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Equimolar Hg-Se Complex Binds to Selenoprotein P

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Binding of equimolar mercury (Hg) and selenium (Se) to a specific plasma protein in the detoxification of Hg was studied in vitro by the HPLC/inductively coupled argon plasma-mass spectrometry (ICP-MS) method with use of an enriched stable isotope. Hg and ^{82}Se became co-eluted with endogenous ^{78}Se on a size exclusion column by incubation of 0-200 μM $HgCl_2$ and ^{82}Se-enriched selenite with rat serum in the presence of glutathione at 37°C for 10 min. The endogenous ^{78}Se peak was the most abundant plasma Se-containing protein, and it showed the affinity to heparin, indicating it to be selenoprotein P (Sel P). The ^{82}Se/endogenous Se ratio of (Hg-Se)-Sel P complex changed with doses of $HgCl_2$ and ^{82}Se-enriched selenite and amounted to more than 100, suggesting that more than 1,000 units of (Hg-Se) bind to Sel P based on the fact that there are 10 selenocysteinyl residues per Sel P. These results indicate that equimolar Hg and Se bind to Sel P to form the $\{(Hg\text{-}Se)_n\}_m$-Sel P complex, where n is the number of Hg-Se complexes and m the number of binding sites in Sel P.

Accumulation of mercury (Hg) in tissues of marine mammals (1) and mercury mine workers (2) is known to be accompanied by the co-incidental accumulation of equimolar selenium (Se). Toxicity of each element is reduced by their co-administration known as "interaction" (3). Se in selenite reacts with Hg in the bloodstream after being processed in red blood cells to form a stable complex with a specific plasma protein. The resulting complex contains the two elements at an equimolar ratio, and has been assumed to play a role in preventing the toxicity of Hg (4, 5). Our previous study suggested the formation of the equimolar $(Hg\text{-}Se)_n$ complex first, followed by binding to a specific plasma protein (6).

Se is an essential element which mainly functions in the body through selenoproteins such as glutathione peroxidase (7). Selenoprotein P (Sel P) is the major plasma selenoprotein containing approximately 65% of the plasma Se (8). Sel P contains 10 selenocysteinyl residues (9) and has an affinity to heparin (10). This protein is presumed to have a protective role against oxidative injury to the liver plasma membrane (11).

In the present study, the HPLC/inductively coupled argon plasma - mass spectrometry (ICP-MS) method was applied to determine the mechanism leading to the formation of the complex containing equimolar Hg and Se with a specific plasma protein using an enriched stable isotope. The result suggested that this specific plasma protein is Sel P, and the (Hg-Se)-Sel P complex is formed in the bloodstream by the co-administration of these elements. Effective use of HPLC/ICP-MS was further demonstrated in estimating the number of Hg and Se atoms bound to Sel P.

MATERIALS AND METHODS

Chemicals. ^{82}Se-enriched sodium selenite ($Na_2{}^{82}SeO_3$) was prepared by oxidation of the enriched metal (97.02 %) (Oak Ridge National Laboratory, Oak Ridge, TN, USA) (12). $Na_2{}^{82}SeO_3$ and mercuric chloride ($HgCl_2$) (natural abundance) were dissolved in Dulbecco's phosphate buffered saline (PBS).

Animals. Male Wistar rats (Clea Japan Co., Tokyo, Japan) weighing 350 - 400 g were maintained in an air-conditioned room (22 ± 2°C; relative humidity, 50%) and given a commercial diet (CE-2, Clea Japan Co.) and tap water *ad libitum*.

In vitro formation of the complex. Blood was collected from the abdominal aorta under light ether anesthesia, and centrifuged at 12,000 *g* for 10 min to separate serum. $Na_2{}^{82}SeO_3$ and/or $HgCl_2$ were added to the serum in the presence of glutathione (GSH, 2 mM), and each mixture was incubated at 37°C for 10 min.

