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Comparison of Properties of Two Isometallothioneins in Oxidation and Metal Substitution Reactions

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The reactivity of two isometallothioneins (two isoforms of rat liver zinc- and cadmium-thioneins) was compared *in vitro*. Reactions characteristic of metal-thiol mercaptide bonds were selected as follows; oxidative formation of intramolecular disulfide bonds by 5,5′-dithiobis(2-nitrobenzoic acid), intra- and inter-molecular oxidations by air and ligand substitution reaction with ethylenediaminetetraacetic acid (EDTA). The relative ratio of the two isometallothioneins and the reaction products were determined by high performance liquid chromatograph-atomic absorption spectrophotometry. Metallothionein-I was shown to be more reactive than metallothionein-II in all reactions tested in this study.

Keywords——metallothionein; isometallothionein; oxidation of metallothionein; ligand substitution reaction; 5,5′-dithiobis(2-nitrobenzoic acid); reconstitution of metallothionein; ethylenediaminetetraacetic acid

Two isoforms with the same number of amino acids (61 amino acid residues) and the same distribution of 20 cysteinyl residues along the polypeptide chain but with partially different amino acid compositions other than cysteine have been recognized as common amino acid sequences of mammalian metallothioneins.[1] Although several biological functions such as protection from harmful heavy metals and regulation of essential metals, zinc and copper, have been proposed for metallothionein since the first characterization of the protein,[2] the differences in the biological roles and chemical properties of the two isoforms have not been studied in detail.

Recently we reported the changes in the relative isometallothionein levels during induction and degradation of metallothionein by loadings of zinc, cadmium and copper.[3] Replacement *in vitro* of zinc and cadmium in metallothionein with cupric ion indicated that the reactivity in the replacement reaction is dissimilar between the two isoforms.[4] Winge and Miklossy also reported differences in the reactivities of the two isoforms in the reconstitution reaction of apo-carbonic anhydrase with metals in metallothionein and in the ligand substitution reaction with ethylenediaminetetraacetic acid (EDTA).[5] These observations suggest that the two isoforms have different chemical properties and may have different biological roles.

The present study was performed to clarify the differences in the chemical properties of the two isoforms of metallothionein. The following representative reactions, which seem to be characteristic of mercaptide bonding in metallothionein, were selected; oxidation of the thiol group by substitution with the disulfide reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), oxidation with air, and the ligand substitution reaction with EDTA. Although the reaction of metallothionein with DTNB[6] and the ligand substitution reaction of metallothionein with EDTA[7] have already been reported, the difference in reactivity of the two isoforms was not studied in those experiments.

Recently a radioimmunoassay became available to detect small amounts of metallothionein, especially in circulating fluid and urine.[8] However, the detectability of metallo-

thionein with different oxidation states and/or with different metal compositions has not been studied so far. Therefore, although the present study was performed mainly to compare the reactivity of the mercaptide bond in the two isometallothioneins, we were also interested in the changes in chromatographic behavior induced by oxidation of thiol groups and also by the ligand substitution reaction.

Materials and Methods

Preparation of Zinc- and Cadmium-thioneins——Zinc-thionein was induced in the livers of female rats (Wistar strain, mean body wt. 198 g) by injecting the rats intraperitoneally with zinc acetate once at a dose of 12 mg Zn/kg body weight. The rats were killed after 18 h. The livers were homogenized in 3 volumes of 0.1 M Tris-HCl buffer (pH 7.4)/0.25 M glucose (gassed with N_2 before use) with a Polytron homogenizer (Kinematica) in an atmosphere of N_2 under ice-water cooling. The homogenate was centrifuged at 170000 *g* for 60 min in a Beckman L8-55 ultracentrifuge (Ti 70 rotor; 50000 rev./min; 0—2 °C). A 10 ml portion of the supernatant was applied directly or after mixing with $CdCl_2$ (1000 μg Cd/ml) to a column (2.6 cm × 90 cm; Pharmacia) of Sephadex G-75 that had been pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.6, degassed under reduced pressure at 80 °C before use), and the column was eluted with the same buffer. The metallothionein fractions which contained zinc-thionein or cadmium-thionein at a 1 : 1 ratio of the two isometallothioneins were pooled. The ratio of the two isometallothioneins in each fraction on a Sephadex column was determined by high performance liquid chromatography-atomic absorption spectrophotometry (HPLC-AAS). Replacement of zinc in zinc-thionein with cadmium was confirmed by analyzing zinc in the metallothionein fraction by HPLC-AAS.

