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SUBSTITUTION OF GLUTAMIC ACIDS FOR THE CONSERVED LYSINES IN THE α DOMAIN AFFECTS METAL BINDING IN BOTH THE α AND β DOMAINS OF MAMMALIAN METALLOTHIONEIN

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SUMMARY: Lysine residues are highly conserved in mammalian metallothioneins(MTs). Recombinant mutant Chinese hamster MT2 in which all of the lysines (K) in the α-domain were substituted by glutamic acids (E) was assayed with, expressed in and purified from a cadmium sensitive strain of yeast *Saccharomyces cerevisiae*. Circular dichroism analyses of the mutated protein, mutein K43,51,56E, revealed that the overall structure remained unchanged. However, a 1-D ^{113}Cd NMR study detected significant differences in the chemical shifts of the corresponding resonances between wild type protein and the recombinant mutein. Reduction of integrated intensity in the NMR spectra was also observed for resonances from the four-metal cluster (I and V-VII) in the α-domain of the mutein. At various temperatures, facile intermolecular exchange of metals in the β-domain of the mutein was also observed, which was unexpected and was different from wild type. Our results thus demonstrate that replacing all three lysines by glutamic acids in the α–domain changed metal-thiolate interactions in both domains of the recombinant mutein. This may explain the reduced ability of the mutein to convey cadmium resistance. We propose that while the lysine residues in the α-domain of wild type MT are not critical for maintaining protein structure, they play a role in regulating the microenvironment and stability of both metal-binding clusters, a feature critical to metal detoxification.

Lysine residues are highly conserved in metallothioneins (MTs), a family of metal binding proteins enriched in cysteine. In all MT sequences thus far examined, lysines are juxtaposed next to cysteine residues, which are invariant. Lysine, which is positively charged at neutral pH, may play a role in neutralizing the excess negative charge of the metal thiolate complexes in MT (Kojima et al. 1976) and in restraining the structural expansion of the MT molecule (Vasak et al. 1985). Both ^{1}H NMR and pH titration studies of MTs containing chemically modified lysines support a model of electric interaction in which it is proposed that the protonated side chains of lysines interact with the threefold negatively charged metal-thiolate clusters (Vasak et al. 1985, Pande et al. 1985). By site-

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directed mutagenesis, Cody and Huang (1993) showed that both lysine residues in the hinge between two domains are important for MT expression since replacement of both by glutamates results in decreased MT level and detoxification function. In contrast, single or double substitution of lysines to glutamates in the α–domain cause no obvious change in either the structure or detoxification function of MT (Cody and Huang 1994).

A triple MT mutein, i.e. mutated protein, has been generated in which all three lysines in the α domain were replaced by glutamic acids. In this mutein, a significant reduction in the detoxification capacity was observed (Cody and Huang 1994). This effect was not due to a reduced half life of the molecule. We suspect that the triple mutation affected metal affinity. This study was designed to examine possible changes in the stability of the metal clusters.

MATERIALS AND METHODS

Mutein preparation. Recombinant K43,51,56E of Chinese hamster ovary (CHO) MT-2, was purified from a yeast expression system using *S. cerevisiae* AB-DE1 as the host. Details for the site-directed mutagenesis, cloning, MT purification, and ^{113}Cd replacement has been described elsewhere (Cody and Huang 1993, Johnson, Hou and Huang, in preparation).

Characterization. MT concentration was determined using the value ε_{220}=47,300 $M^{-1}cm^{-1}$ for the apoprotein (Buhler and Kagi, 1979). Electronic absorption (UV) and circular dichroic (CD) spectra were determined following procedures described previously (Cismowski and Huang 1991).

Nuclear magnetic resonance. ^{113}Cd NMR spectra were recorded at 66.5 MHz on a Bruker MSL-300 spectrometer, under conditions of broad band proton decoupling. ^{113}Cd-enriched MT (0.5 ml of 1 mM) was run in 5-mm NMR tubes in 10 mM sodium phosphate, pH 7.5, containing 10% D_2O to provide the field-frequency lock. Typical acquisition parameters were 70° pulse, 1-s pulse repetition rate, and 6-10x 10^4 transients. Chemical shifts are in ppm downfield from the ^{113}Cd resonance of 0.1 M $Cd(ClO_4)_2$.

