Effect of Cysteine Replacements at Positions 13 and 50 on Metallothionein Structure†

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ABSTRACT: Recombinant wild-type and mutant Chinese hamster metallothioneins, purified from the yeast *Saccharomyces cerevisiae*, were analyzed for their chemical and spectroscopic properties. The mutant proteins contain cysteine to tyrosine replacements at positions 13 and 50. Wild-type and mutant metallothioneins, in their cadmium-bound forms, all showed characteristic ultraviolet absorption spectra with shoulders at 245–250 nm due to cadmium–thiolate charge transfer. Upon acidification, these absorption shoulders were abolished. In all cases, two distinct titrations were seen, presumably corresponding to two independent cadmium binding domains in each of the proteins. Analysis of domain structures was performed both with the sulfhydryl reagent 5,5′-dithiobis(2-nitrobenzoic acid) and with the protease subtilisin. These studies indicated that both mutations affected domain structure by disrupting the normally tight protein clusters. Circular dichroism spectra obtained for wild-type and mutant metallothioneins showed unique structural rearrangements in mutants containing a cysteine-50 to tyrosine alteration. These data, along with previously obtained ^{113}Cd NMR data, were incorporated into a model which can account for the in vivo and in vitro properties of these mutant proteins.

Metallothioneins (MT)[1] comprise a class of low molecular weight cysteine-rich metal binding proteins found in a variety of plant and animal species. The major intracellular functions of MT appear to be detoxification of heavy-metal ions (e.g., Cd^{2+} and Hg^{2+}) and homeostasis of essential metal ions (e.g., Zn^{2+} and Cu^{+}) [recent reviews include Hamer (1986) and Kägi and Schäffer (1988)]. Mammalian MTs contain 61 or 62 amino acids, including 20 cysteines at invariant positions, and characteristically lack aromatic amino acids and histidine. MTs bind up to 7 mol equiv of divalent metal ions in two separate metal clusters, and all cysteines participate in metal coordination within the clusters, necessitating that cysteine sulfhydryls act as either univalent (terminal) or bivalent (bridging) ligands. Early spectroscopic studies led to the proposal that metal binding occurs through tetrahedral tetrathiolate coordination (Otvos & Armitage, 1980; Vašák et al., 1981; Vašák & Kägi, 1983). This coordination was unambiguously verified by one-dimensional and two-dimensional ^{1}H and ^{113}Cd NMR analysis (Boulanger et al., 1983; Otvos et al., 1985; Vašák et al., 1987; Arseniev et al., 1988; Schultze et al., 1988). The N-terminal β-domain binds three divalent metals via three bridging and six terminal cysteines; the C-terminal α-domain binds four divalent metals via five bridging and six terminal cysteines.

Because of its unique amino acid composition and metal binding properties, MT has many unusual chemical and spectroscopic features. Fully metalated MT is highly resistant to proteolysis (Nielson & Winge, 1983; Stillman & Zelazowski, 1989), presumably due to tight protein folding within the domains. Metal-chelating agents, such as EDTA, remove zinc from Zn_7-MT in a multiphasic reaction (Li et al., 1980a). It has also been shown that EDTA can selectively remove zinc from the β-domain of Cd_5Zn_2-MT (Winge & Miklossy, 1982), and can remove cadmium from the β-domain of either mixed Cd,Zn-MT or Cd_7-MT (Nielson & Winge, 1983; Stillman & Zelazowski, 1989). In all cases, the removal of metal from the β-domain makes this domain susceptible to proteolytic digestion. From these data, it has been inferred that metal is bound more dynamically to the β-domain than to the α-domain. Saturation-transfer ^{113}Cd NMR spectroscopy confirms the dynamic nature of metal in the β-domain. ^{113}Cd rapidly exchanges within the β-domain via intermolecular interactions, while ^{113}Cd in the α-domain remains static (Nettesheim et al., 1985; Otvos et al., 1987).

Ultraviolet absorption spectroscopy has been useful in understanding the nature of metal binding (especially cadmium binding) to MT. Cd-MT shows a strong absorbance at approximately 245–250 nm, due to cadmium–thiolate charge transfer, which imparts a broad shoulder to the otherwise featureless spectrum (Kägi & Vallee, 1961; Vašák & Kägi, 1983; Willner et al., 1987). Acidification of MT leads to release of bound cadmium, which can be monitored as a decrease in the absorption at 245–250 nm (Kägi & Vallee, 1961; Law et al., 1984). Circular dichroism (CD) has also been an important method for elucidating MT structural information. The spectropolarimetric features of Cd-MT above 220 nm are largely due to cadmium–thiolate interactions (Rupp & Weser, 1987; Vašák & Kägi, 1983; Law et al., 1984). From CD measurements made on isolated α- and β-domains reconstituted with cadmium, it was proposed that the characteristic features of Cd-MT are largely due to contributions from the α-domain (Stillman et al., 1987). This is also true in the intact protein, where titrations of apo-MT with cadmium have been monitored by CD. It was proposed that the change from individual metal–tetrathiolate sites to metal–thiolate clusters

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[1] Abbreviations: MT, metallothionein; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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containing bridging cysteines (at approximately 3–4 metal equiv) is what gives MT its characteristic CD spectrum (Stillman et al., 1987; Willner et al., 1987). Thus the α-domain, which contains proportionally more bridging cysteines, predominates in the CD spectrum of MT.

