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A streptavidin–metallothionein chimera that allows specific labeling of biological materials with many different heavy metal ions

(fusion protein/biotin-binding protein/metal-binding protein/genetic engineering)

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ABSTRACT **We have designed a streptavidin–metallothionein chimeric protein in which the streptavidin moiety provides a means of binding the metallothionein moiety tightly to specific biological targets. A gene fusion of streptavidin with mouse metallothionein I was efficiently expressed in *Escherichia coli*, and the expressed chimeric protein was purified to homogeneity by a simple procedure. The purified chimera, consisting of four identical subunits, bound one biotin and approximately seven Cd^{2+} ions per subunit (19.5 kDa). This indicates that both the streptavidin and the metallothionein moieties are fully functional. The high binding affinity of the chimera both for biotin and for heavy metal ions allows the specific labeling or conjugation of any biological material containing unhindered biotin with a variety of different heavy metal ions and their isotopes, thereby opening the way for simultaneous assay systems for a large number of biological targets.**

Metallothionein is a small cysteine-rich protein that is found in a broad range of eukaryotic species and in many different tissues (1, 2). This protein binds a variety of heavy metal ions with extremely high affinity through coordination bonds to its cysteine residues (1, 2). The heavy metals that can be bound by metallothionein include Cd, Zn, Cu, Hg, Co, Pb, Ni, Fe, Bi, Sn, Tc, Au, and Ag (2). Since various sensitive detection methods are available for heavy metals, such as x-ray fluorometry, polarography, atomic absorption spectrometry, mass spectrometry, anodic stripping voltammetry, inductively coupled plasma emission spectrometry, and NMR (3–9), as well as for various radioisotope species, bound heavy metal ions to metallothionein should be detected with high sensitivity by such methods. Many useful assay systems for specific biological targets could be developed if there were a way to attach metallothionein specifically to these targets. To provide metallothionein with the potential for labeling or conjugating biological materials with various heavy metal ions and their isotopes, we have designed a streptavidin–metallothionein chimeric protein.

Streptavidin, a protein produced by *Streptomyces avidinii*, specifically binds the water-soluble vitamin D-biotin with remarkably high affinity (10–12). The tight and specific binding affinity of streptavidin for biotin and the potential ability of biotin to be easily incorporated into various biological materials have made the streptavidin–biotin system a useful tool for detection and characterization of such materials (13–18). We have developed an expression system for the cloned streptavidin gene, which efficiently produces streptavidin in *Escherichia coli* (19). The establishment of the expression system allowed the design and preparation of streptavidin-containing chimeric proteins. More recently, we have designed a streptavidin–protein A chimera (20) in which the streptavidin moiety provides antibody molecules bound to the protein A moiety with recognition capability in addition to their natural antigen recognition—i.e., binding to biotin, biotin derivatives, and biotinylated biological materials. This work showed that streptavidin-containing chimeric proteins are capable of retaining both the biotin-binding activity of the streptavidin and the ligand-binding activity of the proteins fused to it.

Here we describe the design and expression of a streptavidin–metallothionein chimeric protein. We have expressed the chimera in *E. coli* and purified it to homogeneity. The purified chimera was able to label biological materials containing unhindered biotin specifically with heavy metal ions. These results imply that the chimera has the potential to serve as a tool for tagging a wide variety of biological materials with many different heavy metal ions and their isotopes.

MATERIALS AND METHODS

Materials. L-[^{35}S]Cysteine, D-[*carbonyl*-^{14}C]biotin, and $^{109}CdCl_2$ were obtained from Amersham. 2-Iminobiotin-agarose was from Sigma. Sephacryl S-300HR, PD-10 columns, and molecular mass standard proteins for SDS/PAGE and gel filtration chromatography were from Pharmacia LKB. Chelex 100 and prestained molecular mass standard proteins for SDS/PAGE were from Bio-Rad. Biotinylated horseradish peroxidase was from Boehringer Mannheim. Other reagents were analytical grade.

