of bound cadmium has been shown to cause a larger decrease in the 258-nm peak intensity than we observed (Willner et al., 1987). In fact, the ellipticity profile of the

mutant was similar to that observed for arylated MT, with an average of 1.4 lysines nonspecifically modified by trinitrobenzenesulfonic acid (TNBS), which exhibits normal metal binding stoichiometry (Pande et al., 1985).

The MTs in our study can be separated into five groups on the basis of net interdomain charge. They are +2 (wild type), +1 (K30Q and K31Q), 0 (K30,31Q; K30E; and K31E), −1 (K30Q,K31E and K30E,K31Q), and −2 (K30,31E), assuming that lysine contributes a net charge of +1, glutamine 0, and glutamic acid −1 at pH 8.8. K30,31E, the most negatively charged mutant, exhibits the same metal binding stoichiometry as wild-type MT. The other mutants, which were of intermediate charge, should also bind metals with the same stoichiometry. If all of the MTs in this study bound the same number of metals and had the same conformation, then the only variation in mobility expected on a nondenaturing gel should be caused by changes in interdomain charge and the expected order of migration on a nondenaturing gel should be $-2 > -1 > 0 > +1 > +2$. The predicted order of mobility was generally observed (Figure 2). Our electrophoresis conditions were sensitive enough to resolve different mutants with the same charge; however, the minor variations observed between different mutant MTs in the same charge group were much less than the mobility differences exhibited between mutant MTs in different charge groups (Figure 2). The mobility differences observed within a charge group were too small to be due to differences in metal binding since the loss of a single divalent metal would be expected to alter the net charge by −2, which is comparable to the change in charge when a lysine is replaced by a glutamate residue.

Previous studies have indicted that the two domains of MT can function independently following selective proteolysis with subtilisin (Winge & Miklossy, 1982; Nielson & Winge, 1985; Stillman & Zelazowski, 1989). However, amino acid analyses indicate that at least one of the hinge lysines remains on isolated $\alpha$ (Nielson & Winge, 1985) or $\beta$ (Winge & Miklossy, 1982) domain peptides. Amino acid inserts of four or more residues following lysine-31 render MTs unstable in vivo (Rhee et al., 1989). Our study is the first to show that a stable MT which binds metals normally can form in the absence of both highly conserved lysines between the domains.

Our results support the notion that individual lysines contribute differentially to the structure and function of MT, and may explain why earlier studies showed that chemical modification of lysines does not always diminish metal binding. The hinge lysines do not appear to be involved in charge neutralization of MT. Modification which does not affect metal binding of MT, such as mild alkylation with TNBS (Pande et al., 1985) or polymerization (Templeton & Cherian, 1984), may involve only lysines-30 and -31. There are six other conserved lysines in MT which could be involved in charge neutralization of the thiolates. The juxtaposition of lysines within the domains to the metal-chelating cysteines may predispose them for charge balancing by forming outer-sphere complexes, in which the positive charge of these lysines would restrain, electrostatically, the structural expansion of the MT molecule (Pande et al., 1985). We speculate that chemical modification of these intradomain lysines could be responsible for the decreased metal binding observed following intense TNBS treatment (Pande et al., 1985).

## ACKNOWLEDGMENT

We thank Drs. Gunther Eichhorn and Y. Shin for the use of their JASCO 500C spectrophotometer.