We have been studying series of mutants of Chinese hamster MT-2, which contains proteins with single or multiple amino acid replacements, in order to elucidate the relative contributions individual residues make toward the structural and functional integrity of MT (Cismowski et al., submitted for publication; Chernaik & Huang, 1991; Cody & Huang, submitted for publication). In particular, we have analyzed several mutants containing cysteine to tyrosine alterations, for both their in vivo and or in vitro properties. Three of these mutants, containing cysteine to tyrosine alterations at position 13, position 50, or both position 13 and position 50 (C13Y, C50Y, and C13,50Y, respectively), and wild-type MT were analyzed for their ability to function in vivo in a cadmium detoxification capacity. It was found that, while the mutant C13Y was able to protect a metal-sensitive *Saccharomyces cerevisiae* strain from cadmium as well as wild-type MT, the mutants C50Y and C13,50Y were not (Cismowski et al., submitted for publication). These proteins have been purified to homogeneity from *S. cerevisiae* in quantities large enough for structural analysis (Cismowski & Huang, 1991). All of the *S. cerevisiae* cultures were grown in low levels of $CdCl_2$, producing purified protein with cadmium as the predominant bound metal species. Cadmium binding stoichiometries were determined for each protein. Each mutant protein stably bound approximately one less Cd^{2+} than the wild-type MT (Cismowski et al., submitted for publication; see Materials and Methods). One-dimensional ^{113}Cd NMR spectroscopy was performed on these proteins (Cismowski et al., submitted for publication). This analysis confirmed the structural integrity of the wild-type protein, and showed that each mutant protein had undergone substantial rearrangement within its mutated domain. In this study, we further explore the nature of these changes. Susceptibility to chemical or proteolytic reagents was analyzed, as well as the spectroscopic and spectropolarimetric features of these proteins. These studies have allowed us to propose a model which incorporates the in vivo and in vitro properties of these mutants, and to infer some of the features important for the functioning of wild-type MT.

Materials and Methods

Protein Purifications. Wild-type and mutant MTs (C13Y, C50Y, and C13,50Y) were expressed and purified from the *S. cerevisiae* host AB-DE1. Expression vectors, growth of yeast in media containing 10 μM $CdCl_2$, purification of MTs, protein quantitation, and determination of metal stoichiometries were all as described (Cismowski & Huang, 1991; Cismowski et al., submitted for publication). The calculated stable metal binding stoichiometries for each protein are as follows: for wild-type MT, 6.4 ± 0.1 Cd, 0.6 ± 0.1 Zn, <0.2 Cu; for C13Y, 4.8 ± 0.1 Cd, 1.0 ± 0.1 Zn, <0.2 Cu; for C50Y, 4.8 ± 0.1 Cd, 1.4 ± 0.1 Zn, <0.2 Cu; for C13,50Y, 5.2 ± 0.1 Cd, 0.9 ± 0.1 Zn, <0.2 Cu.

Ultraviolet Absorption Spectroscopy and pH Titrations. All absorption spectra were acquired on a Hitachi 2000 spectrophotometer at ambient temperature using a scan rate of 100 nm/min. Matched 10-mm quartz cuvettes were used.

Three nanomoles of each purified MT was diluted to 1 mL with thoroughly degassed and N_2-bubbled 10 mM NaH_2PO_4, pH 7.5. A UV absorption spectrum was recorded using 1 mL of 10 mM NaH_2PO_4, pH 7.5, as a blank. An aliquot of degassed 0.5 N HCl was added to both sample and blank, followed by rapid mixing by inversion. The pH of the sample solution was then read directly with a PHM82 standard pH meter equipped with a combined microelectrode (Radiometer Copenhagen). A new UV absorption spectrum was recorded, and this process was repeated until a final pH of approximately 2.0 was obtained (27–43 individual scans). The HCl additions required for these titrations led to volume increases in the samples of <10%. To control for any effects caused by the presence of locally high concentrations of HCl during the titrations, MTs were diluted directly into degassed 10 mM phosphate buffer which had previously been titrated to various pHs, and absorption spectra were acquired. No significant differences were seen between spectra recorded in this manner and spectra acquired by addition of concentrated HCl (data not shown).

Raw data were transferred to Sigma-Plot Version 4.0 (Jandel Scientific) for analysis. Absorbance reading at 5-nm intervals were used to derive UV spectra at various pH readings, and the curves were smoothed by using the splining function of the program. No qualitative differences were seen between data processed in this way and data plotted at every nanometer with or without curve smoothing.