Construction of an Expression Vector. An expression vector was constructed by inserting a mouse metallothionein I cDNA (a gift from R. D. Palmiter, University of Washington) (21) into an expression vector for streptavidin-containing chimeric proteins, pTSA-18F (22). A 300-base-pair *Bgl* I–*Bam*HI fragment of the mouse metallothionein I cDNA that carries the entire coding sequence was cloned into the *Bam*HI site of pTSA-18F. The clone, in which the metallothionein gene has the same orientation as the streptavidin gene, was used as the expression vector. The resulting expression vector pTSAMT-2 (Fig. 1) encodes a 19.5-kDa protein in which the metallothionein moiety follows the C terminus of streptavidin with 10 additional amino acid residues between the two moieties.

Expression of a Streptavidin–Metallothionein Chimeric Protein. Expression of the gene fusion of streptavidin with metallothionein was carried out according to the method previously described (19, 20, 22). Lysogen BL21(DE3)(pLysE) (23, 24) carrying the expression vector pTSAMT-2 was grown at 37°C with shaking in M9 minimal medium (25) supplemented with 1 mM $MgSO_4$, 0.2% glucose, 1.5 μM thiamin, 0.5% Casamino acids (Difco), 8.2 μM biotin, 150 μg of ampicillin per ml, and 25 μg of chloramphenicol per ml. When the OD_{600} of the culture reached 0.6, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM to induce the T7

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Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TBS, Tris-buffered saline.

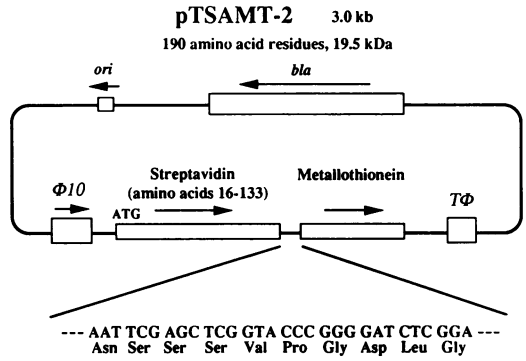

FIG. 1. Expression vector for a streptavidin–metallothionein chimeric protein. A 300-base-pair *Bgl* I–*Bam*HI fragment carrying the entire coding region of the mouse metallothionein I cDNA (21) was cloned into the *Bam*HI site of pTSA-18F (22). The coding sequence is flanked by the bacteriophage T7 *Φ10* promoter (23, 24) and the TΦ transcription terminator (23). *bla*, β-Lactamase gene; *ori*, replication origin; kb, kilobase pairs.

RNA polymerase gene. After the induction, the cells were incubated at 37°C with shaking.

For pulse-labeling of expressed proteins with [^{35}S]cysteine, the culture medium contained, instead of Casamino acids, each natural amino acid at 40 μg/ml except for cysteine. Pulse labeling was carried out by incubating a 1.0-ml culture with 20 μCi of [^{35}S]cysteine (>600 Ci/mmol; 1 Ci = 37 GBq) at 37°C for 10 min. Total cell protein from 167 μl of culture was subjected to SDS/PAGE analysis. The gel was immersed in 20% methanol/10% acetic acid for 20 min and dried under reduced pressure. The dried gel was exposed to Kodak XAR-5 film.

Purification of a Streptavidin–Metallothionein Chimeric Protein. Purification of the expressed streptavidin–metallothionein chimeric protein was carried out at 4°C or on ice, unless otherwise stated. We used BL21(DE3)(pLysE)(pTSAMT-2) incubated for 5 hr after induction as the source. The culture (100 ml) was centrifuged at 2900 × *g* for 10 min, and the cell pellet was suspended in 10 ml of 2 mM EDTA/30 mM Tris Cl, pH 8.0/0.1% Triton X-100/10 mM dithiothreitol (DTT)/0.1 mM phenylmethylsulfonyl fluoride (PMSF) to lyse the cells. The cell lysate was stored frozen at −70°C until used.