Gel Permeation Chromatography on an SW Gel Column——The outlet of an HPLC machine (Toyo Soda HLC 803A) equipped with a gel permeation column (TSKgel 3000 SW, 7.5 mm × 600 mm with a precolumn of 7.5 × 75 mm) was directly connected to a nebulizer tube of a flame AAS (Hitachi 170-50A).[9] The column was eluted with 50 mM Tris-HCl buffer, pH 8.0 at 25 °C (degassed by heating at 80 °C under reduced pressure) at a flow rate of 1.0 ml/min. Molecular absorbances at 254 and 280 nm were continuously monitored in a flow cell with a dual-wavelength ultraviolet (UV) detector (Altex 152).

Results and Discussion

Reaction of Cadmium-thionein with DTNB

Although a large excess of DTNB relative to thiol groups has to be used to quantify the thiol groups and in fact the thiol groups in metallothionein were also determined in the presence of excess DTNB,[6] less than a molar equivalent of DTNB (0.42 molar equivalent to thiol groups in metallothionein) was reacted with cadmium-thionein in the present study to trace the reaction.

The time-course of the reaction was monitored by measuring the absorbance at 412 nm (absorption maximum of 5-thio-2-nitrobenzoate) as shown in Fig. 1. The reaction was over

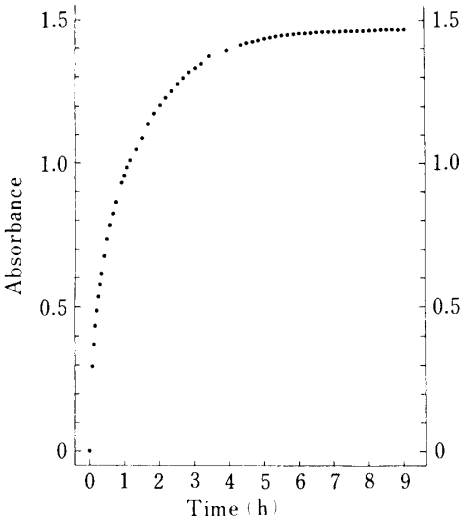

Fig. 1. Time Course of Reaction between Cadmium-thionein and DTNB

A 1.0 ml aliquot of cadmium-thionein solution (6.1 μg Cd in 1.0 ml of 50 mM Tris-HCl buffer, pH 8.6) was mixed in a quartz cell (light path 10 mm) with 6.4 μl of 10 mM DTNB in 0.1 M phosphate buffer (pH 7.0), and allowed to stand in an atmosphere of N_2. The absorbance at 412 nm was determined on a spectrophotometer (Hitachi 220A) using the same mixed solution without cadmium-thionein as a reference solution.

after about 7 h. As the calculated optical density at 412 nm was 0.73 on the assumption of formation of one molar equivalent of 5-thio-2-nitrobenzoate, the result shown in Fig. 1 indicates that two molar equivalents of 5-thio-2-nitrobenzoate were detected after completion of the reaction. This result indicates that not one but two molecules of 5-thio-2-nitrobenzoate were formed from one molecule of DTNB and that intermolecular disulfide bonds between thiol groups of metallothionein and 5-thio-2-nitrobenzoate were not present. The time-course of the reaction also indicates that the reaction was fast and linear up to the formation of one molar equivalent of 5-thio-2-nitrobenzoate.

Distribution profiles of cadmium during the reaction of metallothionein and DTNB changed with time as shown in Fig. 2. The cadmium peak of metallothionein-I decreased faster than that of metallothionein-II and cadmium peaks having larger retention times than the two isometallothioneins appeared. Changes in the distribution profiles of cadmium were apparent at first (during the fast and linear reaction as shown in Fig. 1), but were no longer obvious after about 40 min. Figure 2 also shows that cadmium peaks were not observed at retention times smaller than those observed for the original metallothionein. When 5-thio-2-nitrobenzoate groups are bound to the reacted metallothioneins through disulfide bonds, the reaction products should be bigger in molecular weight and the electric charges of the reaction products seem to be more negative than that of the original metallothionein. The column used in the present study has both gel filtration and cation exchange chromatographic properties under the conditions employed.[9] Therefore, the cadmium eluted more slowly than the original metallothionein is probably not bound to the products of intermolecular disulfide bond formation between metallothionein and 5-thio-2-nitrobenzoate, but bound to intramolecular oxidation products (intramolecular disulfide bond formation).

