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Effects of amino acid replacements on cadmium binding of metallothionein α-fragment

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Abstract. To evaluate whether only 20 cysteine residues at invariant positions are needed to bind and coordinate the metals in metallothioneins (MTs), and whether changing the positions of cysteine residues in the sequence affects the metal-binding capacity and the coordination of MTs, we examined the cadmium-binding affinities of seven mutant MTαs using an *Escherichia coli* expression system. Five mutant MTαs in which the constitutive amino acid residues other than cysteines of the α-fragment were replaced with glycine residues, and the remaining two mutant MTαs in which the invariant positions of the cysteine residues of the α-fragment were shifted, were analysed for their ability to be expressed as cadmium-binding forms and for their biochemical properties. The results showed that extreme alteration of the constitutive amino acid residues other than cysteines in the MT α-fragment leads to disruption of their cadmium-binding abilities and of their structure. However, mutant MTαs containing changes of the invariant positions of the cysteine residues were expressed in a cadmium-binding form in *Escherichia coli*, although the invariant positions of 20 cysteine residues in the MTs are thought to be important for their metal-binding abilities. These results suggest that the position of cysteine residues and the chemical nature of the other amino acids in the amino acid sequence of MTs are less critical than expected.
Key words. Cadmium; cysteine; *Escherichia coli*; expression; metal; amino acid replacement; α-fragment; metallothionein.

MTs comprise a family of low molecular weight cysteine-rich metal-binding proteins in a variety of plant and animal species [1]. The major intracellular functions of MTs are the homeostasis of essential heavy metals, such as zinc and copper, and the detoxification of heavy metals, such as cadmium [2, 3]. Most mammalian MTs consist of 61 or 62 amino acid residues, including 20 cysteine residues at invariant positions and highly conserved basic amino acid residues [3]. The mammalian forms of MTs have the characteristic three Cys-Cys, seven Cys-x-Cys and three Cys-x-y-Cys sequences, where x and y are amino acid residues other than cysteine. The conserved cysteine residues bind a series of transition IB and IIB metals. MTs are composed of two metal-thiolate fragments, termed β- and α-fragments. The β-fragment consists of amino acids one to 30 and contains nine cysteine residues that bind to three cadmium and/or zinc ions. The α-fragment consists of amino acids 31 to 61 or 62 and contains 11 cysteine residues that bind four cadmium and/or zinc ions [4]. This has been demonstrated by ^{113}Cd NMR [5, 6], homo- and heteronuclear 2D NMR [7, 8], and X-ray crystallography [9].

Recently, expression systems using *E. coli* and yeast were used to investigate the physiological roles and metal-binding abilities of vertebrate MTs [10, 11, 12, 13], MTs mutated by using site-directed mutagenesis [14, 15, 16, 17, 18, 19, 20], and chimeric constructs [21]. The replacement of all three highly conserved lysine residues with glutamic acid residues in the α-fragment of MTs does not change the overall structure, but it alters the dynamics of metal-binding in both α- and β-fragments [19]. The individual cysteines are critical to metal-binding stoichiometry and each residue differs in its contribution to the biological and physical integrity of MTs [16]. The existence of a wide range of isoforms of MTs, which obviously differ in amino acid composition but retain the same fundamental metal-binding properties, was reported [22]. Nevertheless, differences have also been reported in the strength of metal-binding between rat MT-I and MT-II [23]. However, as far as we know, no reports have defined the importance of the constitutive amino acid residues, other than lysine, serine and cysteine, or of the invariant positions of the 20 cysteine residues to the metal-binding capacity.