To the thawed cell lysate (10 ml), PMSF, pepstatin A, and leupeptin were added to final concentrations of 1 mM, 1 μM, and 1 μM, respectively. The addition of the proteinase inhibitors was indispensable to prevent proteolysis of the expressed chimera during purification. The cell lysate was then treated with DNase I (10 μg/ml) and RNase A (10 μg/ml) in the presence of 12 mM $MgSO_4$ at room temperature (≈20°C) for 30 min, followed by centrifugation at 39,000 × *g* for 15 min. The precipitate was dissolved in 5 ml of 6 M guanidine hydrochloride, pH 1.5/10 mM DTT and dialyzed against the same solution to remove bound biotin. To reduce the protein concentration, the dialyzed sample was diluted with the same solution to a total volume of ≈100 ml and then dialyzed against 0.2 M ammonium acetate, pH 6.0/0.5 mM $CdCl_2$/0.1 mM EDTA/1 mM PMSF/1 μM pepstatin A/1 μM leupeptin/0.02% NaN_3. To achieve slow removal of guanidine hydrochloride, the dialysis bag containing the diluted sample was left overnight in the solution (≈800 ml) without stirring, followed by several changes of the dialysis solution and dialysis with stirring. The dialyzed fraction was centrifuged at 39,000 × *g* for 15 min, and the supernatant was briefly dialyzed against 1.0 M NaCl/50 mM sodium carbonate, pH 10.5/1 mM PMSF/1 μM pepstatin A/1 μM leupeptin. The dialyzed sample was centrifuged at 39,000 × *g* for 15 min, and the supernatant was adjusted to pH 10.5 with 1 M NaOH if necessary. This fraction was applied to a 2-iminobiotin-agarose (26) column (1.2 × 1.5 cm) previously equilibrated with 1.0 M NaCl/50 mM sodium carbonate, pH 10.5/1 mM PMSF/1 μM pepstatin A/1 μM leupeptin. After unbound proteins were removed by washing the column with the same solution, the bound protein was eluted with 6 M urea/50 mM ammonium acetate, pH 4.0/0.5 mM $CdCl_2$/0.1 mM EDTA/1 mM PMSF/1 μM pepstatin A/1 μM leupeptin. The eluted protein fraction was dialyzed against 0.2 M ammonium acetate, pH 7.0/0.5 mM $CdCl_2$/0.1 mM EDTA/1 mM PMSF/1 μM pepstatin A/1 μM leupeptin and then against 0.2 M ammonium acetate (pH 7.0). The dialyzed sample was filtered through a poly(vinylidene difluoride) filter (pore size, 0.22 μm; Millex-GV, Millipore), and the filtrate was stored at 4°C or at −70°C, for long-term storage.

Determination of Biotin-Binding Ability. Biotin-binding ability was determined by a gel filtration method (27) using a PD-10 column and D-[*carbonyl*-^{14}C]biotin (53 mCi/mmol).

Determination of Metal-Binding Ability. Quantitative x-ray fluorescence analysis was employed to determine the metal-binding ability. The purified streptavidin–metallothionein chimeric protein (2.3 μg, 120 pmol of subunits) was dialyzed at 4°C against 0.2 M ammonium acetate (pH 7.0) that had been treated with Chelex 100 to remove heavy metal ions. The dialyzed fraction was lyophilized and then was dissolved in 18 μl of formic acid (Aldrich). The dissolved sample (4 μl) was spotted on a polypropylene membrane (thickness, 6 μm; Chemplex, Eastchester, NY) and air-dried. The dried sample was subjected to quantitative x-ray fluorescence analysis to determine the amount of metals in the sample spot. The dialysis solution was used as the control. Standard $CdCl_2$ solution was used for calibration.

The x-ray fluorescence analysis system was described previously (28). The W-anode x-ray tube was operated at 80 kV. A single secondary target, Tb, with a 10-μm Ta prefilter was used to provide the excitation radiation, which consisted primarily of Tb K_α (44.2 keV) and Tb K_β (50.7 keV) x-rays.