RESULTS

Spectral features of purified K43,51,56E mutein were similar to those of wild type MT. The UV absorption envelope around 245-250 nm, which is due to tetrahedral Cd-thiolate complexes, was characteristically reduced with decreasing pH, mirroring the loss of bound Cd as MT sulfhydryls were protonated (Figure 1a). The CD spectrum of the Cd-mutein was also qualitatively similar to that of wild-type Cd-MT at neutral pH, with maxima at 259 (+), 239 (-), and 227 (+) nm (Figure 1b). In comparison to wild type MT (Cismowski and Huang 1991), the spectrum of the mutein at pH 7.64 had less intensity at 259 (+) nm. At various pHs, corresponding to different states of metal binding, spectral changes for the mutein were similar to those for wild-type. Both wild type and mutein MTs, exhibited essentially featureless spectra at a pH near 2, where no Cd was bound.

Measuring the ^{113}Cd chemical shifts allowed us to detect subtle changes in metal coordination and ligand exchange dynamics. Wild type MT exhibits seven major resonances between 600 and 670 ppm, corresponding to seven specific metal-thiolate clusters. As shown in Figure 2, only six could be resolved in the mutein spectrum at ambient temperature. These resonances have been assigned by the first order splitting patterns using ^{113}Cd-^{113}Cd spin coupling, based on two-bound

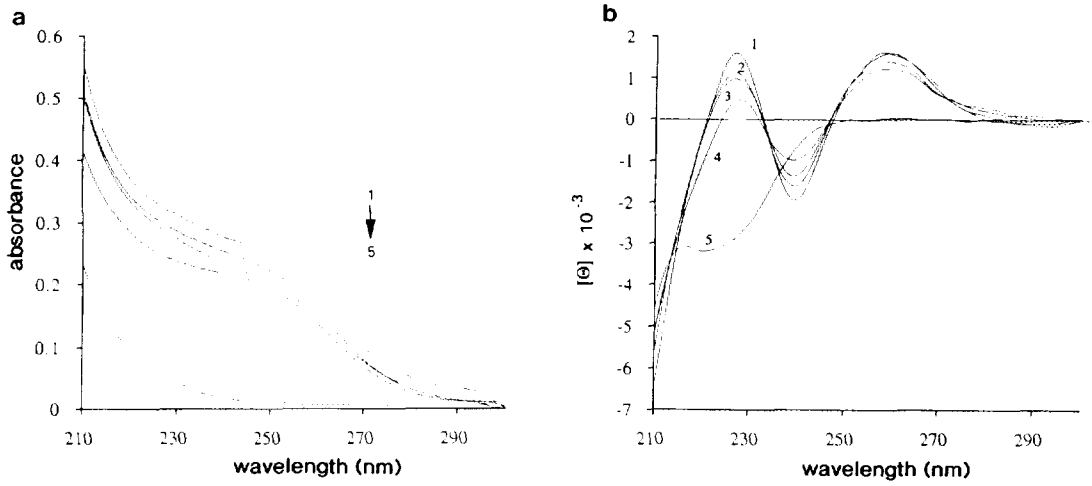

Figure 1. Effect of pH on the ultraviolet absorption spectra (a) and the circular dichroism (b) of Cd7 mutein (K43,51,56E) of CHO MT2. The spectra were recorded in a 2mM solution of protein in 10 mM Tris-HCl, at pH (1) 7.64; (2) 4.52; (3) 3.96; (4) 3.35; (5) 2.14.

interactions between adjacent ^{113}Cd ions in the metal-thiolate clusters. Resonances III and IV overlapped at ambient temperature, but could be resolved into two peaks at 279°K (see later).

Significant upfield chemical shifts of corresponding resonances were observed in the mutein spectrum when compared with that of wild type MT (Table I). Resonance VII, shifted about 10.7