Reaction with 5,5′-Dithiobis(2-nitrobenzoic Acid). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was converted from the free acid to the dipotassium salt (Wilson et al., 1980) and was provided by Dr. Michael Washabaugh. Ten nanomoles of each purified MT was diluted into 0.5 mL of thoroughly degassed and N_2-bubbled 10 mM NaH_2PO_4, pH 7.5, and placed in a 10-mm quartz cuvette. Two nanomoles of DTNB was added, and the solution was quickly mixed by inversion. A blank cuvette containing 0.5 mL of 10 mM NaH_2PO_4, pH 7.5, and 2 nmol of DTNB was also prepared. Absorbance values at 25 °C and 412 nm were read on a Hitachi 2000 spectrophotometer at 10-s intervals for 10 min, beginning approximately 20 s after the addition of DTNB. Data were processed by using a curve-fitting program (MASTER 87; courtesy of Dr. Michael Washabaugh) to obtain pseudo-first-order rate constants. All data fit the derived first-order curves with χ values less than or equal to 1×10^{-6}. Data were plotted as the log of the end-point absorbance minus the absorbance at each time point versus the time of reaction.

Proteolysis Experiments. Two nanomoles of each purified protein was digested in 25 μL of 10 mM Tris-HCl, pH 7.5, at ambient temperature (approximately 20 °C) for 60 min with the protease subtilisin (Boehringer Mannheim). Digestions were performed in the presence or absence of 20 nmol of EDTA, and the subtilisin to MT ratio was approximately 1:20 (w/w). Samples, and nonproteolyzed controls, were then electrophoresed on 19% nondenaturing polyacrylamide gels run at pH 8.8 in 0.25 M Tris-HCl, 0.25 M glycine, and 50 mM sodium thioglycolate. Protein and peptides were visualized by silver staining using the Bio-Rad silver stain kit and protocol, with the modifications of Otsuka et al. (1988).

To determine metal content in gel slices, the wild-type Cd-MT was digested as described above for 40 min and electrophoresed on a 19% nondenaturing polyacrylamide gel. The lane containing the digestion products was then cut with a razor blade into 20 gel slices of 6 mm each. Gel slices were solubilized as described (Stillman & Zelazowski, 1989), and cadmium content was determined by atomic absorption spectroscopy using metal reference solutions (Fisher Scientific) in 5% nitric acid to generate a standard curve. The slope of the standard curve was calculated by linear regression analysis.

Circular Dichroism. Ellipticity measurements were obtained on 8 nmol of each purified MT in 1 mL of 10 mM Tris-HCl

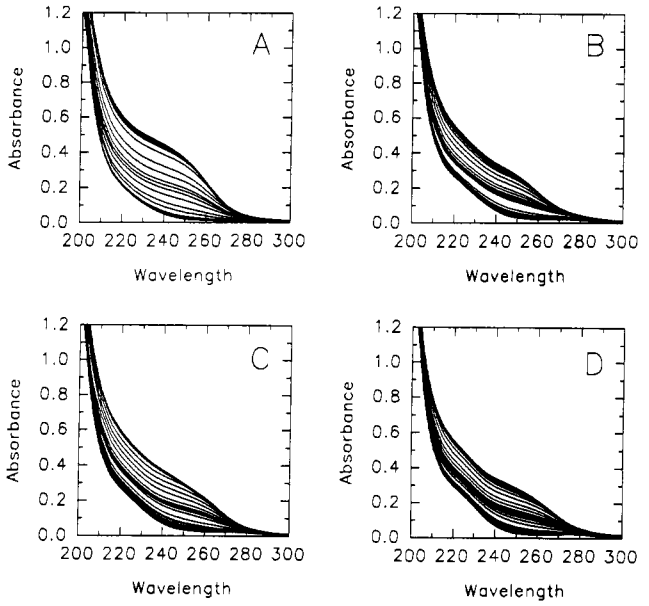

FIGURE 1: Effect of pH on the ultraviolet absorption spectra of purified wild-type and mutant Chinese hamster metallothioneins. For conditions, see Materials and Methods. Plots are derived from absorbance readings taken every 5 nm. The upper curve in each figure represents the spectrum at pH 7.5, and the lowest curve represents the spectrum at a pH of approximately 2.0. (A) Wild-type Cd-MT; (B) Cd-C13Y; (C) Cd-C50Y; (D) Cd-C13,50Y.