Labeling of a Streptavidin–Metallothionein Chimeric Protein with Radioactive Cd^{2+}. The purified streptavidin–metallothionein chimeric protein (68 μg, 3.5 nmol of subunits) was dialyzed at 4°C against 10 mM DTT and then against 10 mM acetic acid in which Chelex 100 was present. To the dialyzed protein, 10 μCi of $^{109}CdCl_2$ (44 Ci/mmol, 0.23 nmol) in 0.1 M HCl and 25 nmol of $ZnCl_2$ in 0.1 M HCl were added. The addition of Zn^{2+} was to saturate the metal-binding sites of the chimera and was indispensable to avoid aggregation of the chimera due to intermolecular disulfide formation by free sulfhydryl groups. The mixture was then dialyzed at 4°C against 0.2 M ammonium acetate (pH 7.0) that had been treated with Chelex 100. The dialyzed fraction was used as the sample. By this procedure, ≈0.8% of the metal-binding sites contained $^{109}Cd^{2+}$, as determined from liquid scintillation counting, with the biotin-binding assay to estimate the protein concentration. Since natural metallothionein has 10^4-fold higher binding affinity for Cd^{2+} than for Zn^{2+} (2), this result suggested that the heavy metal ions that had been bound to the chimera during the purification step were not completely removed under the conditions described above.

Targeting a Streptavidin–Metallothionein Chimeric Protein Containing $^{109}Cd^{2+}$ to Biotinylated Macromolecules. All the procedures were carried out at room temperature, unless otherwise stated. Various amounts (0–5 μg) of biotinylated peroxidase in Tris-buffered saline (TBS: 150 mM NaCl/20 mM Tris Cl, pH 7.5/0.02% NaN_3) were spotted on a nitrocellulose membrane (0.8 × 9 cm; pore size, 0.45 μm). The membrane was incubated with 3% gelatin dissolved in TBS for 60 min to block free binding sites on the membrane and then washed with TBS containing 0.02% Tween 20. The membrane was then incubated for 60 min in 1.5 ml of TBS containing 0.02% Tween 20 in which ≈5 μg of the streptavidin–metallothionein chimeric protein containing $^{109}Cd^{2+}$,

prepared as above, was included. To remove unbound chimera, the membrane was extensively washed with TBS containing 0.02% Tween 20 and was air-dried. The dried membrane was exposed to Kodak XAR-5 film at −70°C with intensifying screens (Lightning*Plus*, DuPont).

Gel Filtration Chromatography. Gel filtration chromatography was carried out at room temperature using a Sephacryl S-300 HR column. Detailed conditions are given in the legend to Fig. 4.

SDS/PAGE Analysis. SDS/PAGE was carried out with a discontinuous buffer system (29) in a 15% polyacrylamide gel. Proteins were stained with Coomassie brilliant blue R-250 dissolved in 45% methanol/10% acetic acid.

RESULTS AND DISCUSSION

Expression of a Streptavidin–Metallothionein Chimeric Protein. A gene fusion of streptavidin with metallothionein was constructed by inserting a mouse metallothionein I cDNA (21) into the polylinker of an expression vector for streptavidin-containing chimeric proteins, pTSA-18F (22). The resulting expression vector pTSAMT-2 (Fig. 1) encodes a streptavidin–metallothionein chimeric protein (19.5 kDa), in which the metallothionein moiety follows the core region of streptavidin. The encoded chimera, consisting of 190 amino acid residues, contains 20 cysteine residues, which are derived solely from the metallothionein moiety.

To express the gene fusion of streptavidin with metallothionein, we used the T7 expression system (23), with which we had previously successfully expressed a cloned streptavidin gene in *E. coli* (19). SDS/PAGE of the total cell protein during expression (Fig. 2*A*) showed a major band at 22 kDa after the induction of the T7 RNA polymerase gene. The apparent molecular mass of the major band was higher than that estimated from the deduced amino acid sequence (19.5 kDa). To determine whether the 22-kDa protein was the streptavidin–metallothionein chimeric protein, the proteins were pulse-labeled with [^{35}S]cysteine during expression. Because of the high cysteine content, the metallothionein moiety of the expressed chimera should be strongly labeled with radioactive cysteine. Autoradiography of the gel (Fig. 2*B*) showed that the [^{35}S]cysteine was incorporated almost exclusively into the 22-kDa protein. This result reveals that the 22-kDa protein is cysteine-rich and indicates that it is the streptavidin–metallothionein chimeric protein. The discrepancy in the molecular masses of the chimera estimated by SDS/PAGE and from the deduced amino acid sequence will be discussed below.