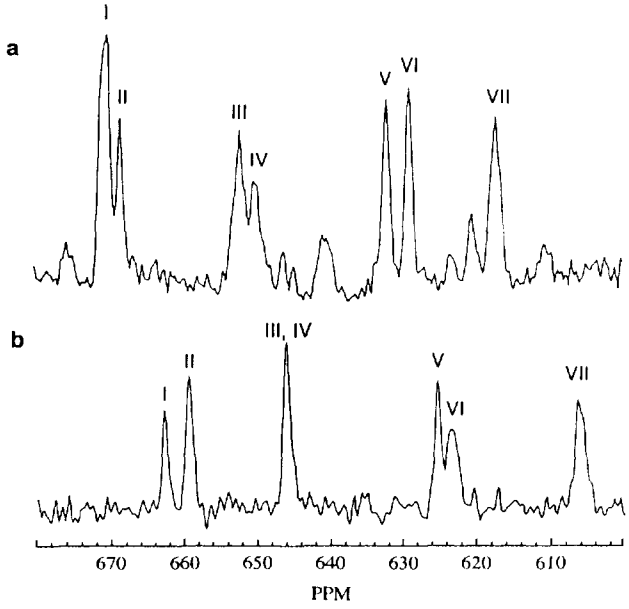

Figure 2. Proton-decoupled 66.5 MHz one-dimensional ^{113}Cd NMR spectra of ^{113}Cd enriched wild type (a) and mutein (K43,51,56E) (b) CHO MT2 purified from yeast. Spectrum (a) was adapted from Cismowski et al. (1991). Spectrum (b) was acquired at 298°K from approximately 0.1 mM protein in 10 mM phosphate buffer, pH 7.5 (93800 pulses). Chemical shifts relative to 0.1 M $Cd(ClO_4)_2$(aq) at 298°K.

Table I. ^{113}Cd NMR chemical shifts for wild type MT at ambient temperature and recombinant mutein K 43,51,56E at various temperatures

Resonance assignment	wild type[a]	K43,51,56E				
	298°K	279°K	288°K	298°K	308°K	317°K
			δ (ppm)			
I	670.3	665.9	664.7	662.8	660.7	658.6
II	668.3	660.3	660.0	659.4	658.8	658.6
III	651.9	648.8	647.7	646.1	644.7	643.3
IV	650.0	646.7	--	646.1	644.7	643.3
V	632.1	627.3	626.3	625.3	624.2	622.9
VI	628.9	625.1	624.4	623.4	622.6	621.6
VII	617.2	608.1	607.4	606.5	604.9	603.6

[a] Data from Cismowski *et al.*, 1991.

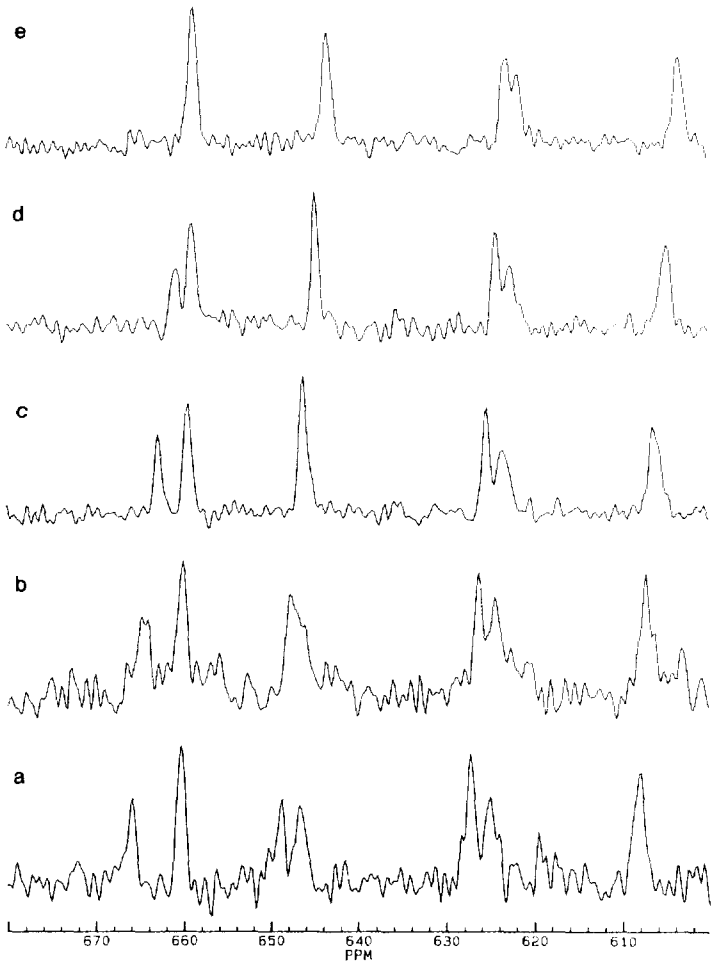

Figure 3. Effect of temperature on the proton-decoupled ^{113}Cd NMR spectrum at 66.5 MHz of ^{113}Cd-enriched mutant form (K43,51,56E) of CHO MT2 purified from yeast in 10 mM phosphate buffer, pH 7.5, at (a) 279°K, (b) 288°K, (c) 298°K, (d) 308°K, (e), and 317°K. Chemical shifts were referenced to extemal aqueous 0.1 M $Cd(ClO_4)_2$ at 298°K.

