A 14-Kilodalton Selenium-binding Protein in Mouse Liver Is Fatty Acid-binding Protein*

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In a previous study, we purified three selenium-binding proteins (molecular masses 56, 14, and 12 kDa) from mouse liver using column chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The aim of the present study was to determine the amino acid sequence of the 14-kDa protein thereby establishing any relationship with known proteins. Although the amino terminus of the 14-kDa protein was blocked, separate in situ digestions of the protein with endoproteinases Glu-c and Lys-c gave overlapping peptides that provided a continuous sequence of 93 amino acids. This sequence exhibited a 92.5% sequence homology with rat liver fatty acid-binding protein. In situ enzymatic digestion and partial sequencing of a 12-kDa selenium-binding protein revealed identical homology to the 14-kDa protein. The 14-kDa protein bound specifically to an oleate-affinity column from which the protein and 76Se coeluted. Delipidation or sodium dodecyl sulfate treatment failed to remove 76Se from the protein, indicating that the selenium moiety was tightly bound to the protein. These observations confirm that the mouse liver selenium-binding 14-kDa protein is a fatty acid-binding protein. The nature of the selenium linkage to the protein still needs to be explored.

The understanding of the biological functions of selenium has continually evolved over the past 40 years. Originally recognized for its highly toxic properties, selenium has changed from being perceived as a carcinogenic trace element to an essential nutrient exhibiting potent anticarcinogenic properties (1, 2). Of the numerous publications that have examined the effects of selenium as a chemopreventive agent, the vast majority (83%) have demonstrated an inhibitory effect of selenium supplementation on tumorigenesis in mice, rats, and hamsters (reviewed in Refs. 3 and 4). Selenium compounds also inhibit the growth of mammalian cells in vitro (4-6). The mechanisms of action underlying the chemopreventive and growth inhibitory effects of selenium are not understood.

It has been proposed that the chemopreventive effects of selenium might be mediated by selenium-binding proteins other than glutathione peroxidase (4, 6–9). The presence of mammalian selenium-binding proteins has been documented in numerous organs by the administration of selenium 75 (4, 10–18). Selenium in most of these proteins has been shown to be retained when the proteins were separated in SDS–PAGE. To date, the physiological function of these proteins has not been elucidated.

A few attempts have been made to purify and characterize these proteins. Yang et al. (16) have purified a 57-kDa selenium-binding protein from rat plasma and generated specific antibodies against it. Recently, we purified three proteins (molecular masses 56, 14, and 12 kDa) from mouse liver cytosol and raised polyclonal antibodies against these proteins (19). The organ distribution and specificity of these antibodies were characterized with an immunoblot assay using mouse and rat tissues (19, 20). The antibody to the 56-kDa protein recognized the cellular 56 kDa in a variety of organs (gastrointestinal, male and female endocrine) from both species but did not recognize the 57-kDa plasma protein or any protein in muscle. The antibody to the 14-kDa protein recognized a 14-kDa protein only in rat and mouse liver. The antibody to the 12-kDa protein recognized both the 12- and 14-kDa liver proteins.

Since the function of these proteins was unknown, we wished to characterize these proteins by determining their amino acid sequences. In the present study, we report approximately 75% of the amino acid sequence of the 14-kDa protein that was isolated from liver after fractionation on a DEAE-Sephadex A-50 column (see Ref. 19 for details of complete purification). The 14-kDa selenium-binding protein shows 92.5% sequence homology to rat liver fatty acid-binding protein.

MATERIALS AND METHODS

Treatment of Mice with 76Se

Normal virgin 6-week-old female BALB/c mice were maintained in a closed conventional mouse colony at the Baylor College of Medicine with a 12/12-h light/dark cycle and fed Wayne Lab-Blox and water ad libitum. Each mouse was given a single intraperitoneal injection of 0.5 μCi of 76Se as selenious acid (specifc activity = 100.65 mCi/mg selenium; Du Pont-New England Nuclear). After 40 h, the animals were anesthetized with ether and exsanguinated by heart puncture. The liver was removed rapidly from each mouse, cut into pieces, and washed in ice-cold 0.9% NaCl solution containing diatomium-EDTA (1 mM), diethyethol (1 mM), aprotinin (0.5 μg/ml), and phenylmethylsulfonyl fluoride (1 mM) to inhibit protease activity. All steps were done at 4 °C, and all buffers contained protease inhibitors as described above. After washing, the livers were blotted dry and homogenized.