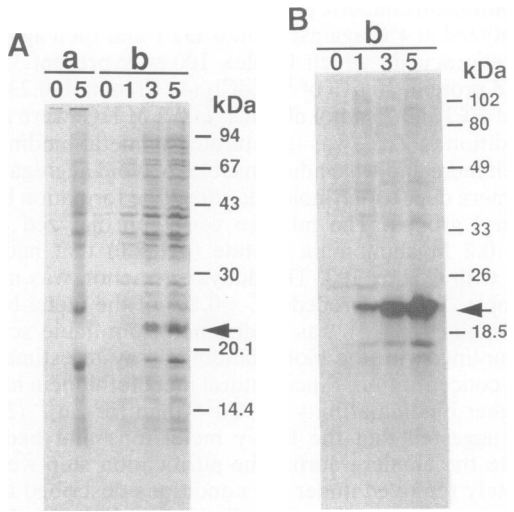

FIG. 2. Expression of streptavidin–metallothionein chimeric protein. Total cell protein of *E. coli* BL21(DE3)(pLysE) with or without pTSAMT-2 was subjected to SDS/15% PAGE. Proteins were detected by staining with Coomassie brilliant blue R-250 (*A*) or by autoradiography of the gel containing proteins pulse-labeled with [^{35}S]cysteine (*B*). Lanes a, BL21(DE3)(pLysE); lanes b, BL21(DE3)(pLysE)(pTSAMT-2). The number above each lane is the time (in hours) after induction. Arrows indicate the 22-kDa protein. Positions of the molecular mass standards for *A*, and those of the prestained standards for *B*, are indicated. For *A*, each lane contained the total cell protein from 167 μl of culture except for the lane at 5 hr for lanes a, where 83 μl of culture was used. For *B*, total cell protein from 167 μl of culture was applied to each lane.

Expression of the chimeric protein in minimal medium had the advantage that proteolysis of the expressed chimera in the host cells was considerably reduced. When LB medium (25) was used, instead, a major smeared band appeared at around 19 kDa, and no intact chimera was observed on SDS/PAGE. *E. coli* BL21(DE3)(pLysE) expressed the chimera more efficiently than the equivalent strain carrying pLysS (23).

Purification and Characterization of a Streptavidin–Metallothionein Chimeric Protein. The expressed streptavidin–metallothionein chimeric protein was purified to homogeneity (Fig. 3*A*) by a simple purification procedure including affinity chromatography using 2-iminobiotin as the ligand. After complete denaturation of the expressed chimera, which formed inclusion bodies in the cells, renaturation in the presence of heavy metal ions such as Cd^{2+} and Zn^{2+} provided the chimera with additional stability, which allowed frozen storage and lyophilization. By this procedure, the yield of the purified chimera ranged from 0.8 to 1.2 mg per 100 ml of culture.

In addition to monomer, the subunit dimer and tetramer were also observed on SDS/PAGE (Fig. 3*A*), though the protein sample was heated in boiling water for 5 min in the presence of 3% SDS and 10 mM DTT. Although natural streptavidin shows subunit oligomers on SDS/PAGE (30–33), the amounts of such molecules for the chimera seem greater than those for natural streptavidin. It is not clear, however, why the subunit association of the chimera in the presence of SDS is tighter than that of natural streptavidin. It is possible that intersubunit disulfide bonds, which are not completely cleavable even by DTT, are associated with the subunit oligomer formation.

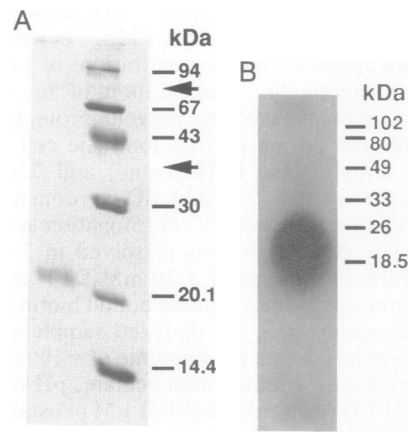

FIG. 3. SDS/PAGE analysis of purified streptavidin–metallothionein chimeric protein. (*A*) Approximately 6 μg of the purified chimera was applied to a 15% acrylamide gel. The subunit dimer and tetramer are indicated by arrows. Proteins were stained with Coomassie brilliant blue. The right lane contains the molecular mass standard proteins. (*B*) Approximately 4 μg of the purified chimera containing $^{109}Cd^{2+}$ was applied to a 15% acrylamide gel. The gel was exposed to Kodak XAR-5 film without intensifying screens at −70°C to prevent diffusion of proteins. Autoradiograph of the gel is shown. The positions of prestained protein molecular mass standards are indicated.