In order to determine whether only the 20 cysteine residues at invariant positions are required to bind metals and to coordinate the metals in MTs, and whether changing the positions of the cysteine residues in the MT sequence affect the metal-binding capacity and coordination of MTs, we constructed expression plasmids of five mutants where the constitutive amino

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acid residues other than the cysteine residues were replaced with glycine, and of two mutants in which the invariant positions of the cysteine residues were shifted. In this study, the α-fragment gene was used as a model of MTs to construct mutant MTα expression vectors, because the α-fragment of human MT-2 has been independently expressed as the cadmium-binding form without participation of the β-fragment in *E. coli* [24]. The expression of these seven mutant MTαs as the cadmium-binding form in *E. coli* was examined, and the cadmium-binding ability of these seven mutant MTαs was analysed.

Materials and methods

Materials. *E. coli* strains JM105 and MV1184 were obtained from Pharmacia Biotech and Takara Shuzou, respectively. Plasmids pEXPα for expression of α-fragment of human MT-2 [24] and pEXPMTII for expression of human MT-2 [13] were derivatives of pKK223-3 (Pharmacia Biotech). Sequence vectors, pUC118 and pUC119, and Dye Deoxy Terminator Cycle Sequencing Kit were purchased from Takara Shuzou and Applied Biosystems, respectively. The restriction enzymes, DNA modifying enzymes and cell culture reagents were bought from Toyobo, Takara Shuzou, Boehringer Mannheim or Difco Laboratories. Isopropyl β-D-thiogalactoside (IPTG) used as an inducer of *tac* promoter, Sephadex G-50 and Silver Staining Kit were products of Pharmacia Biotech. Centricon-3 and Centricon-10 were obtained from Grace Japan Company. All other chemicals were analytical grade and available commercially.

Cloning strategy. Two pairs of complementary oligonucleotides used to construct expression plasmids of the mutant MTα fragments A, B, C, D, E, F and G described below were synthesized with a DNA synthesizer, model 391EP (Applied Biosystems), and were then purified with an oligonucleotide purification cartridge (Applied Biosystems). The purified oligonucleotides were phosphorylated at the 5′-ends by polynucleotide kinase and were annealed with the complementary oligonucleotides.

Figure 1 shows the construction of the expression plasmids containing the structural genes of the mutant MTαs. Each 107 bp fragment was inserted into the vector pEXPMTII digested with *Eco*RI and *Hind*III to yield the expression plasmid of each mutant MTα. To ensure the inserted sequences of the mutant MTαs were correct, they were sequenced using a 373A DNA sequencing system (Applied Biosystems).

Mutant MTαs. The characters of the seven mutant MTαs are described as follows (fig. 2A, B):

1. Fragment A: all constitutive amino acids of the α-fragment, other than the cysteine were replaced with glycine.
2. Fragment B: the constitutive amino acids of the α-fragment other than the amino acids containing serine, glutamine and cysteine were replaced with glycine.
3. Fragment C: the constitutive amino acids, other than lysine, aspartic acid and cysteine of the α-fragment, were replaced with glycine.
4. Fragment D: the constitutive amino acids, other than serine, glutamine, lysine, aspartic acid and the cysteines of the α-fragment, were replaced with glycine.
5. Fragment E: the constitutive amino acids, other than the nonpolar amino acids and cysteine of the α-fragment, were replaced with glycine.

