Identification of S100b Protein as Copper-binding Protein and Its Suppression of Copper-induced Cell Damage*

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We have isolated from bovine brain a protein with a high capacity to inhibit the copper ion-catalyzed oxidation of L-ascorbate and identified it as S100b protein, an EF-hand calcium-binding protein, by sequencing its proteolytic peptides. Copper binding studies showed that this protein has four copper-binding sites per dimeric protein molecule with a dissociation constant of 0.46 μM and that in the presence of L-ascorbate, copper ions bind to a total of six binding sites with a great increase in affinity. Furthermore, we examined whether S100b protein can prevent copper-induced cell damage. Bovine S100b protein was found to suppress dose-dependently the hemolysis of mouse erythrocytes induced by CuCl₂. We transformed Escherichia coli cells with pGEX-5X-3 vector containing a cDNA for rat S100b protein, so that this protein could be expressed as a fusion protein with glutathione S-transferase. The transformed cells were demonstrated to be markedly resistant to a treatment with CuCl₂ plus H₂O₂ as compared with the control cells expressing glutathione S-transferase alone. These results indicate that S100b protein does suppress oxidative cell damage by sequestering copper ions.

The transition metal copper is essential to a variety of cell functions; however, intake of its excess are toxic to living organisms. The toxicity is thought to result, at least in part, from the Fenton or Haber-Weiss reaction, in which copper ions participate in the production of OH· radical from H₂O₂ (1). OH· radical is a powerful oxidant that can damage various cellular components, such as lipid, nucleic acid, and protein. Therefore, copper ions in vivo should be sequestered such that their redox cycling could be hampered, thereby copper ions being unable to participate in the production of OH·. Considering the occurrence of 0.1–10 mg of copper/100 g of wet tissue or body fluid in human body, the copper sequestration should be recognized as an important mechanism for prevention of oxidative damage.

Under normal conditions copper ions may be tightly bound to proteins, but there are certain oxidant stress conditions where copper ions are released from the tightly binding sites of proteins and become redox-active, e.g. ischemia-reperfusion injury (2), fulminant hepatic inflamation in Wilson’s disease (3), Parkinson’s disease (4), and rheumatoid arthritis (5). Sequestration of copper ions is also important to prevent of L-ascorbate oxidation that is extremely accelerated by free copper ions (6, 7).

To understand the importance of copper sequestration in prevention of oxidant-induced tissue damage, we have initiated a study to isolate copper-binding proteins from bovine brain and found that S100b protein, an EF-hand calcium-binding protein (8), was one such protein. As a matter of fact, S100b protein was observed to prevent copper-induced hemolysis. By expressing recombinant S100b protein in Escherichia coli cells, we also demonstrated that the S100b protein markedly increased the resistance to H₂O₂ in the cells that had previously been treated with copper salt.

EXPERIMENTAL PROCEDURES

Materials—Commercial sources of materials are as follows: Sephadex G-75 and pGEX-5x-3 vector from Pharmacia Biotech, Upgsala, Sweden; DE52 cellulose from Whatman International, Ltd., Madison, United Kingdom; pGEM-3Zf (+) vector from Promega Corp.; Pfu DNA polymerase from Stratagene, La Jolla, CA; synthetic oligonucleotides and a SuperScript preamplification system from Biotechnologies, Inc., Gaithersburg, MA; restriction enzymes from New England Biolabs, Beverly, MA; E. coli strain BL21(DE3) pLysS from Novagen, Inc., Madison, WI; Achromobacter protease 1 (lysid endopeptidase) from Wako Pure Chemical Industries, Ltd., Osaka, Japan; and bovine S100b protein from Sigma. The S100b protein was dissolved in 20 mM Tris-HCl, pH 7.5, and the trace EDTA included was removed by the desalting procedure using a Microcon-3 concentrator (Amicon, Inc., Beverly, MA). Its concentration was estimated in view of the facts that the protein has absorbance of 1.0 at 280 nm at a concentration of 5.4 mg/ml (9) and that its dimeric molecular weight is 21,000 (10). All other chemicals were of analytical grade. Distilled water was purified by passage through a Milli-Q water purification system (Millipore Corp., Bedford, MA). The resistance of the water was 1.8 × 10¹⁰ Ω·cm at 20 °C.