Removal of heparin-binding proteins. A 3 ml aliquot of serum was incubated with 0.75 g (dry weight) of heparin-Sepharose resins (Pharmacia, Piscataway, NJ) at room temperature for 30 min to remove heparin-binding proteins, and centrifuged at 400 *g* for 1 min to separate the supernatant. Then, the resins were washed three times with 50 mM Tris-HCl (pH 7.4), and mixed with the same buffer containing 1 M NaCl at room temperature for 30 min to desorb heparin-binding proteins from the resins. The solutions removed of and containing heparin-binding proteins were reacted with the same mixture of $Na_2{}^{82}SeO_3$, $HgCl_2$ and GSH as described above.

Partial purification of selenoprotein P. A 0.2 ml aliquot of serum was applied to an Asahipak GS 520 column (7.5 × 500 mm, Showa

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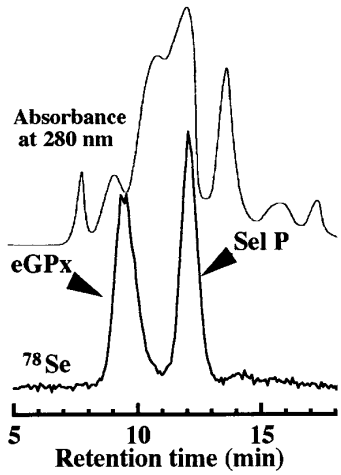

FIG. 1. Distribution profile of endogenous ^{78}Se in rat serum determined by HPLC/ICP-MS. A 0.1 ml portion of rat serum was applied to an Asahipak GS 520 column and the distribution profile of ^{78}Se was determined by HPLC/ICP-MS. The profile of the absorbance at 280 nm was also determined. Abbreviations: extracellular glutathione peroxidase (eGPx) and selenoprotein P (Sel P).

Denko, Tokyo), and the column was eluted with 50 mM Tris-HCl (pH 7.4) at a flow rate of 1 ml/min. As selenoprotein P was eluted at a retention time of about 12 min, the fraction with retention times ranging from 11.6-12.4 min was collected. This procedure was repeated ten times, and a 7.2 ml portion of the combined fractions was loaded on a heparin-affinity column (7.5 × 150 mm). The column was washed with 50 mM Tris-HCl (pH 7.4) at a flow rate of 0.5 ml/min, and then eluted with the same buffer containing 0.5 M NaCl.

HPLC/ICP-MS. A 0.1 or 0.2 ml aliquot of each sample was applied to a GS 520 column, and the column was eluted with 50 mM Tris-HCl buffer (pH 7.4) at a flow rate of 1 ml/min. The eluate was introduced directly into the nebulizer tube of an ICP-MS (PMS 2000, Yokogawa Analytical Systems, Musashino, Japan) to detect Se (m/z 78 and 82) and Hg (m/z 202), and the distributions of endogenous ^{78}Se, and exogenous ^{82}Se and Hg were determined. The relative natural abundances of ^{78}Se, ^{82}Se and ^{202}Hg are 23.8, 8.7 and 29.9 %, respectively. Profiles of ^{82}Se/endogenous Se ratios were obtained as reported previously (12).

RESULTS

Two major Se peaks were observed in rat serum in the distribution of endogenous ^{78}Se by HPLC/ICP-MS (Fig. 1), the faster eluting peak being designated as extracellular glutathione peroxidase (eGPx), and the slower one as Sel P (13). Figs. 2a and 2b show the distributions of endogenous ^{78}Se, and exogenous ^{82}Se and Hg in serum after the addition of either ^{82}Se-enriched selenite or Hg^{2+} in the presence of GSH, respectively. ^{82}Se mainly distributed to albumin at a retention time of about 14.5 min as shown in Fig. 2a (13), while Hg was eluted at 15.2 min by forming a complex with GSH. Figs. 2c and 2d show the distributions of the three elements in serum after the addition of 40 and 120 μM of both ^{82}Se and Hg in the presence of GSH, respectively. GSH was added to reduce selenite, because the complex containing Se and Hg is formed after the interaction between Hg and a reduced metabolite of selenite (16). Although no change in the distribution of ^{78}Se was observed after the addition of either one of ^{82}Se or Hg, the distribution of one of the two ^{78}Se peaks, namely Sel P, was altered by the simultaneous addition of both ^{82}Se-enriched selenite and Hg, and the peaks of ^{82}Se and Hg became co-eluted by maintaining the equimolar ratio together with the altered ^{78}Se peak at 10.9 min. Intensities of the three peaks in the new complex were dependent on the doses of ^{82}Se and Hg. These results suggest that Hg and Se bind as a $(Hg\text{-}Se)_n$ complex to Sel P to form the $(Hg\text{-}Se)_n$-Sel P complex.