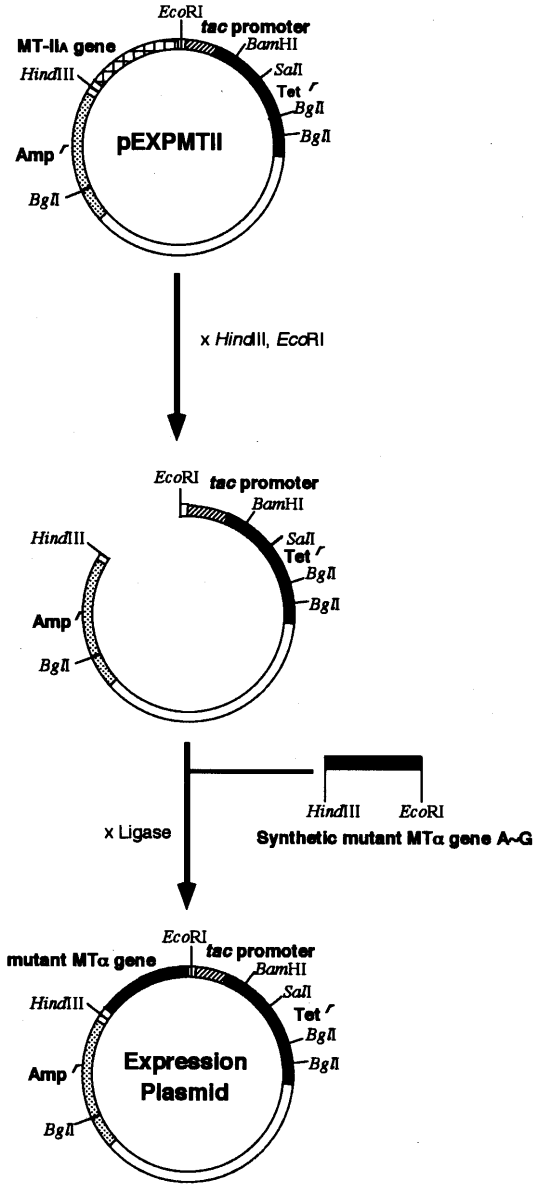

Figure 1. Construction scheme of the expression plasmids. Schematic representation of the cloning strategy. Details of the construction are provided in Materials and methods.

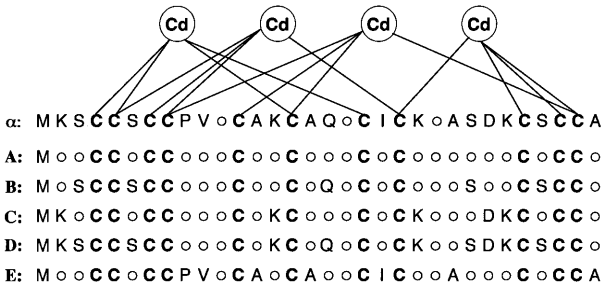

Figure 2A. Amino acid sequences of five mutant MTαs. (A): fragment A, (B): fragment B, (C): fragment C, (D): fragment D, (E): fragment E and (α): α-fragment. Cys is indicated by bold characters and Gly is marked by a circle. Line shows cadmium-thiolate bonds.

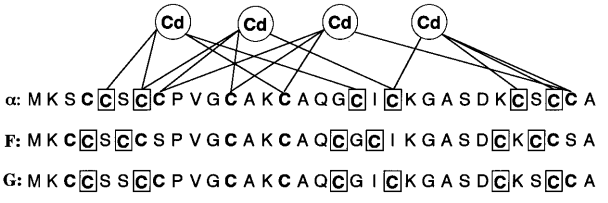

Figure 2B. Amino acid sequences of two mutant MTαs. (F): mutant F, (G): mutant G and (α): α-fragment. Cys is indicated by bold characters and shifted Cys is marked by bold characters in a square. Line shows cadmium-thiolate bonds.

6. Fragment F: three Cys-x-Cys sequences of the α-fragment were shifted one residue in the direction of the N-terminal.
7. Fragment G: all Cys-x-Cys-y sequences of the α-fragment were replaced with Cys-x-y-Cys sequences.

Expression and purification procedures. These mutant MTαs and the α-fragment of human MT-2 were expressed by the method of Odawara et al. [13]. The culture medium contained 30 μg/ml of cadmium. After 2 h of induction of mutant MTα by IPTG, the cultured cells were harvested and then resuspended in 50 mM Tris, 12.5 mM HCl. The cells obtained were disrupted by sonication five times using a Branson Sonifier 250. The sonic extracts were applied to a column (2.6 × 100 cm) of Sephadex G-50 equilibrated with 10 mM Tris, 5 mM HCl. The effluent was collected in 10-ml fractions and measured for cadmium concentration with an atomic absorption spectrophotometer (Hitachi, model 180-30). The fraction containing low molecular weight cadmium-binding protein was filtered using a Centricon-10 at 4600 × g for 40 min. The filtrate was subsequently applied to a Centricon-3 at 7000 × g for 60 min. The concentrated fraction with molecular weight 3000 ~ 5000 Da was washed with 10 mM Tris, 5 mM HCl five times and pooled for further experiments.