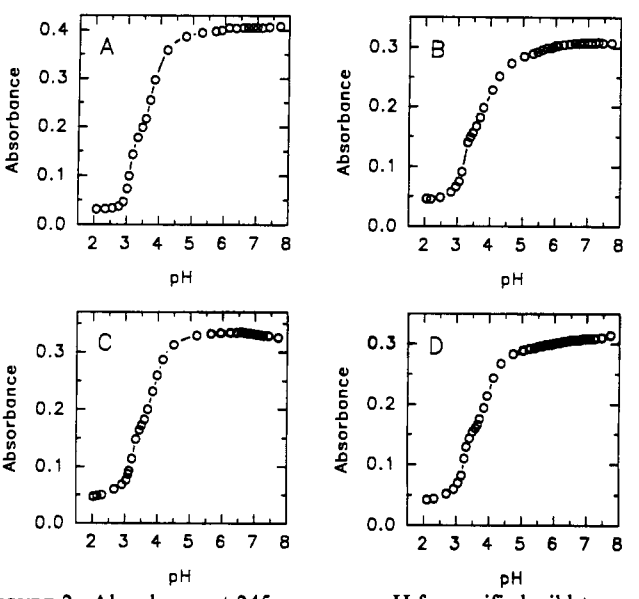

FIGURE 2: Absorbance at 245 nm versus pH for purified wild-type and mutant Chinese hamster metallothioneins. For conditions, see Materials and Methods. Plots are derived from absorbance readings taken at 245 nm for each scan made in Figure 1. (A) Wild-type Cd-MT; (B) Cd-C13Y; (C) Cd-C50Y; (D) Cd-C13,50Y.

at the indicated pH using a Jasco 500C spectropolarimeter run at 5 nm/min at 20.5 °C. A 1-cm quartz cuvette was used for all readings. A background reading was obtained at each pH using 1 mL of 10 mM Tris-HCl. Molar ellipticities (in degrees centimeter squared per decimole) were calculated, on the basis of an average amino acid molecular mass of 111.1 g/mole, from readings made every 2 nm of the ellipticity trace. Data were plotted by using Sigma-Plot Version 4.0 (Jandel Scientific), and curves were smoothed by using the splining function of the program.

Results

Purified samples of wild-type and mutant MTs were characterized by ultraviolet absorption spectroscopy. The absorption spectra of 3 μM solutions of each cadmium-bound protein in 10 mM NaH_2PO_4 buffer at neutral pH are shown in the upper traces of Figure 1. All of the MTs, including the mutants, had an absorption envelope centered around 245–250 nm. As this envelope is characteristic of tetrahedral cadmium–thiolate complexes (Vašák et al., 1981), it was clear that the mutants did not have gross structural disruptions. This result was consistent both with the stable metal binding capacities of the mutants (see Materials and Methods) and with ^{113}Cd NMR data which indicated that, although domain rearrangements had occurred in the mutants, there was still conservation of tetrahedral coordination chemistry (Cismowski et al., submitted for publication). The Cd–thiolate absorption envelopes of the mutant proteins, however, were slightly red-shifted relative to wild-type MT. This may be indicative of minor perturbations of the Cd–thiolate binding geometry.

Removal of cadmium bound to the purified wild-type and mutant MTs was accomplished by titration of each of the proteins with aliquots of 0.5 M HCl. Absorption spectra were taken at each point in the titration (Figure 1). These spectra showed a clear loss of the absorption envelope at 245–250 nm as the pH was lowered, mirroring the loss of bound cadmium as MT sulfhydryls were protonated. Mutant MTs also showed this loss of bound cadmium. A plot of absorbance at 245 nm versus pH shows that all MTs lost cadmium below pH 4.4 (Figure 2). Though there were no major differences in the titration profiles between wild-type and mutant MTs, the mutants all showed small upward shifts in their titration profiles by approximately 0.1–0.2 pH unit.

Table I: Calculated Rate of Reaction of Purified Wild-Type and Mutant Chinese Hamster MTs with 5,5′-Dithiobis(2-nitrobenzoic Acid)

protein	rate constant ($s^{-1} \times 10^{-3}$)	relative rate
Cd-MT	2.56 ± 0.95 (3)[a]	1.0
Cd-C50Y	5.74 ± 0.32 (2)	2.2
Cd-C13Y	10.24 ± 0.52 (2)	4.0
Cd-C13,50Y	10.51 ± 1.89 (3)	4.1

[a] Values in parentheses are the number of determinations made.

It is interesting to note that, in following the release of bound cadmium at 245 nm, there was clear indication of two independent titrations occurring (Figure 2). This presumably is due to the cooperative loss of metal from each of the two domains. Other qualitative differences between the absorption spectra of the wild-type and mutant MTs (Figure 1) could be accounted for by the absorption properties of the introduced tyrosine residues. An absorption shoulder at approximately 225 nm was seen in each mutant UV spectra, corresponding to protonated tyrosine hydroxyls (Greenstein & Winitz, 1961). A minor absorption peak was also present at approximately 280 nm in the mutants, due to the presence of this aromatic amino acid. Neither of these absorptions appeared to interfere with the cadmium–thiolate absorption at 245 nm.