One of the ^{78}Se peaks, Sel P, completely disappeared by the removal of heparin-binding proteins from rat serum (Fig. 3a), and the specific binding of ^{82}Se and Hg to Sel P was no longer observed when the serum was reacted after the removal of heparin-binding proteins

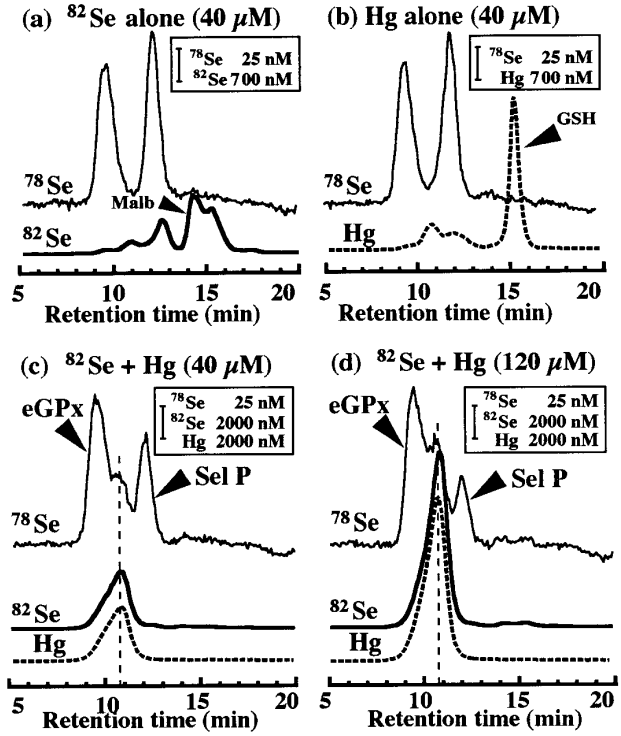

FIG. 2. Distribution profiles of endogenous ^{78}Se and exogenous ^{82}Se and/or Hg reacted with rat serum in the presence of glutathione. Rat serum was reacted with (a) ^{82}Se-enriched selenite alone, (b) $HgCl_2$ alone, (c) ^{82}Se-enriched selenite + $HgCl_2$ (40 μM), or (d) ^{82}Se-enriched selenite + $HgCl_2$ (120 μM) in the presence of GSH (2 mM) at 37°C for 10 min. A 0.1 ml portion of each sample was applied to an Asahipak GS 520 column and the distribution profiles of ^{78}Se and ^{82}Se and/or Hg were determined by HPLC/ICP-MS. Abbreviations: mercaptoalbumin (Malb), glutathione (GSH), extracellular glutathione peroxidase (eGPx), and selenoprotein P (Sel P). The vertical bars denote the calibration equivalence for each element with the indicated values.