SDS polyacrylamide gel electrophoresis. Electrophoresis of each mutant MTα fraction, eluted in the same elution volume as the α-fragment on Sephadex G-50 gel filtration, was performed on a 0.1% SDS and 15% polyacrylamide gel at pH 8.8 with the Tris-tricine buffer system of Schägger and Jagow [25]. The gel was stained with a silver staining kit.

Ultraviolet absorption spectroscopy and amino acid and metal analysis. The ultraviolet absorption spectra of the purified proteins were scanned on a Beckman spectrophotometer, model DU-65, at room temperature using a scan rate of 500 nm/min with a 10 mm quartz cuvette using 100 μl of 10 mM Tris, 5 mM HCl as a blank. Three μl of 1 N HCl were added to both the sample and the blank.

Two hundred pmol of the purified protein was oxidized by the method of Schram et al. [26]. The oxidized samples were dried and then hydrolysed with 6N HCl in vacuo at 110 °C for 24 h. The cysteine and cadmium contents of the purified proteins were determined with a Hitachi 835 amino acid analyzer and a Hitachi flame atomic absorption spectrophotometer, model 180-30, respectively.

The cadmium-binding ratio of the mutant MTαs was calculated from the contents of cadmium and cysteine.

Reaction with DTNB. To study their cadmium-binding strength, the mutant MTαs and the α-fragment were reacted with 5-5′-dithio bis (2-nitrobenzoic acid) (DTNB). The competitive reaction with DTNB was carried out by the method of Emoto et al. [20] with slight modifications. One nmole of the purified mutant MTαs and α-fragment in 100 μl of 100 mM Tris, 50 mM HCl was put into a quartz cuvette. The reaction was started by adding 25 nmol DTNB. Absorbance at 412 nm was recorded on a Beckman DU-65 spectrophotometer at 10 s intervals for 90 min at room temperature, beginning 30 s after adding DTNB. As a blank, distilled water containing 25 nmol of DTNB was used. The pseudo-initial velocity of each mutant MTα was obtained from each calculated inverse number (1/Vt) of the velocity versus the reaction time (t), (min). The pseudo-first order rate constant of each mutant MTα was determined by the method of Cismowski and Huang [15].

Results

Confirmation of the expression vectors. The coding region of each mutant MTα was synthesized on the basis of the DNA sequence of the α-fragment of human MT-2 as described by Karin and Richards [27]. The ATG initiation codon of translation was added to the 5′-end of the nucleotide sequences of the mutant MTαs. Figure 1 shows the scheme of the construction of each expression plasmid containing the coding region of the mutant MTαs.

Plasmids carrying the coding regions for the mutant MTαs were confirmed by gel electrophoresis after digestion with restriction enzymes (data not shown). To ensure that no insertion, deletion, or substitution in the mutant MTαs occurred during their synthesis and con-