Sephadex G-150 and DEAE-Sephadex Fractionation of Liver Cytosol

Chromatography of selenium-binding proteins was carried out as described recently in (19). Briefly, a 20% homogenate of pooled livers was dialyzed against 0.05 M phosphate buffer (pH 7.1) and applied to a 6 ml column of Sephadex G-150 equilibrated with the same buffer. The flow rate was 15 ml/h. The column was eluted with the same buffer, and 1 ml fractions were collected. The fractions were then applied to a 2 ml column of DEAE-Sephadex A-50 equilibrated with 0.05 M phosphate buffer (pH 7.1) and eluted with a 0-1 M NaCl gradient. The flow rate was 2.5 ml/h. The fractions were collected every 0.5 ml and ultracentrifuged for 1 h at 105,000 g. The protein content of the fractions was determined by absorbance at 280 nm.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; FABP, fatty acid-binding protein; TBS, Tris-buffered saline.
from 10–20 mice was prepared and centrifuged for 1 h at 100,000 × g in an SW-40 rotor using a Beckman LS-75 ultracentrifuge. Five-ml aliquots of liver cytosol were applied to a Sephadex G-150 (2.5 × 75-cm) column and scanned for $^{75}$Se activity. Five peaks (designated I–V) of $^{75}$Se were observed and characterized for the presence of different selenium-binding proteins.

To minimize the possibility of modification of reactive amino acid residues or of amino termini of the peptides during electrophoresis, 0.1 mM sodium thioglycolate was added to the running buffer in the well for 15 min and then run to the stacking/separating gel interface at a constant current of 15 mA. At this point, the current was increased to 30 mA/gel, the gels were rapidly stained and destained as described by Hunkapillar et al. (25) with few modifications. Gels were sliced into 3-mm sections, dried, and exposed to Cronex 4 x-ray film (Du Pont-New England Nuclear) for 10 days. For one-dimensional PAGE of column fractions, a concentrated protein fraction was mixed with an equal volume of double-strength sample buffer, placed in a boiling water bath for 5 min, and run on SDS-PAGE as indicated above.

Preparation of Proteins from SDS-PAGE for Amino Acid Sequencing

SDS-PAGE—Discontinuous SDS-PAGE was performed as described by Laemmli (24) with minor modifications. Fifteen percent separating gels were allowed to polymerize for 16 h. The gels were stained with Coomassie Brilliant Blue R-250, dried, and exposed to Cronex 4 x-ray film (Du Pont-New England Nuclear) for 10 days. For one-dimensional PAGE of column fractions, a concentrated protein fraction was mixed with an equal volume of double-strength sample buffer, placed in a boiling water bath for 5 min, and run on SDS-PAGE as indicated above.

Protein concentrations in samples were determined by a microassay method of Bradford (23).

Electroblotting

Peptides were blotted onto a polyvinylidene difluoride membrane (Millipore), which was then stained with Coomassie Blue essentially as described by Matsudaira (28). Briefly, the gels were soaked for 10 min in transfer buffer containing 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 10% methanol, pH 11. Polyvinylidene difluoride (PVDF) membranes were cut to size, wetted with 100% methanol, and rinsed with transfer buffer. Electroblotting was carried out in a Hoefer transfer electrophoresis unit at 1.5 A for 45 min (Hoefer Scientific Instruments). After transfer, the blots were washed with deionized water for 5 min, stained with 5 min with 0.1% Coomassie Blue in 50% methanol, and then destained with 50% methanol, 10% acetic acid.

Affinity Chromatography for FABP

The procedure for oleate affinity chromatography was performed as described in (29) with some modifications. Sodium oleate was coupled by stirring 1 g equivalent of AH-Sepharose 4B (Pharmacia LKB Biotechnology Inc.) gel in 1.5 volume of a 0.1 M sodium acetate, pH 4.5, containing 40 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; ethanol (25%) was also included to increase the solubility of sodium oleate. The mixture was stirred for 24 h, and the pH was maintained between 4.5 and 6.