The streptavidin–metallothionein chimeric protein containing $^{109}Cd^{2+}$ was also subjected to SDS/PAGE. Autoradiography of the gel (Fig. 3*B*) indicated that the chimera retained the bound heavy metal ions even after the heat treatment indicated above. This result demonstrates the extreme stability of the chimera–heavy metal ion complexes. The higher apparent molecular mass of the chimera on SDS/PAGE can be explained by the presence of the bound heavy metal ions, which provide the chimera with additional positive charges resulting in slower migration.

The purified streptavidin–metallothionein chimeric protein bound 0.99 molecule of biotin per subunit (19.5 kDa), indicating that the chimera had full biotin-binding ability. In addition, the purified chimera bound 6.7 ± 1.0 Cd^{2+} ions per subunit, as determined by quantitative x-ray fluorescence analysis. Since mammalian metallothioneins bind a maximum of seven Cd^{2+} ions per molecule (1, 2, 34–36), this result indicates that the metallothionein moiety of the chimera is also fully functional.

On gel filtration chromatography (Fig. 4), the molecular mass of the chimeric protein under nondenaturing conditions was estimated to be ≈85 kDa, indicating that the chimera forms a subunit tetramer. This result also reveals that the subunit association of the chimera is determined by the streptavidin moiety. Therefore, one streptavidin–metallothionein chimera consisting of four subunits binds four biotin molecules and 28 Cd^{2+} ions. Although aggregation of the chimera was observed when frozen-stored samples were used, the amount of such aggregates was <15% of the total protein. However, such aggregated molecules also retained biotin-binding ability.

Targeting a Streptavidin–Metallothionein Chimeric Protein Containing $^{109}Cd^{2+}$ to Biotinylated Macromolecules. To demonstrate the capability of the streptavidin–metallothionein chimeric protein to attach heavy metal ions specifically to biological macromolecules containing biotin, we attempted to target the chimera containing $^{109}Cd^{2+}$ to biotinylated peroxidase immobilized on a nitrocellulose membrane. Autoradiography of the membrane (Fig. 5*A*) showed that the biotinylated peroxidase was specifically labeled with $^{109}Cd^{2+}$. This result clearly indicates that the bound heavy metal ions of the metallothionein moiety of the chimera were specifically attached to the biotinylated peroxidase by the chimera (Fig. 5*B*). The result also documents that the chimera can bind biotin and heavy metal ions simultaneously. Since extensive washing steps were included in this system, the result indicates that the chimera retains the tight binding affinity both for biotin and for heavy metal ions that the natural streptavidin and metallothionein molecules possess. This is also supported by absence of a background signal from nonspecific binding of the chimera or the bound $^{109}Cd^{2+}$ to the blocker molecules (gelatin).

In conclusion, the streptavidin–metallothionein chimeric protein is capable of labeling or conjugating biological materials containing unhindered biotin with various heavy metal ions and their isotopes. The potential ability of biotin to be incorporated easily into various biological materials, including proteins, nucleic acids, carbohydrates, lipids, cells, and tissues (17, 18), will allow the application of this chimera to a broad range of biological materials. Such applications include multiple simultaneous labeling of various biological materials with different heavy metal ions or their isotopes, because the binding affinity of this chimera both for biotin and for heavy metal ions is extremely tight, so that exchange reactions should be prevented.