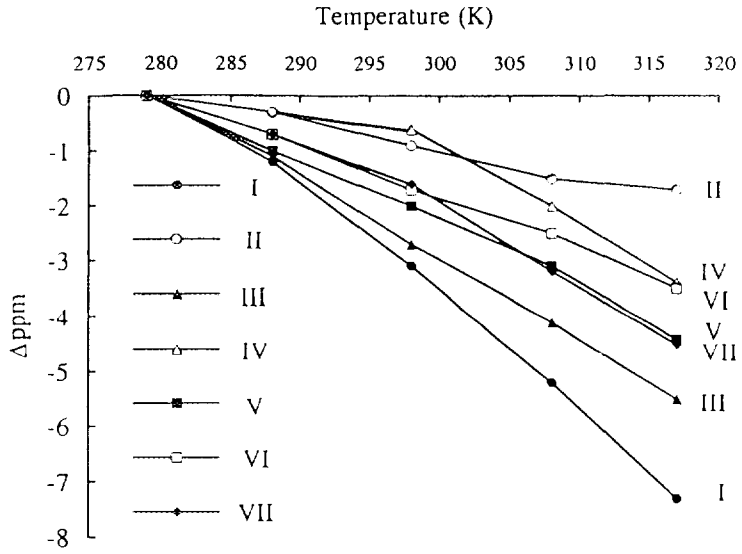

Figure 4. Change in ^{113}Cd NMR chemical shifts of resonances of ^{113}Cd NMR-enriched mutein (K43,51,56E) of CHO MT2 on increasing temperature from 279°K to 317°K.

ppm, was the most perturbed, followed by resonances I and II. A reduction in integrated intensity was seen in resonances I, V, VI and VII, all of which are derived from the α-domain metal-thiolate cluster.

All resonances shifted upfield when the temperature was raised (Figure 3 and Table I). The slopes of chemical shift versus temperature plots for individual resonances of the mutein were nearly constant, except for resonances IV and VII (Figure 4). Resonances III and IV of the mutein could be resolved into two separate peaks at 279°K, merged into a broad peak at 288°K, and totally overlapped each other, forming a sharp peak, when the temperature was higher than 298°K. Resonance I was also sensitive to temperature; it shifted to overlap resonance II when the temperature was elevated to 317°K (Figure 3e).

DISCUSSION

A unique feature of MT structure is that amino acid residues form abundant helical turns and wrap around the metal cores, so that the metal clusters are buried within the protein. Results of x-ray crystallography (Robbins et al. 1991) and 2-D NMR (Messerle et al. 1992) show that all MT lysine side chains are exposed to the surface. Accordingly, one would have predicted *a priori* that replacement of these residues would be inconsequential despite their high conservation. Thus the observations in this study that both the function and structure of the K43,51,56E mutein were perturbed is of considerable interest and significance.

The results of the UV and CD spectral analyses in this study suggest that the lysine residues in the α-domain of MT are not essential to overall MT structure, since both mutein and wild type MTs exhibited similar pH dependent spectra. However, a more rigorous analysis by ^{113}Cd NMR

showed that some of the resonance signals of the mutein were altered in both chemical shift and intensity relative to the wild type protein. Since the resonance signals reflect the state of individual metal binding, these changes are indicative of a lowered metal-binding capacity in the α-domain.

We also noted a differential chemical shift of resonances III and IV, which was nonlinear with temperature changes at about 298°K. This feature might be caused by the transition from slow-exchange to fast-exchange regimes between 279°K and 298°K, which is attributable to intramolecular fluctuation of the environment of the three-metal cluster (β-domain) of the mutein. As a weighted average of the chemical shifts of resonances III and IV in rapid equilibrium when the temperature is above 298°K, the shift reflects the lifetime of metal ion exchange between the sites of Cd III and Cd IV. This time has been estimated to be less than 10^{-3} s when the temperature is above 298°K (Armitage and Boulanger, 1983). It should be pointed out that resonances III and IV from wild-type MT can be resolved into two peaks at 298°K, setting a lower limit of about 10^{-2} s on the lifetime of the metal ions in these sites (Nettesheim et al., 1985). On this basis, the rate of intramolecular metal exchange in the β-domain may be considered to remain in the slow-exchange regime for wild type MT at 298°K, instead of being rapid as in the mutein. The altered rate of intramolecular metal exchange in the β-domain could influence intermolecular metal-ligand exchange, resulting in formation of MT dimers (Otvos et al. 1993). These observations lend credence to the inference that lysine substitutions in the α -domain have an effect on the β-domain.