Measurement of Copper Binding Capacity—Copper ions in the micromolar range linearly increases the rate of L-ascorbate oxidation, and the increase in the rate is suppressed in proportion to the amount of EDTA, a copper chelator, added. This phenomenon is the basis for the measurement of copper binding capacity that is obtainable from the degree of suppression of the copper ion-catalyzed L-ascorbate oxidation. The standard reaction mixture contained 20 mM Tris-HCl (pH 7.5), 2.0 μM CuCl₂, 50 μM L-ascorbate, and sample to be tested for copper binding. Oxidation of L-ascorbate was measured spectrophotometrically at 265 nm at 20 °C with a Shimadzu UV-Vis-visible recording spectrophotometer UV-160 (Shimadzu, Kyoto, Japan).

Purification of Copper-binding Protein—The purification procedure employed is in principle the same as used to purify copper-containing proteins by Sharoyan et al. (11). The following procedures were carried out at 4 °C unless otherwise stated. Acetone powder (45 g) prepared from bovine brain was extracted with 10 ml sodium acetate buffer (pH 6.0). The extract (350 ml) was applied onto a DE52 cellulose column (5 × 12 cm) that had been equilibrated with 10 mM sodium acetate buffer (pH 6.0). The column was washed with 760 ml of 100 mM sodium phosphate buffer (pH 6.0), and eluted with a 800-ml linear gradient, from zero to 1.0 M, of NaCl containing 100 mM sodium acetate buffer (pH 6.0). Fractions of 2.3 ml were collected, and those containing copper binding capacities above 43 nmol of copper bound/mg of protein were combined and concentrated by use of Centricon-3 concentrators (Amicon, Inc.). The resulting solution was applied onto a Sephadex G-75 column (3 × 100 cm) that had been equilibrated with 100 mM sodium...
acetate buffer (pH 6.0), and then the column was eluted with the same buffer. Fractions of 2.3 ml were collected, and those containing high specific copper binding capacities were combined and concentrated. Portions (100 µl) of the pooled fractions were further subjected to reverse-phase high performance liquid chromatography (HPLC) on a Shodex Amino 10 column (6 x 150 mm, Showadenko, Tokyo) with a linear gradient, from 0 to 70%, of acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1.0 ml/min, and the elution was followed spectrophotometrically at 230 nm.

Amino Acid Sequencing Analysis—The purified protein was digested with Achromobacter protease 1 in 10 mM Tris-HCl (pH 9.0) at 57 °C for 15 min. The resulting peptides were separated by reverse-phase HPLC as described above. Two peptides were sequenced using an Applied Biosystems 477A gas-liquid phase protein sequencer equipped with an on-line 120A PTH analyzer.

Construction of an Expression Plasmid for Production of a Fusion Protein of Rat S100β Subunit with Glutathione S-Transferase—Rat cerebellum RNA was prepared by the method of Chomczynski and Sacchi (13), and a first strand cDNA was synthesized from this RNA with random hexamers as primers. The synthesis was carried out by use of a SuperScript premplification system according to the manufacturer's manual. A rat S100β subunit cDNA was amplified from this cDNA by polymerase chain reaction with a pair of primers that had been synthesized on the basis of the reported nucleotide sequence of rat S100β subunit cDNA (14). The primers were a sense primer (5'-AGCT-TCTGCTCTAACCCTCT-3') and an antisense primer (5'-CGGAGTTGTATCCCG-3'), corresponding to the sequence from nucleotide 4 to 16 and that from nucleotide 548 to 568, respectively. Polymerase chain reaction was carried out in a MiniCycler (MJ Research, Inc., Watertown, MA) with Pfu DNA polymerase under the conditions as described previously (15) except that the temperature of the annealing step was 55 °C. After isopropyl alcohol precipitation, the amplified cDNA was ligated into the HindIII site of pGEM-3Zf(+). Further polymerase chain reaction was carried out, with the resulting construct as a template, for preparation of a cDNA fragment to be inserted into the mutiple cloning site of the prokaryotic expression vector pGEX-5X-3. The sense primer was 5'-GGGATCCGGATCTGCT-GACGGAGGAGG-3', which would generate a BamHI site at the 5' end and contains the nucleotide sequence surrounding the initiation codon (nucleotide 119 to 138 of the reported cDNA), and the antisense primer was 5'-CCGGAACGACGCGCAGG-3', which corresponds to a part of the LacZα sequence within the vector. The polymerase chain reaction product was digested with BamHI and ligated into the BamHI site of pGEX-5X-3, and the resulting plasmid with the correct orientation (designated pGEX-5X-3/S100β) was selected by restriction mapping. The final construct can produce a fusion protein of glutathione S-transferase and rat S100β subunit.