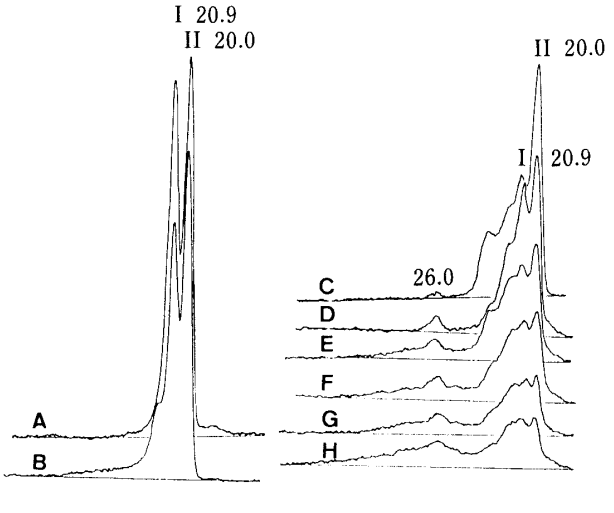

Fig. 2. Change in Distribution Profiles of Cadmium with Time on Reaction between Cadmium-thionein and DTNB

Distribution profiles of cadmium in the mixed solution of cadmium-thionein and DTNB mentioned in Fig. 1 were determined by HPLC-AAS. A 0.1 ml aliquot of the reaction mixture was applied to an SW column. The profile A corresponds to the control (cadmium-thionein before addition of DTNB). The profiles B to H were obtained after mixing cadmium-thionein with DTNB as follows; B (1.5 min), C (6.5 min), D (16 min), E (26 min), F (40 min), G (60 min), and H (100 min). I and II correspond to metallothionein-I and -II, respectively.

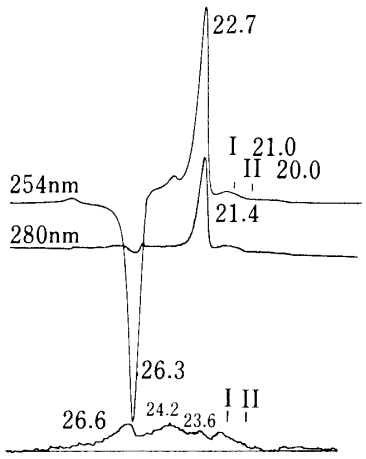

Fig. 3. Distribution Profile of Cadmium 20 h after Mixing Cadmium-thionein with Less than a Half Molar Equivalent of DTNB Relative to Thiol Groups

Cadmium-thionein and DTNB were mixed as indicated in Figs. 1 and 2, and the mixture was allowed to stand for 20 h at room temperature in an atmosphere of N_2. The distribution profile of cadmium was determined by HPLC-AAS. I and II correspond to the retention times of original metallothionein-I and -II, respectively.

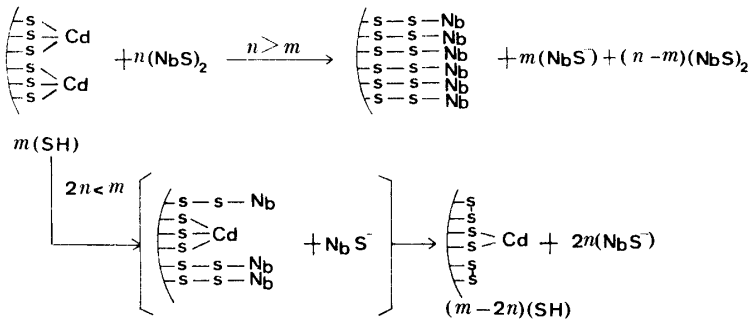

Fig. 4. Schematic Presentation of the Reaction between Cadmium-thionein and DTNB

A solution of cadmium-thionein which contains m mol of thiol groups was assumed to react with n mol of DTNB (represented as $(N_bS)_2$) to give N_bS^- (which shows an absorption maximum at 412 nm) according to the ratios of m and n.