In metallated MT, negative charges are generated by metal-thiolate linkages. In this connotation, the juxtaposition of the lysines within the two domains to the metal chelating cysteines may predispose them for charge balance by forming outer-sphere complexes, in which the positive charges of these lysines would restrain possible structural expansion of the MT molecule. Substitutions of three lysines by glutamic acids at position 43, 51, and 56 would alter the net charge of the α–domain from -3 to -9, which could contribute to the lowered stability of the α-domain .

We have used the coordinates established for MT in the Protein Data Bank to calculate the electrostatic potential energy difference between wild type and this mutein. The position of an amino acid residue was approximated by its Cα, and the dielectric constant ε was taken as a sigmoidal distance-dependent function (Daggett et al. 1991). According to this calculation, substitution of all three lysines would alter the energy of the four-metal cluster (α-domain) by more than 20 kcal/mol, sufficient to collapse the structure (unpublished data). This suggests that the mutein attains a more flexible structure by reducing the electrostatic energy change of the α-domain and lowering its metal-binding affinity. This energy alteration could be responsible for the observed decrease in metal detoxification capacity of the mutein (Cody and Huang 1994). We note that the mutein is stable in vivo, binds 7 metal atoms per molecule, and maintains an overall structure similar to wild type MT.

Earlier studies showed that effects of cysteine substitution are confined to the domain in which the residue is replaced; thus each domain in MT seemed to be independent in its structure and function (Cismowski et al. 1991). In this study, although effects were also seen in the unmodified domain, the greatest perturbations in the one-dimensional ^{113}Cd NMR spectra of the mutein were observed in the α-domain resonances. Resonances of Cd I and Cd VII showed a significant difference in chemical shifts and those of Cd I and Cd VI showed an obvious reduction in their integrated intensity compared with wild-type MT. The fact that the resonance of Cd V was less affected might be due to its being further away from the lysine residues.

Our mutagenesis should have eliminated the intramolecular hydrogen bond interaction between the K43 Nζ and the C59 O (Robbins et al., 1991) and altered the structural stability of the α-domain, since C59 is a terminal cysteine whose ligand is Cd I. This is consistent with our NMR results which showed that the resonance of Cd I in mutein MT had lower integrated intensity and thermal stability than in wild type MT.

The charged side-chains on lysine residues may be indirectly involved in the exchange of metal ions in MT. The net charge alteration of the substitutions could also disturb the outer-sphere complexes and rearrange the distribution of the positive charges on the side chains of the other lysine residues, i.e., those in the β domain (K20, K22, and K25) and between domains (K30, and K31). This would perturb the intramolecular hydrogen bond interactions between K31 Nζ to the sulfur atom of C19 and to the carbonyl groups of C19 and C21. Both C19 and C21 are terminal cysteines connecting Cd II and Cd IV, respectively (Robbins et al. 1991). This may explain why the ^{113}Cd NMR resonance of Cd II had different chemical shifts in mutant and wild-type spectra.

In conclusion, we observed in this study that replacing all three lysines with glutamic acids in the α-domain of MT did not change the overall structure of MT. However, the dynamics of metal binding in both the α- and β-domains were altered. In the α-domain metal binding was weakened, while intramolecular exchange of metals in the β-domain were enhanced. We surmise that the shielding of the metal nuclei of both metal-binding clusters was affected. Thus, the positively charged residues located on the outer-sphere indirectly affect the metal affinity of MT, but they exert little effect on metal cluster formation and overall protein conformation. The effects of lysine replacement may be the consequence of perturbation of the normal charge-balancing, within the domain microenvironment, that is important to the stability of both metal clusters. Our results demonstrate for the first time that substitution of lysines in one domain alters the intramolecular-exchange and the shielding of metal nuclei in the other domain of MT. It suggests that lysine replacement might enhance interactions between the α- and β-domains.

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