The replacement of a single cysteine residue in a domain can potentially alter either the liganding strength or the coordination state of the remaining cysteines in that domain. Therefore, the normally tight clustering of the domain may be disrupted. These effects were assayed by reacting wild-type and mutant proteins with two reagents, the sulfhydryl reagent DTNB and the protease subtilisin. Under conditions of limiting DTNB, pseudo-first-order rate constants were derived from the initial rates of release of product, thionitrobenzoate, which was followed spectrophotometrically at 412 nm (Figure 3 and Table I). The rate of reaction with DTNB was higher for each mutant than for wild-type, reflecting a greater ac-

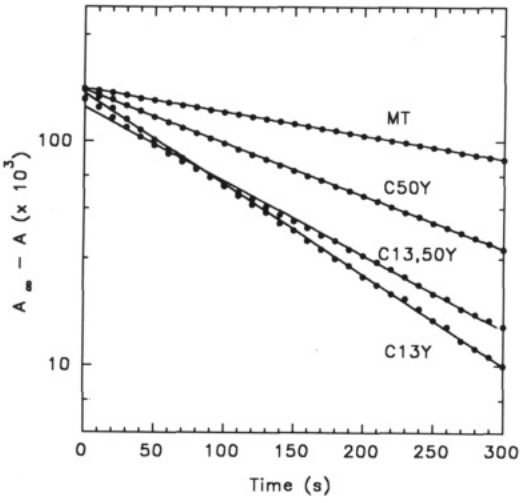

FIGURE 3: Reaction of purified wild-type and mutant Chinese hamster Cd-metallothioneins with 5,5′-dithiobis(2-nitrobenzoic acid), pH 7.5. For conditions, see Materials and Methods. Data are plotted as the log of the end-point absorbance at 412 nm minus the absorbance ($A_\infty - A$) versus the time of reaction. The data are representative of one experiment.

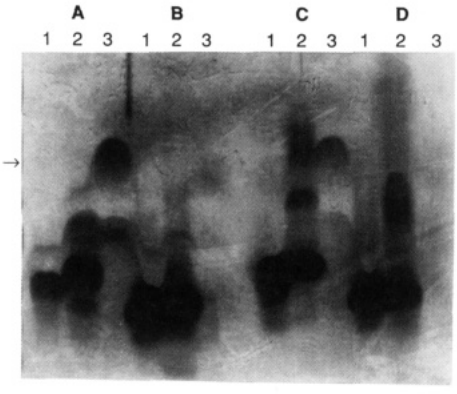

FIGURE 4: Reaction of purified wild-type and mutant Chinese hamster metallothioneins with subtilisin, pH 7.5. For reaction and electrophoretic conditions, see Materials and Methods. (A) Wild-type Cd-MT; (B) Cd-C50Y; (C) Cd-C13Y; (D) Cd-C13,50Y. (1) No subtilisin, no EDTA; (2) subtilisin added, no EDTA; (3) subtilisin and EDTA added. The arrow indicates the location of the EDTA-stable proteolytic product which is presumably the α-domain.

cessibility of this relatively bulky reagent toward sulfhydryls in the mutants. Mutant C50Y had an initial rate of reaction approximately 2-fold higher than wild type, while mutants which share a cysteine to tyrosine alteration at position 13 (C13Y and C13,50Y) had initial rates of reaction approximately 4-fold higher than wild type.

Fully metal-bound MT is highly resistant to degradation by the protease subtilisin; the metal chelator EDTA will selectively remove metal from the β-domain, allowing subtilisin to digest MT to a stable peptide, the α-domain (Winge & Miklossy, 1982; Nielson & Winge, 1983; Stillman & Zelazowski, 1989). Reactivity toward subtilisin was assayed in our purified wild-type and mutant MTs (Figure 4). Wild-type MT, as expected, was resistant to subtilisin in the absence of EDTA (lane A2); in the presence of a 10-fold molar excess of EDTA, wild-type MT was degraded to a peptide which likely corresponds to the α-domain (lane A3). This peptide was still capable of binding metal, as detected by analysis of metal content in polyacrylamide gel slices containing the reaction products (data not shown). Mutant C50Y was also resistant to subtilisin digestion in the absence of EDTA (lane B2); in the presence of EDTA, however, the protein was