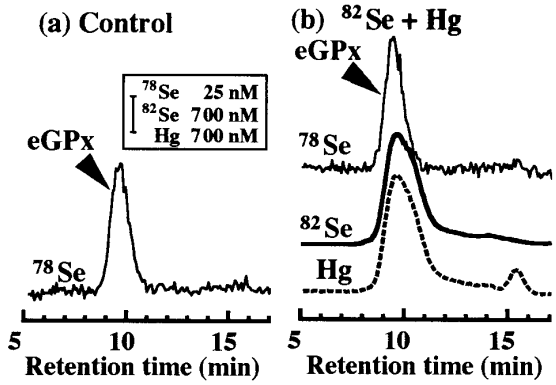

FIG. 3. Effects of removal of heparin-binding proteins on the distribution of Hg and Se. Heparin-binding proteins in rat serum were removed by incubation with heparin-Sepharose resins at room temperature for 30 min (a). 40 μM ^{82}Se-enriched selenite and $HgCl_2$ was added to the serum in the presence of GSH (2 mM), and the mixture was incubated at 37°C for 10 min (b). A 0.1 ml portion of each sample was applied to an Asahipak GS 520 column and the distribution profiles of ^{78}Se ^{82}Se and Hg were determined by HPLC/ICP-MS. Abbreviation: extracellular glutathione peroxidase (eGPx). The vertical bar denotes the calibration equivalence for each element with the indicated value.

with a mixture of ^{82}Se-enriched selenite, Hg^{2+} and GSH. Instead, ^{82}Se and Hg were eluted diffusely at different retention times (Fig. 3b), indicating that ^{82}Se and Hg were bound to other plasma proteins in the absence of heparin-binding proteins.

In the fraction containing only heparin-binding proteins, only the ^{82}Se peak of Sel P was observed, while eGPx was not (Fig. 4a). Changes in the profile of the ratio of exogenous ^{82}Se to endogenous Se, in addition to the distributions of endogenous ^{78}Se, exogenous ^{82}Se and Hg after additions of different doses of equimolar ^{82}Se-enriched selenite and Hg^{2+} to the fraction, are shown in Figs. 4b-4e. The complex containing ^{82}Se and Hg was eluted at the same retention time as that formed in whole serum (Figs. 2c, 2d), and a dose-dependent shift was observed for the ^{78}Se peak of Sel P. The $(Hg\text{-}Se)_n$-Sel P complex was eluted slightly faster at doses of 100 (Fig. 4d) and 200 μM (Fig. 4e) of ^{82}Se-enriched selenite and Hg. The ratio of ^{82}Se/endogenous Se at the peak top was plotted against the doses of ^{82}Se-enriched selenite and Hg in Fig. 5. The ratio increased with doses of the two elements, indicating that the amount of the (Hg-Se) complex bound to one molecule of Sel P also increased with their doses. A similar change in the ratio was also observed for the shoulder peak in Fig. 4.

Heparin-binding proteins eluted at the retention time ranging from 11.6 - 12.4 min in rat serum were separated as described in "Materials and Methods". Sel P occupies approximately 0.3 % of total proteins in this fraction. Chromatograms of absorbance at 280 nm and endogenous ^{78}Se in this fraction are shown in Fig. 6a. By the addition of ^{82}Se-enriched selenite and Hg^{2+}, while the ^{78}Se peak of Sel P was completely shifted, little change was observed in the distribution of UV absorbance (Fig. 6b). This result indicates that the binding of Sel P is specific for $(Hg\text{-}Se)_n$ among heparin-binding proteins.

DISCUSSION

We have applied the HPLC/ICP-MS method as a means of speciating Se in biological materials (13-15). Effective use of an enriched stable isotope was also demonstrated by use of enriched ^{82}Se as a tracer (13), and this at the same time enhanced the detectability. Thus, simultaneous speciation of endogenous and exogenous Se has been demonstrated in both metabolic and mechanistic studies. With this new methodology, it was revealed that exogenous Se administered as ^{82}Se-enriched selenite binds to Sel P together with Hg in a form of $(Hg\text{-}Se)_n$ by maintaining a molar ratio of ^{82}Se/Hg = 1.

Our previous study (6) revealed that Hg reacts firstly with a reduced form of selenite at an equimolar ratio to form the $(Hg\text{-}Se)_n$ complex followed by its binding to the specific plasma protein showing an extremely high affinity and capacity to the $(Hg\text{-}Se)_n$ complex. The present study suggested that this specific protein is Sel P.