## REFERENCES

Agros, P. (1990) *J. Mol. Biol. 211*, 943–958.
Arseniev, A., Schultze, P., Worgotter, E., Braun, W., Wagner, G., Vasak, M., Kagi, J. H. R., & Wuthrich, K. (1988) *J. Mol. Biol. 201*, 637–657.
Bowie, J. U., Reidhaar-Olson, J. F., Lim, W. A., & Sauer, R. T. (1990) *Science 247*, 1306–1310.
Buhler, R. H. O., & Kagi, J. H. R. (1979) *Experientia, Suppl. 34*, 211–220.
Chernaik, M. L., & Huang, P. C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3024–3028.
Cismowski, M. J., & Huang, P. C. (1991a) *Biochemistry 30*, 6626–6632.
Cismowski, M. J., & Huang, P. C. (1991b) *Methods Enzymol. 205*, 312–319.
Cismowski, M. J., Narula, S. N., Armitage, I. M., Chernaik, M. L., & Huang, P. C. (1992) *J. Biol. Chem. 266*, 24390–24397.
Ecker, D. J., Butt, T. R., Sternberg, E. J., Neeper, M. P., Debouck, C., Gorman, J. A., & Crooke, S. T. (1986) *J. Biol. Chem. 261*, 16895–16900.
Griffith, B. B., Walters, R. A., Enger, M. D., Hildebrand, C. E., & Griffith, J. K. (1983) *Nucleic Acids Res. 11*, 901–910.
Kagi, J. H. R., & Kojima, Y. (1987) *Experientia, Suppl. 52*, 25–61.
Kagi, J. H. R., & Schaffer, A. (1988) *Biochemistry 27*, 8509–8515.
Kojima, Y., Berger, C., Vallee, B. L., & Kagi, J. H. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3413–3417.
Kunkel, T. A., Roberts, J. D., & Zakour, R. A. (1987) *Methods Enzymol. 154*, 367–382.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Messerle, B. A., Schaffer, A., Vasak, M., Kagi, J. H. R., & Wuthrich, K. (1990) *J. Mol. Biol. 214*, 765–779.
Morris, S., & Huang, P. C. (1987) *Mol. Cell. Biol. 7*, 600–605.
Nielson, K. B., & Winge, D. R. (1984) *J. Biol. Chem. 259*, 4941–4946.
Nielson, K. B., & Winge, D. R. (1985) *J. Biol. Chem. 260*, 8698–8701.
Otvos, J. D., & Armitage, I. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7094–7098.
Pande, J., Vasak, M., & Kagi, J. H. R. (1985) *Biochemistry 24*, 6717–6722.
Pande, J., Pande, C., Gilg, D., Vasak, M., Callender, R., & Kagi, J. H. R. (1986) *Biochemistry 25*, 5526–5532.
Rakshit, D., & Vasak, M. (1992) *J. Biol. Chem. 267*, 235–238.
Rhee, I. K., Lee, K. S., & Huang, P. C. (1989) *Protein Eng. 3*, 205–213.
Robbins, A. H., McPee, D. E., Williamson, M., Collett, S. A., Xuong, N. H., Furey, W. F., Wang, B. C., & Stout, C. D. (1991) *J. Mol. Biol. 221*, 1269–1293.
Rose, M. D., Winston, F., & Hieter, P. (1990) *Methods in Yeast Genetics*, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Rupp, H., & Weser, U. (1978) *Biochim. Biophys. Acta 533*, 209–226.
Sanger, F., Miklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463–5467.
Schultze, P., Worgotter, E., Braun, W., Wagner, G., Vasak, M., Kagi, J. H. R., & Wuthrich, K. (1988) *J. Mol. Biol. 203*, 251–268.
Sone, T., Yamaoka, K., Minami, Y., & Tsunao, H. (1987) *J. Biol. Chem. 262*, 5878–5885.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Stillman, M. J., & Zelazowski, A. J. (1989) *Biochem. J. 262*, 181–188.
Templeton, D. M., & Cherian, M. G. (1984) *Biochem. J. 221*, 569–575.
Vasak, M., McClelland, Ch. E., Hill, H. A. O., & Kagi, J. H. R. (1985) *Experientia 41*, 30–34.
Vasak, M., Worgotter, E., Wagner, G., Kagi, J. H. R., & Wuthrich, K. (1987) *J. Mol. Biol. 196*, 711–719.
Willner, H., Vasak, M., & Kagi, J. H. R. (1987) *Biochemistry 26*, 6287–6292.
Winge, D. R., & Miklossy, K. A. (1982) *J. Biol. Chem. 257*, 3471–3476.