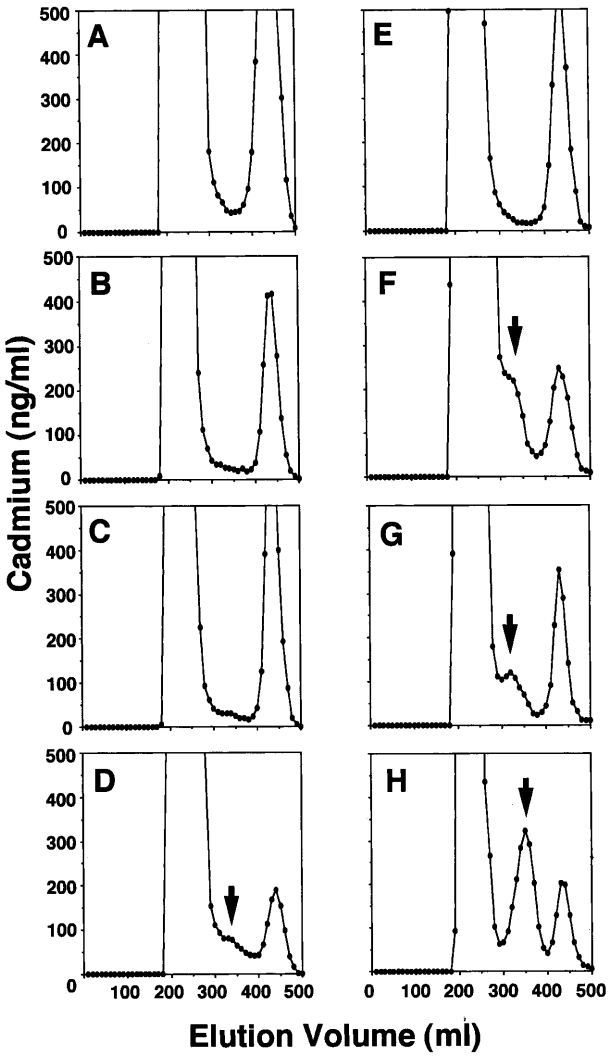

Figure 3. Sephadex G-50 chromatography of seven mutant MTαs and α-fragment. (*A*) mutant A, (*B*): mutant B, (*C*) mutant C, (*D*) mutant D, (*E*) mutant E, (*F*): mutant F, (*G*) mutant G and (*H*): α-fragment. The cadmium contents are indicated by filled circles. Arrow shows the fraction of expressed mutant proteins and the α-fragment.

struction, the entire region extending from the *Eco*RI to the *Hind*III sites of each mutant MTα gene was inserted into the sequence vectors pUC118 and pUC119 and was sequenced. The sequences of these genes were verified.

Expression of mutant MTαs. Figure 3 shows the elution profiles of Sephadex G-50 gel filtration for the sonic extracts from *E. coli* containing the expression plasmid of each mutant MTα or the plasmid pEXPα. The cadmium-binding fragments D, F and G were eluted at the elution volume 300 ml to 400 ml (fig. 3). On the other hand, no cadmium-binding MTα appeared to be produced by fragments A, B, C or E (fig. 3).

To confirm whether each mutant MTα was expressed in *E. coli*, SDS-polyacrylamide gel electrophoresis of the fractions equivalent to the α-fragment was performed (fig. 4). These results showed that the silver staining

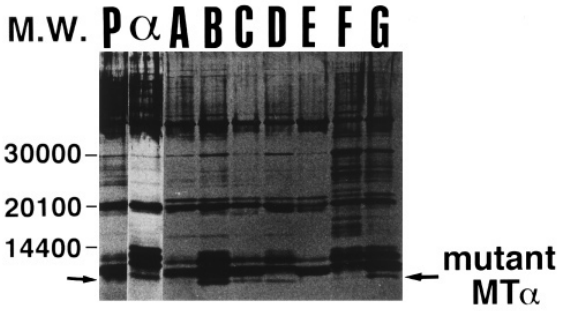

Figure 4. SDS polyacrylamide gel electrophoresis of seven mutant MTαs and α-fragment from the Sephadex G-50 column. Each lane from A to G corresponds to mutant MTαs A to G. α and P represent the α-fragment fraction from Sephadex G-50 column and the fraction having the same Kd value from the Sephadex-G50 column applied with sonic extracts of *E. coli* carrying pKK223-3 as a negative control, respectively. The arrow indicates the position of the expressed protein.

band migrated to the predicted position corresponding to the α-fragment in lanes A to G. The mutant MTαs A, B, C, D, E, F and G were successfully expressed.