As reported previously (19), five different $^{75}$Se peaks were observed on Sephadex G-150 fractionation of mouse liver cytosol. Briefly, peak I contained aggregated material that eluted in the void volume, peak II contained glutathione peroxidase, peak III contained a 56-kDa selenium-binding protein, peak IV contained a 14-kDa selenium-binding protein, and peak V contained low molecular mass selenium compounds unretained by a 10,000-dalton molecular mass cut-off membrane. The peak IV sample was further fractionated on DEAE-Sephadex, and a single major $^{75}$Se peak was obtained in the eluate. SDS-PAGE of this DEAE-Sephadex fraction showed two $^{75}$Se-labeled proteins at 14- and 12-kDa molecular masses (see Fig. 2A). Two-dimensional SDS-PAGE of this fraction showed a major and a minor trailing band at 14 kDa and a single band at 12 kDa with Coomassie Blue (Fig. 1A). The $^{75}$Se activity localized primarily in 14-kDa bands (Fig. 1B). The $^{75}$Se signal was not detected in the 12-
elution of peak IV (from Sephadex G-150 fractionation) using two-
pg that had a PI of 8.5. The protein spot that ran faster and slightly
dimensional SDS-PAGE. The sample applied to the gel contained 25
Coomassie Blue.
ments generated from endoproteinase Lys-c digestion.
was found to be 8.5 and 8.6, respectively.
ments generated from endoproteinase Glu-c digestion of the 14-kDa
protein. The derived 31-
N-terminal was blocked when sub-
The peak IV proteins not bound to the oleate affinity column
were eluted with acid alcohol, pH 2.4, against the 12-kDa protein recognized both the 12- and 14-
digestion. In an earlier study (19), we showed that the antibody raised against the 12-kDa protein recognized both the 12- and 14-kDa proteins on an immunoblot. In order to determine if the two proteins were related proteins, we sequenced one of the peptide fragments from the 12-kDa protein generated by in situ digestion with endoproteinase Glu-c. The derived 31-amino acid sequence matched 100% with amino acid sequence 71-101 of the 14-kDa protein (Fig. 3).
Affinity Chromatography—Liver FABP was recognized initially on the basis of its strong affinity for long chain fatty acids. In order to characterize further the selenium-binding 14-kDa protein, we examined its behavior on an oleic acid affinity column monitoring both the protein and the $^{75}$Se signal. Both the $^{75}$Se and 14-kDa protein in the DEAE-Sephadex fraction peak IV bound to the affinity column and eluted with 30-50% ethyl alcohol (pH 6) as illustrated in the autoradiograph of the SDS-PAGE gel (Fig. 4, lanes B and D). The peak IV proteins not bound to the oleate affinity column included the 31- and 21-kDa proteins that did not bind selenium (Fig. 4C; the Coomassie Blue-stained gel pattern is not shown as part of Fig. 4 but is shown in Fig. 2A). Residual $^{75}$Se and proteins were also eluted with acid alcohol, pH 2.4,

![FIG. 1. (A) separation of proteins from DEAE-Sephadex A-50 elution of peak IV (from Sephadex G-150 fractionation) using two-dimensional SDS-PAGE. The sample applied to the gel contained 25 µg of protein and 2500 cpm of $^{75}$Se. The pH gradient extended from acid (left) to base (right). The arrow identifies the 14-kDa protein that had a pH of 8.5. The protein spot that ran faster and slightly more basic (pI = 8.6) is the 12-kDa protein, NPHGE, nonequilibrium pH gradient electrophoresis; 15% acrylamide gel stained with Coomassie Blue. (B) autoradiograph of the gel shown in (A), 20-day exposure. The arrow points to the single 14-kDa protein band.](image1)