Figure 3 shows the distribution profile of cadmium after completion of the reaction. Cadmium was not found at the retention times of the original metallothionein. The total amount of cadmium found on the chromatogram (peak area) was estimated to be about 16% of the cadmium before addition of DTNB.[9] As 0.42 molar equivalent of DTNB relative to the thiol groups was reacted and two molar equivalents of 5-thio-2-nitrobenzoate were detected, the result indicates that 84% of the thiol groups in metallothionein were oxidized after the completion of the reaction. The amount of cadmium found in the chromatogram was in good agreement with the amount of residual thiol groups in metallothionein calculated on the assumption of 20 thiol groups/7 cadmium atoms in metallothionein.

The reaction of metallothionein with DTNB can be schematically shown as represented in Fig. 4. When an excess amount of DTNB is reacted, the reaction follows the equation for the case of $n>m$. On the other hand, when the amount of DTNB is less than a half molar equivalent, the reaction can be represented by the equation shown for the case of $2n<m$. In the present experiment, 0.42 molar equivalent of DTNB was mixed with metallothionein. Therefore, the reaction can be represented by the latter equation, and the result shown in Fig. 2 can be consistently explained by the latter equation including the slow reaction after liberation of one molar equivalent of 5-thio-2-nitrobenzoate. This result also indicates that intramolecular oxidation products (intramolecular oxidation of thiol groups) can be prepared by mixing metallothionein with less than a half molar equivalent of DTNB relative to the thiol groups.

The reaction of cadmium-thionein with DTNB can be represented as follows: i) metallothionein-I reacts more easily than metallothionein-II, and ii) intramolecular oxidation products are formed when metallothionein is reacted with less than a half molar equivalent of DTNB.

Air–Oxidation of Cadmium- and Zinc-thioneins

The relative reactivity of the two isometallothioneins in the air–oxidation reaction was examined by allowing cadmium- and zinc-thionein to stand for appropriate times either in concentrated or in dilute solution (Figs. 5—7). Metallothionein-I was more easily oxidized than metallothionein-II under all conditions tested in the present experiment. Furthermore, metallothionein was found to be more susceptible to air–oxidation in concentrated than in dilute solution. As air–oxidation products of metallothionein are eluted more slowly than the original metallothionein,[10] cadmium peaks at retention times of 23.3 and 25.8 min were identified as intramolecular oxidation products, while a cadmium peak at a retention time of 18.7 min was identified as being attributable to a metallothionein dimer.

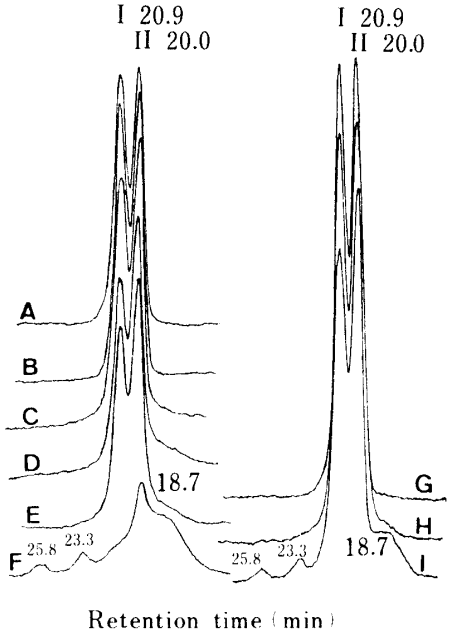

Fig. 5. Air–Oxidation of Cadmium-thionein in Concentrated and Dilute Solutions

Cadmium-thionein was air-oxidized by standing at 24 °C either in concentrated (10 μl of 345 μg Cd/ml) or in dilute (150 μl of 12.2 μg Cd/ml) solution of 10 mM Tris-HCl buffer, pH 8.6. Distribution profiles of cadmium, A to F, and G to I correspond to the oxidation in concentrated and dilute cadmium-thionein solutions, respectively. Each profile was obtained by oxidizing cadmium-thionein for the indicated number of days at 24 °C as follows except for the sample corresponding to profile A which was stored at −20 °C; A (20), B (2), C (3), D (5), E (7), F (20), G (2), H (7) and I (17 d). Metallothionein-I (I) and -II (II) were eluted at 20.9 and 20.0 min, respectively, while intermolecular (metallothionein dimers) and intramolecular oxidation products were eluted at 18.7, 23.3 and 25.8 min, respectively.