To study whether these proteins were obtained as cadmium-binding forms, the ratio of the cadmium content of the fractions containing these proteins was calculated by the following equation:

$$\text{Ratio of cadmium content of each protein} = (a - b)/(c - b)$$

where a: cadmium content of the fractions containing the mutant MTα

b: cadmium content of the fractions at the elution volumes 300 to 400 ml obtained from *E. coli* containing pKK223-3 as a negative control

c: cadmium content of the fractions containing the α-fragment

Table 1 lists the calculated results.

The values of the mutant MTα fragments D, F and G were higher than those of the mutant MTα fragments A, B, C and E. This result demonstrated that mutant MTα fragments A, B, C, and E could barely bind cadmium, although they had the three Cys-x-Cys and three Cys-Cys sequences believed to be important for metal binding.

Characterization of expressed mutant MTαs. The absorption spectra of mutant MTαs at acidic and neutral pH were analysed (data not shown). Each mutant MTα fragment D, F and G had an absorption shoulder at 245-255 nm characteristic of the tetrahedral cadmium-thiolate complex [28]. Direct evidence for cadmium-binding to cysteine was obtained from the absorption spectra of the expressed proteins.

The molar ratios of the cadmium per mutant MTα fragments D, F and G were estimated by calculating the contents of the cadmium from atomic absorption spec-

Table 1. Ratio of cadmium content of mutant MTs.

Mutant MTs	Ratio of cadmium content
A	0.08
B	0.04
C	0.02
D	0.11
E	0.01
F	0.69
G	0.24

Each ratio was calculated from α (=1.00) and pKK223-3 as a control (=0.00).

Table 2. Stoichiometry and kinetic assay of the reaction with DTNB of each protein.

Mutant MTs	Stoichiometry (g atom/molecule)	Initial velocity (n mol/min)	Pseudo first order rate constant
α	3.9	0.76	0.10
D	3.1	7.77	0.16
F	3.7	10.50	0.17
G	3.3	17.21	0.12

trophotometry and of cysteine from amino acid analysis (table 2).

The results showed that the ratio of the mutant MTα fragment F agreed well with that of the α-fragment, but the ratios of the mutant MTα fragments D and G were lower than that of the α-fragment.

Reaction with DTNB. To show whether the expressed mutant MTαs can change the cadmium-binding structure or the cadmium-binding strength, the reactions of mutant MTαs and the α-fragment with DTNB were examined. From the reaction with DTNB, the accessibility of the bulk of the reagent toward sulfhydryls in MT-like proteins can be estimated [15], and hence bound metals can be easily released from the MTs. The reaction rates of the α-fragment and mutant MTαs differed (fig. 5): the rate of the purified α-fragment reacted with DTNB was clearly lower than for the purified mutant MTαs, indicating that the cadmium-binding form of the mutant MTαs was less tight in comparison to the α-fragment. To obtain the initial velocities of the mutant MTαs and the α-fragment , each calculated 1/Vt value versus the reaction time was shown for the reaction period of 0 to 3 min (table 2). The initial velocities of the mutant MTαs with DTNB were approximately 10 to 22-fold higher than for the α-fragment. Under the same conditions, the pseudo-first order rate constants of the mutant MTαs and the α-fragment were estimated from the initial rates of release of the product, thionitrobenzoate, followed by spectrophotometry at 412 nm (table 2). The pseudo-first order rate constant of the α-fragment was approximately 1.2 to 1.7-fold lower than for the mutant MTαs.