![FIG. 2. Coomassie Blue-stained gel patterns of the original peak IV fraction and enzymatically digested 14-kDa protein bands resolved on one-dimensional SDS-PAGE and electroblotted onto PVDF membranes. (A) DEAE-Sephadex A-50 fraction containing the 14-kDa selenium-binding proteins. The 14- and 12-kDa are major proteins. A 10-kDa selenium-binding protein is sometimes seen in this preparation. The 31-kDa protein in this fraction is not a selenium-binding protein. (B) the polypeptide fragments generated from endoproteinase Glu-c digestion of the 14-kDa protein. The dots identify the stained bands from the PVDF membrane, which were cut out and sequenced. (C) the polypeptide fragments generated from endoproteinase Lys-c digestion. KDa protein band due to the low concentration of the 12-kDa protein compared with the 14-kDa protein (19). The pI values of the 14- and 12-kDa proteins on two-dimensional PAGE were found to be 8.5 and 8.6, respectively.](image2)

Amino Acid Sequence of Protein—In the initial studies, electrophoretic digestion of the 14-kDa protein on a PVDF membrane indicated that the amino terminus was blocked when subjected to sequence analysis. Subsequently, in situ cleavage of the protein with endoproteinase Glu-c and endoproteinase Lys-c separately produced polypeptides of different molecular masses (Fig. 2, B and C). These peptides were then transferred to a PVDF membrane and analyzed for amino acid sequence. Treatment with endoproteinase Glu-c yielded overlapping fragments that provided sequence information on 50% of the protein. Digestion of the 14-kDa protein with endoproteinase Lys-C yielded fragments that overlapped at the amido and carboxyl ends of the sequence provided by endoproteinase Glu-c and allowed a continuous sequence of 93 amino acids to be determined. On alignment of the sequences of the different peptide fragments, the final sequence obtained was as illustrated in Fig. 3. The amino acid sequence was examined for sequence homology with known proteins in the GenBank database. The sequence of the 14-kDa selenium-binding protein exhibited a 92.5% (87/93 amino acids) sequence homology with rat liver fatty acid binding protein (FABP) (30). The amino acid sequence of the mouse liver 14-kDa protein correlated with amino acids 21-113 of the rat liver FABP. The 7 amino acids that differed between the mouse and rat proteins are represented in the boxes in Fig. 3 and represented mostly conservative changes. The changes were valine to isoleucine, isoleucine to valine (2), serine to proline, histidine to arginine, methionine to leucine, and phenylalanine to leucine.

In an earlier study (19), we showed that the antibody raised against the 12-kDa protein recognized both the 12- and 14-kDa proteins on an immunoblot. In order to determine if the two proteins were related proteins, we sequenced one of the peptide fragments from the 12-kDa protein generated by in situ digestion with endoproteinase Glu-c. The derived 31-amino acid sequence matched 100% with amino acid sequence 71-101 of the 14-kDa protein (Fig. 3).

![FIG. 3. Amino acid sequences of the mouse liver 14- and 12-kDa selenium-binding proteins derived from enzymatic digestion. The amino acids differing from rat liver FABP are identified by a box. Only 7/93 amino acids differed between the mouse and rat liver protein. The 12-kDa sequence (arrow) was identical to the 14-kDa sequence and showed the same amino acid change in this region of the protein as did the 14-kDa protein (amino acid 85).](image3)
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![ Autoradiograph of DEAE-Sephadex A-50 peak IV proteins that were delipidated or bound to an oleate affinity column before separation on SDS-PAGE. The autoradiograph shows the $^{75}$Se signal comigrating at the 14-kDa level. (A) peak IV protein that was delipidated. The $^{75}$Se signal comigrates with the 14-kDa protein. (B) the 14-kDa-containing peak IV prior to loading on an oleate affinity column. (C) the peak IV proteins not bound to the oleate affinity column. The 14-kDa protein and $^{75}$Se signal bound to the column and were absent in the wash. The 51- and 21-kDa protein bands were prominent in the unbound fraction. The Coomassie Blue-stained gel is not shown as part of the figure. (D) the peak IV bound protein eluted with 50% ethanol, pH 6. Both the 14-kDa protein and $^{75}$Se signal comigrated in the eluate. (E) the peak IV bound protein eluted with 50% alcohol, NaOH (1:1), pH 11. The 14-kDa protein was eluted (and visible by Coomassie Blue staining), however, the majority of the $^{75}$Se dissociated from the protein and was detected in the filtrate upon concentrating the eluted proteins in Centricon-10.

and with 0.05 M NaOH, ethanol, pH 11 (1:1). Under the conditions of high and low pH, $^{75}$Se was not retained bound to the eluted proteins (Fig. 4, lane E) and was found instead in the filtrate upon concentrating the eluted fractions.