There are ≈50 species of heavy metal stable isotopes that can be bound by metallothionein. Thus, the streptavidin–metallothionein chimeric protein offers the potential of simultaneous multi-mass labeling of a mixture of many different biological materials, each with a different metal isotope. In principle, each of such labeled biological targets can be detected and distinguished from the others by mass spectrometry, which discriminates among the tagged metal isotopes. The abundance of the metal stable isotopes that can be bound by the chimera should substantially increase the number of labels that can be attached to different biological

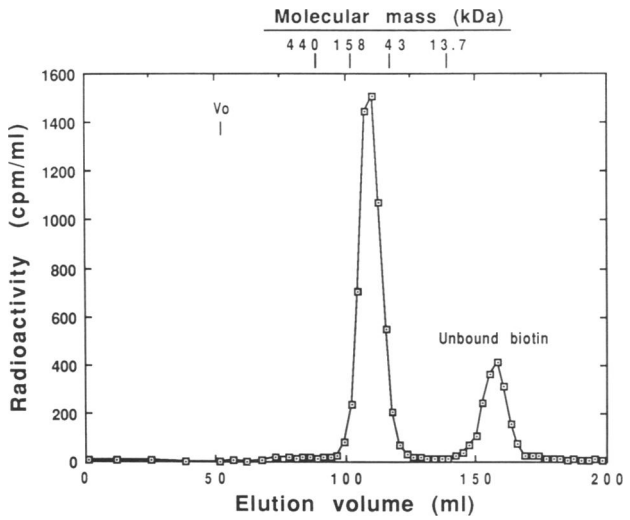

FIG. 4. Gel filtration chromatography of purified streptavidin–metallothionein chimeric protein. The purified chimera (5.4 μg, 0.28 nmol of subunits) was saturated with biotin by adding an excess of D-[*carbonyl*-^{14}C]biotin (53 mCi/mmol) and applied to a Sephacryl S-300 HR column (1.6 × 85 cm) previously equilibrated with TBS containing 0.05% Tween 20. The proteins were eluted at room temperature with the same solution at a flow rate of 24 ml/hr and fractionated. The radioactivity of each fraction (2.66 ml) was determined by liquid scintillation counting. The positions where molecular mass standard proteins were eluted are shown at the top: ferritin, 440 kDa; aldolase, 158 kDa; ovalbumin, 43 kDa; RNase A, 13.7 kDa. V_0, void volume.

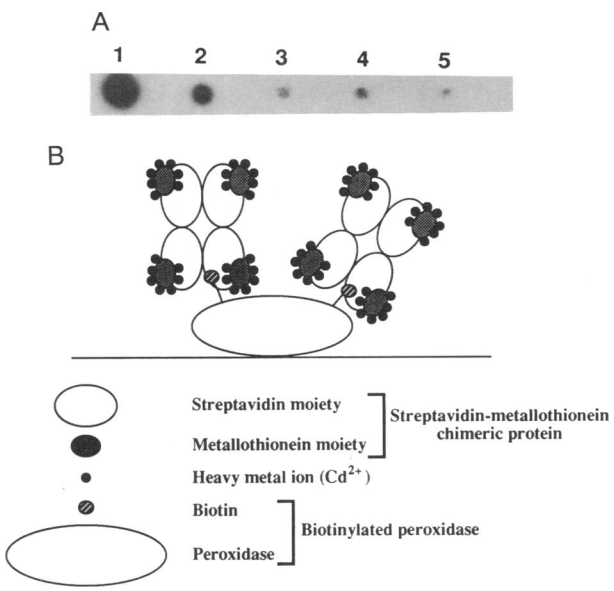

FIG. 5. (*A*) Specific labeling of biotinylated peroxidase with $^{109}Cd^{2+}$ by streptavidin–metallothionein chimeric protein. Various amounts of biotinylated peroxidase were immobilized on a nitrocellulose membrane, and the chimera containing $^{109}Cd^{2+}$ was targeted to the immobilized biotinylated peroxidase. Autoradiography of the membrane is shown. Spots 1–5 contained 5 μg, 1 μg, 200 ng, 40 ng, and 8 ng biotinylated peroxidase, respectively. (*B*) Schematic diagram of a biotinylated peroxidase molecule labeled with heavy metal ions (Cd^{2+}) by the streptavidin–metallothionein chimera.

targets. In addition, resonance ionization spectroscopy coupled with mass spectrometry (37–40) allows accurate detection and discrimination of such heavy metal isotopes down to a subattomole level. This means that a biotinylated biological target should be detectable with extremely high sensitivity upon binding the chimeric protein, each molecule of which can attach many heavy metal ions to the target.

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