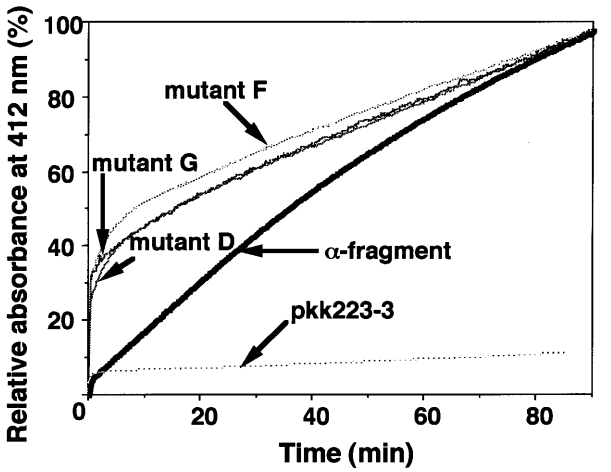

Figure 5. Reactions of each purified mutant protein and an α-fragment with DTNB.

Among the three mutant MTαs, the constant of mutant MTα fragment G was lower than that of the mutant MTα fragments D and F. These results suggest that the mutant MTαs were more unstable than the α-fragment and that fragment G was more stable than the other two mutant MTαs.

Discussion

In this study, the three mutant MTαs, fragment D (where constitutive amino acids other than serine, glutamine, lysine, aspartic acid and cysteine of the α-fragment were replaced with glycines), fragment F (with three Cys-x-Cys sequences of the fragment shifted one residue in the direction of N-terminal), and fragment G (with three Cys-x-Cys-y sequences of the fragment replaced by three Cys-x-y-Cys sequences) were successfully obtained as cadmium-binding forms from *E. coli* carrying the appropriate recombinant gene (fig. 3). On the other hand the four mutant MTαs, fragment A (with all constitutive amino acids of the α-fragment other than cysteine replaced by glycine), fragment B (with constitutive amino acids other than serine, glutamine and cysteine replaced by glycine), fragment C (with constitutive amino acids other than lysine, aspartic acid and cysteine replaced by glycine) and fragment E (with the constitutive amino acid residues other than the nonpolar amino acids and cysteine replaced by glycine) were undetectable as cadmium-binding forms, although the expression of all mutant MTαs was confirmed by gel electrophoresis (table 1, fig. 4). The amount of the expressed MTαs did not depend on their cadmium-binding ability, since electrophoresed fragment B migrated as a band as judged by staining with silver but the fragment B did not bind cadmium. In the purification by gel filtration chromatography the mutant MTα fragments D, F and G were eluted with Kd values of 0.41, 0.38 and 0.38, respectively (fig. 3). The Kd values

were obtained by the method of Winge and Miklossy [29]. The expressed α-fragment was eluted at 0.48 of Kd value. The Kd value of each mutant MTα was lower than that of the α-fragment. The estimated molecular weight of the mutant MTαs seemed to be higher than for the α-fragment. We suggest that the mutant MTαs differ in conformation from the α-fragment due to differences in metal binding.

The absorption spectra of the mutant MTαs are dominated by a transition in the ultraviolet region due to a cadmium-thiolate cluster [28]. The shoulder at 245 to 250 nm was observed in the mutant MTα fragments D, F and G, demonstrating that these three mutant MTαs bound cadmium as a cadmium-thiolate complex.

The stoichiometries of bound cadmium in mutant MTα fragments D, F, G and the α-fragment were calculated from their cadmium and cysteine contents. Nielson and Winge [4] reported that α-fragments from rat MTs generated by in vitro proteolysis bound four atoms of cadmium. As expected, in this study the bound cadmium per α-fragment was approximately four cadmium ions/molecule (table 2). The molar ratios of bound cadmium for the mutant MTα fragments D, F and G were 3.1, 3.7 and 3.3, respectively. The ratio of fragment F agreed well with that of the α-fragment. However, the ratios of fragments D and G were lower than that of the α-fragment. From these results the tetrahedral coordinate structure of fragment F appears to be undisrupted, while that of fragments D and G seems slightly changed.