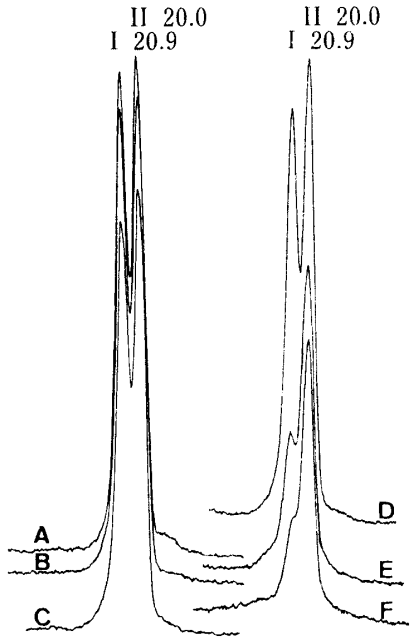

Fig. 6. Air–Oxidation of Zinc-thionein in Concentrated Solution

A 10 μl aliquot of zinc-thionein solution (243 μg Zn/ml of 10 mM Tris-HCl buffer, pH 8.6) was oxidized by allowing it to stand at 24 °C in an air-filled and sealed test tube for the indicated number of days as follows, except for the sample corresponding to profile A, which was stored at −20 °C; A (19), B (2), C (4), D (8), E (15), and F (19 d). Distribution profiles of zinc were obtained by HPLC-AAS. I and II correspond to metallothionein-I and -II, respectively.

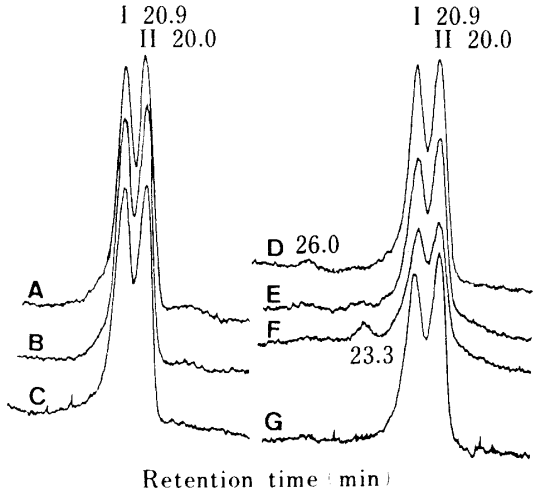

Fig. 7. Air–Oxidation of Zinc-thionein in Dilute Solution

A 150 μl aliquot of zinc-thionein solution (7.6 μg Zn/ml of 10 mM Tris-HCl buffer, pH 8.6) was oxidized by allowing it to stand at 24 °C in an air-filled and sealed test tube for the indicated number of days as follows, except for the sample corresponding to profile G, which was allowed to stand at 24 °C in an N_2-filled and sealed tube; A (1), B (2), C (4), D (6), E (15), F (19) and G (19 d). Distribution profiles of zinc were determined by HPLC-AAS. I and II correspond to metallothionein-I and -II, respectively.

Although air–oxidation products of cadmium-thionein were easily detected as shown in Fig. 5, those of zinc-thionein were usually not observed, and in particular, no peak corresponding to the dimer was detected, as shown in Figs. 6 and 7. The absence of zinc peaks corresponding to the oxidation products of metallothioneins indicates that zinc was liberated

from the original zinc-thionein during the air–oxidation reaction and this is probably due to further oxidation of the partially air–oxidized zinc-thionein.

The results of air–oxidation indicated that i) metallothionein-I is less stable to air–oxidation than metallothionein-II, ii) metallothionein is less susceptible to air–oxidation when the protein is stored in dilute solution, and iii) air–oxidation products of zinc-thionein are less stable than those of cadmium-thionein and the original zinc-thionein.

Ligand Substitution Reaction of Cadmium- and Zinc-thionein with EDTA

The reactivity of the two isometallothioneins was further examined by utilizing the ligand substitution reaction with EDTA as shown in Fig. 8. As the column was eluted with an alkaline buffer, EDTA was eluted faster than expected from its molecular size, and the retention time was close to that of metallothionein-I. Cadmium and zinc bound to metallothionein-I were more easily removed by EDTA than those metals bound to metallothionein-II. The results (shown in Fig. 8) also indicate that zinc can be removed more easily than cadmium by EDTA.