Changes in the ratio of exogenous ^{82}Se/endogenous Se in Sel P (Figs. 4 and 5) indicate that the amount of (Hg-Se) to bind to a Sel P molecule increases with doses of the two elements added to the reaction mixture, and more than 1,000 units of (Hg-Se) bind to Sel P in serum in the presence of 200 μM of the two elements, based on 10 selenocysteinyl residues existing in Sel P (9). Gasiewiz and Smith (17) presumed that a plasma protein solubilizes insoluble colloidal Cd-Se complex, that is, $(Cd\text{-}Se)_n$, as the stability of $(Cd\text{-}Se)_n$ in plasma depends upon the integrity of the native protein components. In this context, Sel P may specifically solubilize the insoluble colloidal $(Hg\text{-}Se)_n$ complex, and this may explain the quantitative relationship between Sel P and the two elements in the present study.

Rat Sel P is a monomer consisting of 366 amino acid residues and is rich in selenocysteinyl (10 residues) and cysteinyl residues (17 residues) (9). Another feature in the deduced amino acid sequence is that Sel P is rich in basic amino acid residues (17.2 %) including two histidine-rich regions (9). In the present study, it was not revealed which residues or regions participate in the formation of the $(Hg\text{-}Se)_n$-Sel P complex. However, formation of the complex was inhibited in the presence of poly-cationic amino acids (6), suggesting that histidine-rich regions in Sel P, at least, may take part in binding of $(Hg\text{-}Se)_n$. We propose that the formation the $\{(Hg\text{-}Se)_n\}_m$-Sel P complex (m = number of binding sites