To check if $^{75}$Se was bound to the FABP (14/12 kDa) in the DEAE-Sephadex fraction peak IV or to fatty acids that might be associated with the FABP, the DEAE-Sephadex fraction was delipidated (31). The fraction was delipidated by extraction for 30 min with 2 volumes of isopropyl ether, the phases were separated, and a second extraction was done for 10 min with an equal volume. After centrifugation, the aqueous phase was obtained and counted for $^{75}$Se activity. All of the $^{75}$Se was retained, bound to the protein in the delipidated protein fraction (Fig 4, lane A).

In another experiment, DEAE-Sephadex fraction peak IV was mixed with 8% SDS in Tris buffer, pH 6.8 (1:1), and dialyzed against water overnight. No $^{75}$Se was lost from the sample after dialysis.

**DISCUSSION**

The purpose of these studies was to purify the selenium-binding proteins from mouse liver and to characterize the amino acid sequence of these proteins, the latter hopefully providing information concerning the function or uniqueness of the proteins. The data so far suggest that the 14-kDa selenium-binding protein is distinct from the prototypic selenoproteins such as glutathione peroxidase and prokaryotic formate dehydrogenase in which selenium is present in the form of selenocysteine, and the incorporation of selenocysteine corresponds to a unique codon (32, 33).

DEAE-Sephadex fractions from peak IV of the Sephadex G-150 column gave a partial purification of two proteins having molecular masses of 14 and 12 kDa. The purity of the fractions was evidenced by the two-dimensional PAGE analysis of the 14- and 12-kDa bands that migrated as single bands and comigrated with the $^{75}$Se signal. Furthermore, the enzymatic digests of these proteins yielded peptide fragments in which a single polypeptide in each fraction was detected during the amino acid sequencing. Finally, polyclonal antibodies raised against each gel-purified protein recognized only the 14- and 12-kDa proteins in total liver cytosol (19). These results support the conclusion that a single selenium-binding protein was present in the 14- and 12-kDa bands.

The evidence for the selenium-binding 14-kDa protein being mouse liver FABP protein is substantial. Both the 14- and 12-kDa proteins were blocked at the amino terminus as analyzed on the gas phase amino acid sequenator. The amino acid sequence of overlapping fragments produced after in situ digestion with endoproteinase Glu-c and Lys-C yielded a sequence of 93 amino acids which was 92.5% homologous with rat liver FABP. Additionally, a polypeptide fragment of the 12-kDa protein yielded a sequence of 31 amino acids which was 100% homologous to an internal portion of the 14-kDa protein. This result coupled with the observation that the 14- and 12-kDa proteins cross-react with the same polyclonal antibody strongly indicates that the 12-kDa protein represents a processed form of the 14-kDa protein.

The mouse 14-kDa protein was specifically retained on an oleate affinity column, which indicates a high affinity for oleic acid similar to biochemically characterized mouse and rat FABP (34, 35). In this experiment (Fig. 4, lanes B and D), the $^{75}$Se signal comigrated and coeluted with the 14-kDa protein band. Since the 14-kDa protein presumably binds fatty acids, the question arises as to the nature of the selenium binding. The delipidation experiments (Fig. 4, lane A) and the SDS exposure experiments indicated that the selenium was tightly bound to the protein and not associated with lipid. However, it is interesting that if the proteins were eluted from the oleate affinity column at extreme pH values (pH 2.4 and pH >10), then the selenium was absent from the proteins (Fig. 4, lane E).