Relative reactivity of the two isometallothioneins in the metal-releasing reaction was also tested by lowering the pH. However, as the relative ratio of the two isometallothioneins had to be determined by eluting the column with alkaline buffer, the metals released from metallothionein at acidic pH were rebound to the apo-protein when the samples were applied to the column. Therefore, the relative reactivity could not be determined. However, the result suggested that the reconstitution of metallothionein from the released metals and apothionein was faster in metallothionein-I than -II (data not shown).

The observations on the substitution reaction with EDTA may be summarized as follows: metallothionein-I is more reactive than metallothionein-II and zinc in metallothionein is less stable than cadmium.

The chemical reactions examined in the present communication are typical of metal-thiol mercaptide bonds. Metallothionein-I was shown to be more reactive than metallothionein-II in all the reactions examined. This is in good agreement with the results reported by Winge and Miklossy as to the reactivities of cadmium,zinc-thionein toward apo-carbonic anhydrase and EDTA.[5] These results suggest that metallothionein-I may be more reactive than metallothionein-II in all reactions in which mercaptide bonds participate.

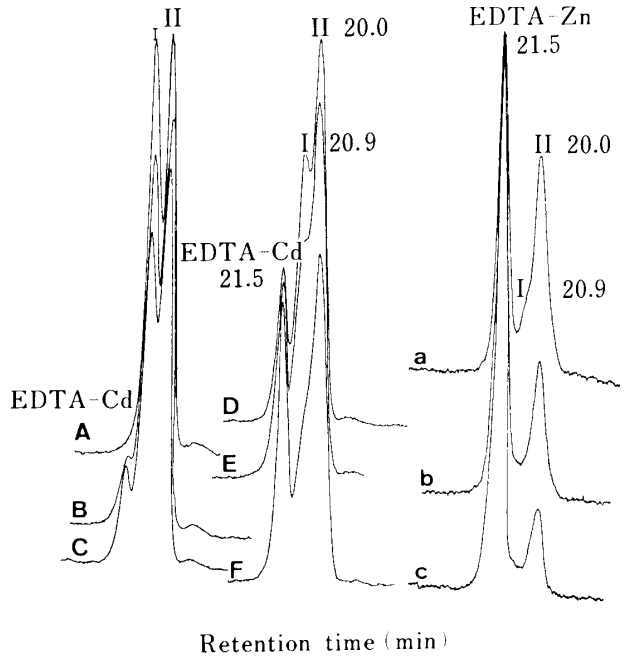

Fig. 8. Ligand Substitution Reaction of Cadmium- and Zinc-thioneins with EDTA

A 92 μl aliquot of cadmium-thionein solution (12.2 μg Cd/ml of 10 mM Tris-HCl buffer, pH 8.6) or an 86 μl aliquot of zinc-thionein solution (7.6 μg Zn/ml of 10 mM Tris-HCl buffer, pH 8.6) was mixed with 0 to 100 μl of 10 mM EDTA, and the mixtures were diluted to 192 or 186 μl with 10 mM Tris-HCl buffer, pH 8.6. The mixed and diluted solutions were allowed to stand for 15 min at 22 °C in an atmosphere of N_2, and then a 100 μl portion was applied to an SW column to determine the distribution profile of cadmium or zinc. Profiles A to F were obtained by mixing cadmium-thionein and EDTA in the following EDTA/Cd ratios; A (10), B (20), C (40), D (60), E (80), and F (100); while profiles a to c were obtained by mixing zinc-thionein and EDTA at EDTA/Zn ratios of a (10), b (40), and c (80). Metallothionein-I and -II, and EDTA–metal complexes were eluted at 20.9, 20.0 and 21.5 min, respectively.

Although the intramolecular oxidation products of the two isometallothioneins were not characterized in detail, they were always eluted more slowly than the original isometallothioneins. This result suggests that the oxidation products are more positively charged than the original isometallothioneins, because the column is negatively charged under the conditions used in the present experiment. Thus, the oxidation products may be different from the original isometallothioneins not only in surface structure due to disulfide bond formation but also in electric charge. Therefore, the oxidation products may be different immunochemically from the original isometallothioneins and it will be important to check the detectability of metallothionein by radioimmunoassay for application to epidemiological and clinical samples.

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