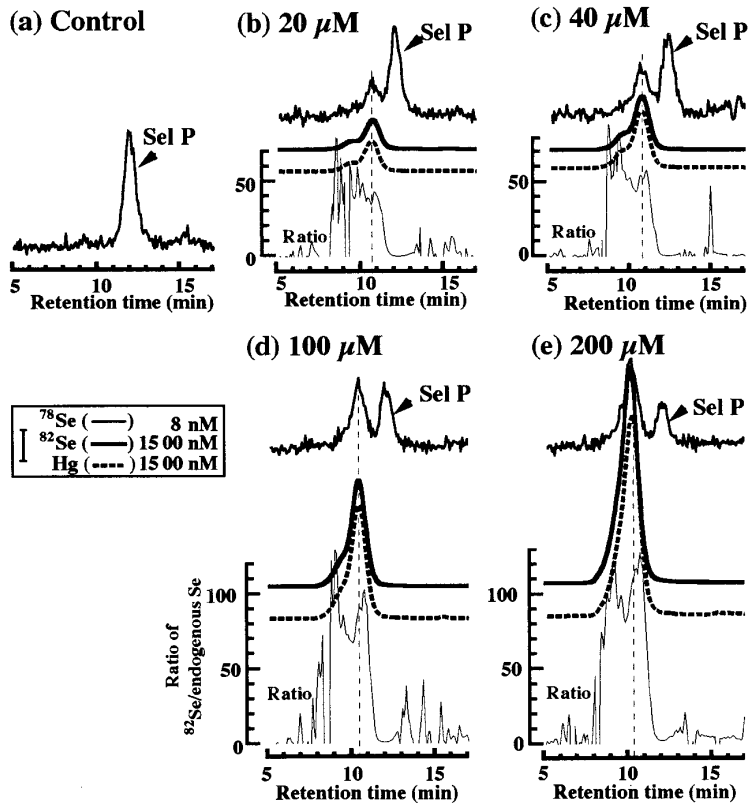

FIG. 4. Dose-related changes in the distributions of endogenous ^{78}Se, exogenous ^{82}Se and Hg, and ratios of ^{82}Se/endogenous Se in reaction mixtures. Heparin-binding proteins in rat serum were separated as described in "Materials and Methods" and reacted with (a) 0, (b) 20, (c) 40, (d) 100, and (e) 200 μM ^{82}Se-enriched selenite and $HgCl_2$ in the presence of GSH (2 mM). A 0.1 ml portion of each sample was applied to an Asahipak GS 520 column and the distribution profiles of ^{78}Se ^{82}Se and Hg were determined by HPLC/ICP-MS. Abbreviation: selenoprotein P (Sel P). The vertical bar denotes the calibration equivalence for each element with the indicated values. The ratios of ^{82}Se/endogenous Se were also plotted against retention time.

in Sel P) is a specific product in serum by the interaction of Hg, Se and the specific plasma protein.

In the present communication, we proposed the hypothesis that Sel P may play an important role in the

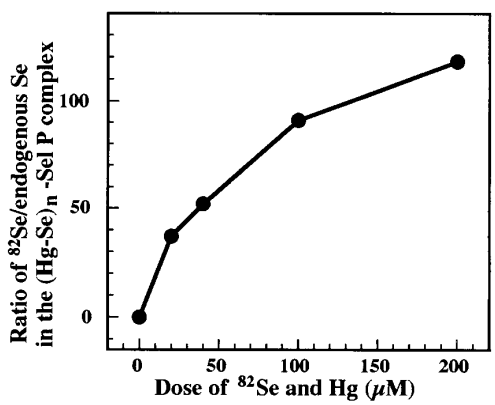

FIG. 5. Dose-related changes in the ratio of ^{82}Se/endogenous Se of the $(Hg\text{-}Se)_n$-Sel P complex. The ratios of ^{82}Se/endogenous Se in the $(Hg\text{-}Se)_n$-Sel P complex in Fig. 4 (at the peak top) were plotted against the doses of ^{82}Se-enriched selenite and $HgCl_2$.

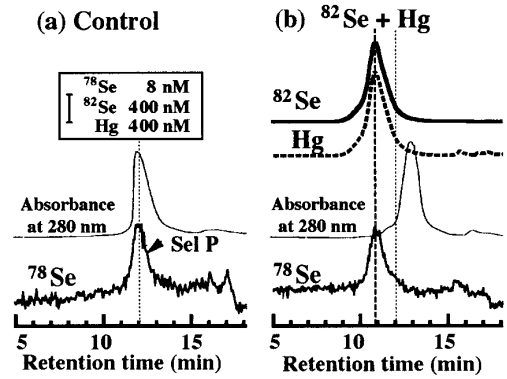

FIG. 6. Effects of formation of the $(Hg\text{-}Se)_n$-Sel P complex on the distributions of absorbance at 280 nm and endogenous ^{78}Se. Sel P was partially purified as described in "Materials and Methods" (a) and reacted with 10 μM ^{82}Se-enriched selenite and $HgCl_2$ in the presence of GSH (2 mM) at 37°C for 10 min (b). A 0.2 ml portion of the sample was applied to an Asahipak GS 520 column and the distribution profiles of ^{78}Se and ^{82}Se and Hg were determined by HPLC/ICP-MS. The profiles of the absorbance at 280 nm were also determined. Abbreviation: selenoprotein P (Sel P). The vertical bar denotes the calibration equivalence for each element with the indicated values.

detoxification of heavy metals such as Hg in plasma by using other Se than those in this protein. Metallothionein is a cytosolic protein in many tissues and is well known to detoxify heavy metals by sequestering these metals as stable complexes (18). On the other hand, Sel P may detoxify these metals by sequestering in the bloodstream. However, as a reduced metabolite of Se is essential for Sel P to sequester selectively, and because it is supplied only in small quantities under normal nutritional conditions, Sel P may be effective primarily against chronic toxicity by consecutive exposure to a low dose of heavy metals rather than to a higher dose resulting in acute exposure. This hypothesis may explain the accumulation of Hg together with Se in tissues of marine mammals (1) and mercury mine workers (2).

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