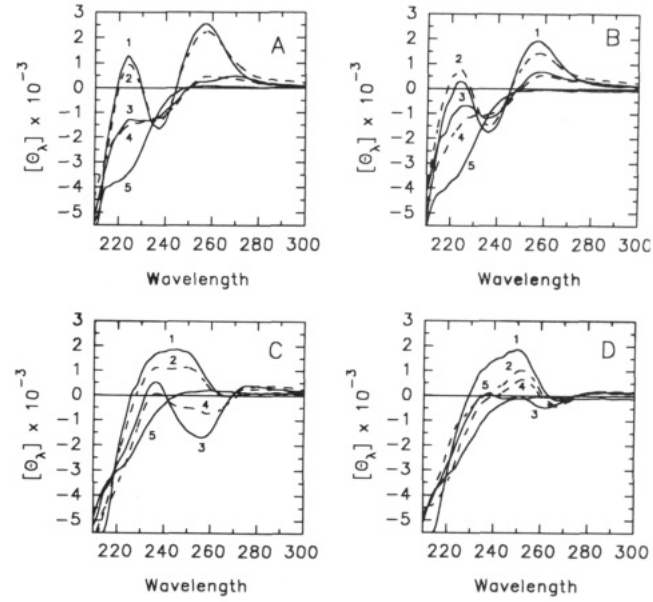

FIGURE 5: Effect of pH on the circular dichroism of purified wild-type and mutant Chinese hamster metallothioneins. For conditions, see Materials and Methods. Plots are derived from readings taken every 2 nm from the ellipticity trace. Molar ellipticities (θ_λ) are expressed in degrees centimeter squared per decimole. The pH of each sample was read directly after the ellipticity trace was recorded. (A) Wild-type Cd-MT: 1 = pH 7.45, 2 = pH 4.40, 3 = pH 3.83, 4 = pH 3.32, 5 = pH 2.11. (B) Cd-C13Y: 1 = pH 7.45, 2 = pH 4.46, 3 = pH 3.65, 4 = pH 3.27, 5 = pH 2.11. (C) Cd-C50Y: 1 = pH 7.45, 2 = pH 4.45, 3 = pH 3.61, 4 = pH 3.28, 5 = pH 2.06. (D) Cd-C13,50Y: 1 = pH 7.45, 2 = pH 4.46, 3 = pH 3.62, 4 = pH 3.26, 5 = pH 2.10.

completely degraded (lane B3). Thus, it appears that the α-domain in the C50Y mutant has been altered such that EDTA can now successfully compete for its bound metal. Mutant C13Y was susceptible to subtilisin even in the absence of EDTA; a peptide comigrating with the wild-type α-domain was released (lane C2). This released peptide was stable to degradation in the presence of EDTA (lane C3). Therefore, like DTNB, subtilisin was apparently capable of directly accessing the β-domain of the C13Y mutant, indicating that this domain may no longer be forming a tight cluster. The double mutant, C13,50Y, was also digested to a peptide in the absence of EDTA (lane D2). This peptide did not comigrate with the wild-type α-domain peptide, presumably due to the presence of a cysteine to tyrosine mutation at position 50. However, this α-domain mutation, like that in the mutant C50Y, did not render the domain directly susceptible to proteolytic attack; only in the presence of EDTA was it degraded (lane D3).

Circular dichroism (CD) was used to further explore the structure of the purified wild-type and mutant MTs. The spectrum of wild-type MT, at pH 7.5, was typical of other Cd-MTs, with maxima at 258(+), 237(−), and 224(+) nm (Figure 5A). The mutant C13Y had a spectrum at neutral pH which was qualitatively similar to wild type [Figure 5B; maxima at 257(+), 236(−), and 224(+) nm], even though the β-domain in this mutant appears to be substantially disrupted (Figures 3 and 4). This is not too surprising as the major features of the CD spectra of MTs seem to be contributed by the α-domain (Stillman et al., 1987). The spectra at neutral pH of mutants sharing the cysteine to tyrosine alteration at position 50 were remarkably different from wild-type MT (Figure 5C,D). In fact, these spectra were qualitatively similar to the spectrum obtained when isolated β-domain is reconstituted with cadmium (Stillman et al., 1987). Thus, it appears that a mutation at position 50 disrupts the α-domain to the extent that it no longer significantly contributes to the char-

acteristic CD spectrum of MT.

CD spectra for all proteins were obtained at various pHs corresponding to different states of metal binding (Figure 5). As expected, all proteins had essentially featureless spectra at a pH near 2, where no cadmium was bound. Wild-type MT and C13Y had spectra which appeared to gradually lose intensity as the pH was lowered. C50Y and, to a lesser extent C13,50Y did not show a gradual diminishing of their spectra as the pH was lowered. Instead, clear spectral transitions were seen during the titrations. For C50Y, this transition occurred between pH 4.4 and 3.6, with a shift from a spectrum with a broad maximum centered at 244(+) nm to a spectrum with sharp maxima at 257(−) and 236(+) nm. These spectral transitions may indicate structural rearrangements occurring as metal is lost from the mutant proteins.

Discussion

The ability to create, express, and purify site-specific mutants of mammalian MT has given us the opportunity to ask fundamental structure–function questions about this protein. In this study and others (Cismowski et al., submitted for publication), we examined the role of individual cysteines in conferring structural and functional integrity to MT. Two sites for mutation were chosen for these initial studies, cysteine-13 and cysteine-50. Cysteine-13 has been shown by two-dimensional NMR to be a terminal cysteine in the β-domain of rat and rabbit MTs, and cysteine-50 has been shown to be a bridging cysteine in the α-domain (Arseniev et al., 1988; Schultze et al., 1988). Because of the absolute conservation in both number and location of cysteine residues within the mammalian MT family, it is likely that these assignments hold true for Chinese hamster MT as well. The similarity of the one-dimensional ^{113}Cd NMR spectra of rabbit, rat, and Chinese hamster MTs substantiates this assumption (Cismowski et al., submitted for publication).