To check for differences in the cadmium-binding strength or the cluster structure of the mutant MTαs, the α-fragment and mutant MTα fragments D, F and G were submitted to oxidation by the sulfhydryl reagent DTNB (table 2). Li et al. [30] showed that upon addition of an excess of DTNB over MT a biphasic reaction occurred and that the relative contributions of metal-thiolate bond cleavage and ligand accessibility to the overall reaction rate could be distinguished in each phase of the reaction curve, which they attributed to the two-fragment structure, the α- and β-fragments of MTs. Since we used the α-fragment as a model of MTs, this study was also made with an excess of DTNB. Under the conditions used a monophasic reaction curve was obtained, possibly corresponding to the initial reaction rate of the α-fragment, since a major difference was observed between the initial velocities of the α-fragment and the mutant MTαs (table 2). A weaker binding, perhaps resulting from a difference in coordination in the mutant MTα fragments D, F and G, could explain the faster rate in the first stage of the reaction with DTNB. However, the initial velocities of mutant MTαs found in this study were almost the same as those of the authentic MT previously reported [24].

On the other hand, from the analysis of the pseudo-first order rate constant of the mutant MTαs, the rate constant of fragment G coincided with that of the α-fragment and was lower than that of fragment D or F (table 2). This study suggests that compared with that of fragment G the cadmium-binding cluster structure of fragments D and F was partially altered and that the cysteine thiolates within the mutated fragment became more accessible to the reagent. This greater accessibility may be the result of a decreased stability of the cadmium-thiolate bonds. In contrast, the mutant MTα fragment G had a more stable cadmium-binding form than fragments D and F. These results suggest that Cys-x-y-Cys sequences, where x and y are amino acid residues other than cysteine residues, enhance the cadmium-binding abilities and thereby coordinative stability of the fragment structure of MTs.

In this study, the replacement of all constitutive amino acid residues other than cysteine by glycines in the α-fragment of MT cause a decrease in cadmium-binding ability and structural changes. On the other hand, changing the positions of the cysteine residues did not show a major effect on cadmium-binding to the mutant MTα. Moreover, we suggested that the contribution of invariant positions of the cysteine residues in MTs to the cadmium-binding abilities and the structural disruption of MTs was not greater than that of the constitutive amino acids of MTs (table 1). We showed that extreme alterations of constitutive amino acid residues in MTs would be more critical than the shifts in invariant positions of cysteine residues in MTs to the cadmium-binding capacity and maintenance of configuration, although the invariant positions of 20 cysteine residues are thought to be important to the metal-binding abilities of MTs. It appears that the position of cysteine residues, and the choice of the other amino acids in the amino acid sequence of MTs is less critical than expected.

The basic amino acids, e.g. lysine, are highly conserved in MTs. Lysine, which is positively charged at neutral pH, may play a role in neutralizing the excess negativity of the metal thiolate complexes in MTs [31] and in restraining the structural expansion of the MT molecule [32]. The mutant MTα fragment D, with all nonpolar amino acid residues replaced by glycines, retained the ability to bind cadmium. From results obtained with fragment D presented here, two explanations were suggested: (i) the polar amino acid residues, e.g. Asp, Lys, are one of the principal factors of the metal-binding abilities of MTs; (ii) the cadmium-binding affinity of fragment D was due to the fact that fragment D contains fewer amino acid substitutions than the other mutant MTαs. It has been reported that the mutant MT in which all three lysines in the α-fragment were replaced by glutamate binds the seven cadmium ions less firmly than the α-fragment of the wild type MT, leading to the conclusion that these intrafragment lysines are important in maintaining the conformational integrity

of MT [18]. However, the cadmium-binding affinity of this MT mutant was due to only three amino acid substitutions; in contrast, fragment D, retaining its cadmium-binding affinity, contained seven amino acid substitutions.

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