Selenium is incorporated as selenocysteine in the mammalian enzyme, glutathione peroxidase (17, 32, 36) and the bacterial enzyme, formate dehydrogenase (33). Chambers et al. (32) have demonstrated recently that the codon for the seleno-cysteine is TGA, normally a stop codon. In glutathione peroxidase, the codon is read through, and a seleno-amino acid (selenocysteine) is incorporated into the elongating protein chain. Scrutiny of the DNA sequence of the rat and human FABPs shows that TGA is not present in the coding portion of the gene (37, 38). This would suggest that selenium is not incorporated into the mouse 14-kDa FABP as selenocysteine in a similar manner as glutathione peroxidase, unless it is in the 25% of the protein which has not been sequenced or there has been a change in the nucleotide sequence. This seems unlikely considering the extensive conservation of sequences in mouse, rat, and human FABPs. Mitzutani and Hitaka (39) have shown recently that phosphorylated proteins (i.e. casein) can be specifically selenylated on a phosphorylated serine residue given the appropriate transfer RNA and enzyme. It remains to be determined if the 14-kDa FABP can be similarly selenylated. If so, this would further substantiate a mechanism by which proteins can be selenylated resulting in selenium tightly bound to the protein.

The surprising result from these experiments is the identification of the 14-kDa selenium-binding protein as liver fatty acid-binding protein. There are two properties of FABP which make the association with selenium interesting for understanding selenium's mechanism of action in the regulation of cell growth. First, liver FABP has multiligand-binding affinities. It binds not only long chain fatty acids but also heme (40) and the carcinogens 2-acetylaminofluorene, 3-methyl-4-dimethylaminoazobenzene, and ethionine (41). It seems possible that selenium should be added to the list of ligands. Second, FABP belongs to a superfAMILY of proteins with various degrees of sequence homology and diverse functions. These proteins include several organ-specific FABPs such as intestinal, heart muscle, and plasma membrane FABP (40), cellular retinoic acid-binding protein (40), cellular retinol-binding protein (40), mouse adipocyte p22 protein (40),

![ Fig. 4. Autoradiograph of DEAE-Sephadex A-50 peak IV proteins that were delipidated or bound to an oleate affinity column before separation on SDS-PAGE. The autoradiograph shows the $^{75}$Se signal comigrating at the 14-kDa level. (A) peak IV protein that was delipidated. The $^{75}$Se signal comigrates with the 14-kDa protein. (B) the 14-kDa-containing peak IV prior to loading on an oleate affinity column. (C) the peak IV proteins not bound to the oleate affinity column. The 14-kDa protein and $^{75}$Se signal bound to the column and were absent in the wash. The 51- and 21-kDa protein bands were prominent in the unbound fraction. The Coomassie Blue-stained gel is not shown as part of the figure. (D) the peak IV bound protein eluted with 50% ethanol, pH 6. Both the 14-kDa protein and $^{75}$Se signal comigrated in the eluate. (E) the peak IV bound protein eluted with 50% alcohol, NaOH (1:1), pH 11. The 14-kDa protein was eluted (and visible by Coomassie Blue staining), however, the majority of the $^{75}$Se dissociated from the protein and was detected in the filtrate upon concentrating the eluted proteins in Centricon-10.](image-url)
human myelin P2 protein (40), mammary gland-derived growth inhibitor protein (42), and fibroblast growth regulator (43). This superfamily of proteins is interesting because both liver FABP and mammary gland-derived growth inhibitor protein have been implicated in the regulation of cell growth. The liver FABP is a target polypeptide of a liver carcinogen and is associated with normal mitosis and overexpression in carcinogen-induced hyperplasias (44). The mammary gland factor MDG1 inhibits proliferation of mammary carcinoma cells in a reversible manner and at low concentrations (42). Interestingly, selenium inhibits proliferation of mammary cells in a reversible manner.

In summary, the identification of the 14-kDa selenium-binding protein as FABP offers new considerations of the mechanisms through which selenium can inhibit cell growth. In addition, the availability of specific antibodies to the 14-kDa protein will allow quantitative studies on the presence of the protein during the evolution of neoplasms. These results also raise questions of how selenium is bound to FABP, which can only be resolved by future experiments.

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