Short Communication

A New Method for Detection of Mercury-Bound Protein with a Combination of Gel Electrophoresis and One Dimensional Synchrotron Radiation X-ray Fluorescence Analysis

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Mercury, a toxic metal, binds readily to sulfhydryl groups of proteins in membranes and enzymes, thereby interfering with membrane structure and function, and with enzyme activity^{1, 2)}. This high affinity of mercury for sulfhydryl groups has been considered as a major factor in the metal and its toxicity. We have recently reported that non-destructive synchrotron radiation X-ray fluorescence (SR-XRF) analysis makes it possible to reveal two-dimensional imaging of mercury in rat brain and kidney after exposure to the metal^{3, 4)}. In this study, with this analysis combined with isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE), we attempted to develop a convenient procedure for detection of mercury bound to protein. Metallothionein (MT) was employed as a model because interaction of mercury with the metalloprotein has been well considered for quantitative analysis of MT⁵⁾.

Experimental Methods

Mercuric chloride and glutathione were obtained from Nacalai Tesque Inc. (Kyoto) and IEF-polyacrylamide gel was obtained from Funakoshi Co. Ltd. (Tokyo). All other chemicals used were of the highest grade commercially available. Purified rat MT-II was generously donated by Dr. C. Tohyama (National Institute for Environmental Study, Tsukuba, Japan).

Reaction mixtures (80 μ l) consisted of 0.15 mM of MT-II, various concentrations of mercury chloride and 50 mM potassium phosphate buffer (pH 7.8)–0.1 mM EDTA in the absence and presence of glutathione. After incubations were carried out for 20 min at 25 °C, each sample was applied onto a slab polyacrylamide gel (9.5 $\times$ 8.8 cm, pH 3.5–

9.5). IEF-PAGE was performed according to the method of Sluyterman and Wijdenes⁶⁾. Isoelectric point (pI) was determined with a calibration kit (pI 3–10, Pharmacia LKB Biotechnology, Inc.).

To detect mercury, the electrophoresed gels were washed with absolute ethanol, followed by drying. Proteins were stained with 0.1% Coomassie Brilliant Blue G-250 (CBB) and then destained with 10% acetic acid–30% ethanol. The resulting gels washed with deionized distilled water were placed on a XY sample stage for SR-XRF analysis. SR-XRF measurements were made at the Photon Factory, National Laboratory for High Energy Physics, Tsukuba, Japan, utilizing an energy dispersive SR-XRF system with monochromatic X-rays obtained by a Si (111) double crystal monochromator. The experimental procedure was based on that described by Shimojo *et al.*³⁾. XRF line analysis of the sample was accomplished in the following manner: Monochromatic X-rays (energy, 16 keV; beam size, 3 mm width, 0.3 mm high) was irradiated on a sample, which was step-scanned (0.3 mm/step) vertically direction and X-ray fluorescence intensity was measured with a Si (Li) solid state detector (counting times, 10 sec/point). The X-ray intensity data for the Hg *L α* fluorescence lines of each point were processed with a personal computer and the results were shown as a plot of normalized data which were calculated by converting the maximum X-ray intensity value to 1,000.

Results and Discussion

Figures 1 and 2 show the results of IEF-PAGE of MT-II in the absence and presence of mercuric chloride and their SR-XRF line analysis. The metalloprotein exhibited a broad pI ranging from 3.7 to 4.7. Interaction of MT-II with relatively small amounts of mercury chloride (equivalent to 0.2 and 2 molar) caused little migration of the pI although appreciable binding of mercury to the protein was detected by SR-XRF. It was confirmed that the CBB staining had no effect on the SR-XRF analysis (data not shown). In contrast, incubation of MT-II with 20 molar equivalent of mercury resulted in a drastic change in its native pI to alkaline pI 9.4. This is not a surprising result because marked migration of pI for MT-II bound to steady amount of mercuric ion has been reported⁷⁾. The migrated band corresponds well with the peak of X-ray intensity data for mercury as shown in Figs. 1d and 2d. The concentration of the metal in the mercury-positive area, where mercury is bound to MT-II on IEF-PAGE, was estimated according to the method of Jacobs *et al.*⁸⁾ to be 0.1 μ g. The change in pI for MT-II by mercury was, however, blocked by pre-

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Fig. 1. IEF-PAGE of rat MT-II interacted with different concentrations of mercuric chloride. Each MT solution containing 12 nmol protein was mixed with 0, 0.2, 2, or 20 molar equivalent of mercury, and they were incubated at 25°C for 24 hr. IEF gel of rat a) MT-II (3 nmol), b) MT-II with 0.2 molar equivalent of mercury, c) with 2 molar equivalent of mercury, and d) with 20 molar equivalent mercury.

Fig. 2. SR-XRF line analysis of rat MT-II interacted with different concentrations of mercuric chloride. Incubations were performed under conditions described in Fig. 1. Plots of HgLa X-ray intensity data for rat a) MT-II, b) MT-II with 0.2 molar equivalent of mercury, c) with 2 molar equivalent of mercury, and d) with 20 molar equivalent mercury.

treatment with glutathione (data not shown), suggesting that such structural changes in the protein are due to modification of the heavy metal.

The present procedure enables us to clarify the

surface structure change in protein by IEF-PAGE and mercury binding by SR-XRF for the same sample. Since an excess of mercury unreacted is successfully removed by IEF-PAGE, electrophoretic alteration in MT-II during interaction of the metalloprotein with mercury appears to be attributable to covalent binding of the heavy metal. And SR-XRF analysis allowed us to analyze the metal on gel directly without destruction, use of radio-labeled compounds and complicated pretreatment. This method will, therefore, become a powerful technique for the better understanding of the interaction between toxic metals and proteins.

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