In our initial studies, the effect of mutations at cysteines-13 and -50 on the in vivo detoxification function of MT was assayed in a metal-sensitive yeast strain (Cismowski et al., submitted for publication). It was found that, while wild-type MT and the mutant C13Y were able to protect against levels of cadmium which would otherwise be toxic to the host, C50Y and C13,50Y were not. This loss of cadmium detoxification function was apparently due specifically to a loss of the cysteine sulfhydryl at position 50, as mutants carrying more conservative serine or alanine substitutions at this position were also defective in vivo (Cismowski et al., submitted for publication). Upon purification of the MTs, it was found that the wild-type protein stably bound approximately seven metal ions per molecule while each mutant stably bound approximately six metal ions per molecule (see Materials and Methods). As each mutant was still able to bind significant amounts of metal, and as the metal binding in each mutant was roughly equivalent, it was clear that the stable metal binding capacities of these mutants did not directly correlate with their detoxification capacities. It seems likely then that more subtle structural features determine the in vivo functionality of these proteins.

One-dimensional ^{113}Cd NMR of the purified wild-type and mutant MTs provided evidence for structural rearrangements in the mutated domains, while in each case the nonmutated domains appeared unaffected (Cismowski et al., submitted for publication). There was no indication in the spectra of the mutants that the tyrosine hydroxyls were participating in cadmium binding. For mutant C50Y, it appeared that the normally static α-domain (Otvos et al., 1987) was altered with respect to resonance positions and intensities. This alteration is consistent with the introduction of more facile metal exchange within this domain. For mutant C13Y, the normally dynamic β-domain appeared to be significantly altered as well. The wild-type β-domain resonances were absent from their normal positions, consistent with an increase in metal lability within this domain. This study is an attempt to probe more deeply into the structures of these mutant MTs in order to derive a model of MT function consistent with this previous in vivo and in vitro data.

Purified wild-type and mutant MTs, in their predominantly cadmium-bound forms, were analyzed by UV absorption spectroscopy at various pHs (Figures 1 and 2). Two distinct titration midpoints were resolved upon acidification of both the wild-type and mutant Cd-MTs. These results confirmed the structural integrity of each of the proteins and were consistent with each mutant protein being comprised of two independent domains. The apparent discrepancy between these results and those of an earlier study on equine Cd-MT (Kägi & Vallee, 1961), in which no obvious inflection point was detected, remains unclear. Inflection points may have been detected in this study as a result of higher resolution curve-fitting analysis or the accumulation of a greater number of data points.

Wild-type and mutant MT structure was also analyzed by chemical and proteolytic reactions using the sulfhydryl reagent DTNB or the protease subtilisin. Both reagents probe the relative tightness of the domains, as neither the peptide backbone nor the cysteine thiolates would be expected to be freely accessible in a correctly folded MT. Under conditions of limiting DTNB, wild-type MT had a slow, but measurable, rate of reaction. As the metal in the wild-type β-domain is known to be labile (Winge & Miklossy, 1982; Stillman & Zelazowski, 1989), DTNB was probably reacting with one or more cysteine thiolates in this domain. This would be consistent with earlier experiments in which the β-domain cysteines of Cd_7-MT were shown to be preferentially alkylated by iodoacetamide (Bernhard et al., 1986). Each of the mutant proteins had a higher rate of reaction with DTNB than the wild-type protein. This is consistent with the mutations causing structural changes such than cysteine thiolates within the mutated domains become more accessible to reagent. This greater accessibility may be the result of an increased rate of Cd–thiolate bond cleavage in the mutant domains or an overall enhancement of solvent accessibility due to domain rearrangement. It has been shown that under conditions of excess DNTB relative to MT a biphasic reaction occurs, and the relative contributions of metal–thiolate bond cleavage and ligand accessibility toward the overall reaction rate can be distinguished in each phase of the reaction curve (Li et al., 1980b). Our initial studies were conducted under conditions of limiting DTNB, such that less than half of the MT molecules on average (approximately 1 in 50 cysteine residues) could potentially be modified. This was done in an attempt to probe for only the most reactive cysteine thiolates in each sample. Under the conditions used here, a monophasic reaction curve was seen, possibly corresponding to the faster initial reaction rate seen in the earlier study. Further work may be needed in order to understand the factors contributing to the enhanced DTNB reaction rates seen in the mutant proteins.

Upon digestion with subtilisin, wild-type MT, as expected, was resistant to degradation in the absence of EDTA but was digested to the α-domain in the presence of EDTA. The data obtained with the mutant MTs are consistent with a mutation at cysteine-13 altering the β-domain such that it becomes susceptible to proteolysis in the absence of EDTA, and a mutation at cysteine-50 altering the α-domain such that it

becomes susceptible to proteolysis in the presence of EDTA. Thus, each mutation again appears to be increasing the solvent accessibility of its respective domain.

Circular dichroism was a useful technique for exploring the structure of the α-domain in the mutants C50Y and C13,50Y. The spectra of these mutants, at neutral pH, did not have the "derivative" peak at 258 nm characteristic of cadmium-bound MTs (Rupp & Weser, 1978; Pande et al., 1986; Willner et al., 1987), but instead appeared qualitatively similar to those of isolated cadmium-reconstituted β-domains (Stillman et al., 1987). The lack of contribution of the β-domain to the CD spectrum of MT has been proposed to be due to an inherent symmetry in this domain and/or a lack of adequate clustering (Stillman et al., 1987; Willner et al., 1987). It is possible, then, that in C50Y and C13,50Y the mutated α-domain can adopt a structure similar to that of the normal β-domain. The simple diminishing of peak intensities observed in wild-type MT and C13Y upon lowering the pH has been proposed to correlate with a single transition from a clustered to a nonclustered state, and may be indicative of a cooperative effect in the formation of the α-domain (Rupp & Weser, 1978; Stillman et al., 1987; Willner et al., 1987). The striking spectral transition seen between pH 4.4 and 3.6 in the mutant C50Y and, to a lesser extent, in the mutant C13,50Y may indicate the occurrence of unique structural rearrangements in these proteins. Further investigation of this phenomenon is currently underway.

All of these results can be accommodated into a model for MT function which makes a distinction between two modes of metal–thiolate liganding. The first mode is the kinetically labile mode seen in the β-domain of the wild-type protein. This mode of binding is proposed to contribute little to the long-term in vivo cadmium detoxification function of MT. Instead, this mode of binding may be important for initial cadmium binding during acute exposure and/or zinc homeostasis (Nettesheim et al., 1985). The α-domain of the wild-type MT, however, binds cadmium in a kinetically stable, rigid cluster (Otvos et al., 1987), and this domain is proposed to be the major contributor to in vivo cadmium detoxification function.

Each of the introduced mutations appears to create a domain which folds less tightly than the wild-type domain. For the β-domain mutant C13Y, the effect is to make a kinetically labile metal binding domain even more so. The β-domain in this mutant now reacts readily with DTNB and is susceptible to proteolytic attack in the absence of metal chelators, indicating the polypeptide backbone is now in a more solvent-accessible state. The protein has a tendency to relinquish cadmium more readily than wild type, as the pH titration midpoints are higher, though the effect is a minor one. The CD spectra of C13Y offer little structural data, as the β-domain presumably does not make a significant contribution to the spectra. However, the one-dimensional ^{113}Cd NMR spectrum of this mutant appears to be consistent with this model, as the normal β-domain resonances are absent, perhaps due to exchange broadening (Cismowski et al., submitted for publication).

The mutation at cysteine-50 appears to alter the kinetically stable domain into a more labile domain, somewhat like the wild-type β-domain. The reaction rate for C50Y toward DTNB is intermediate between that of wild-type MT and C13Y, indicating a somewhat increased solvent accessibility of cysteine thiolates, and the α-domain is now susceptible to proteolysis in the presence of metal chelators. The CD spectra of this mutant indicate an absence of the normal α-domain structure, and the absorbance data obtained during a pH titration indicate that this mutant releases metal somewhat more readily than wild-type MT. The mutated α-domain is apparently not behaving exactly as a "β-domain," however, as there are still two distinct metal titrations seen in the absorbance spectra. Finally, the one-dimensional ^{113}Cd NMR spectrum of C50Y is consistent with a more dynamic metal exchange occurring in the α-domain (Cismowski et al., submitted for publication).

The data on the double mutant C13,50Y are difficult to interpret at present. Though the data presented here are consistent with C13,50Y acting as a simple composite of the two single mutants, the stable metal binding stoichiometry of this protein and its one-dimensional ^{113}Cd NMR spectrum (Cismowski et al., submitted for publication) indicate that this protein may be adopting a highly unique structure. Further work will need to be done on this mutant in order to clarify its structure.

It is interesting to speculate on the structural features of MT that dictate the kinetic stability of the domains. All divalent metal bound to MT is bound in a tetrahedral tetrathiolate coordination, which is an intrinsically high-affinity binding mode. The β-domain contains 9 cysteines and binds 3 metals (a 3:1 cysteine to metal ratio), while the α-domain contains 11 cysteines and binds 4 metals (a 2.75:1 cysteine to metal ratio). However, the affinity of cadmium for the α-domain is higher than it is for the β-domain (Armitage & Boulanger, 1983; Vazquez & Vašák, 1988; Stillman & Zelazowski, 1989). The increase in affinity in the α-domain is not due to an increased ratio of cysteine to metal; therefore, it may be due to a difference in binding mode. The α-domain contains proportionally more bridging cysteines than the β-domain (5 of 11 versus 3 of 9). It is possible, then, that this higher degree of bridging cysteines (a clustering effect) is what gives the α-domain its ability to bind cadmium in a tight, static fashion and, therefore, to act in vivo in a detoxification capacity. The mutant C50Y appears to have lost this ability to bind cadmium statically, and therefore cannot function well in detoxification. This model can be tested by using two-dimensional heteronuclear NMR experiments to determine the number of bridging cysteines in the α-domain of C50Y, and by extending these structural studies to other cysteine substitution mutants. By detailed structural analysis of additional mutants of MT, it may be possible to extend these initial observations into a greater understanding of the structural constraints required for MT detoxification function.

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Registry No. DTNB, 69-78-3; Cd, 7440-43-9; cysteine, 52-90-4.

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