Copper Resistance by Expression of S100β Protein in E. coli Cells—E. coli cells of the strain BL21(DE3) pLyS3 were transformed with pGEX-5X-3/S100β or pGEX-5X-3, and the resulting recombinant clones were cultured at 37 °C in LB containing 50 µg/ml ampicillin. When the A_{600} of the culture became about 0.8, isopropylthio-β-D-galactopyranoside was added to a concentration of 1.0 mM, and the cells were cultured at 37 °C for 2 h, after which time CuCl₂ was added to a concentration of 1 mM and further cultured at 37 °C for 30 min. Then the cells were spun down, washed twice with a solution containing 25 mM Tris-HCl (pH 7.4), 136 mM NaCl, and 2.6 mM KCl and suspended in

1 The abbreviation used is: HPLC, high performance liquid chromatography.

FIG. 1. Sephadex G-75 column chromatography of copper-binding proteins. The pooled fractions (0.2 ml after concentration) from a DE52 cellulose column were subjected to gel chromatography on a Sephadex G-75 gel column (3 x 100 cm). The elution was performed with 100 mM sodium acetate buffer (pH 6.0) at a flow rate of 14.5 ml/h. Fractions of 2.3 ml were collected. The black bar denotes the fractions pooled for further purification by reverse-phase HPLC.

RESULTS

Identification of Bovine S100β Protein as a Copper-binding Protein—Acetone powder of bovine brain was extracted with sodium acetate buffer, and the resulting solution was subjected to DE52 cellulose column chromatography and then to Sephadex G-75 gel chromatography. The purification of copper-binding proteins was followed in terms of copper binding capacity as measured by their inhibitory effect on the copper-catalyzed oxidation of L-ascorbate. The elution profile of the gel chromatography is shown in Fig. 1. A great majority of copper binding capacity was eluted in fractions containing large amounts of protein, and a peak of copper binding capacity, although it was small, appeared in the following fractions whose protein concentrations were very low. The latter fractions with relatively high specific copper binding capacities were combined and further purified by reverse-phase HPLC. Upon elution with a gradient, from 0 to 70%, of acetonitrile containing 0.1% trifluoroacetic acid, there appeared one major and one minor peak at acetonitrile concentrations of 51 and 55%, respectively. Since the major peak showed much higher copper binding capacity than the minor one, we decided to determine the amino acid sequence of the major peak protein. The protein was digested with lysyl endopeptidase, and the resulting peptides were separated by reverse-phase HPLC by elution with the same acetonitrile gradient as used for the protein purification. Two of the peptides eluted at acetonitrile concentrations of 30 and 38% were isolated, and their amino acid sequences were determined to be AVALIDVPQYS-GREGDK and ELINNELSHFLEEIK, respectively. Computer search for these sequences in the available data bases revealed complete coincidence with the partial amino acid sequences (residue 6-24 and 60-71, respectively) of bovine S100β subunit (10), which is a constituent of S100a protein (αβ heterodimer) and S100b protein (αβ homodimer). This identification was confirmed by reverse-phase HPLC: the elution time of the authentic S100b protein (commercially available purified pro-

100 cm). The elution was performed with 100 mM sodium acetate buffer (pH 6.0) at a flow rate of 14.5 ml/h. Fractions of 2.3 ml were collected. The black bar denotes the fractions pooled for further purification by reverse-phase HPLC.
**Suppression of Copper-induced Cell Damage by S100b Protein**

A. The concentration of free copper ion in 20 mM Tris-HCl (pH 7.5) containing S100b protein (0.87 μM) and CuCl₂ (1.25–10 μM) was determined by ultrafiltration as detailed under "Experimental Procedures." II, the copper binding experiment was carried out on mixtures of S100b protein and CuCl₂ including 50 μM L-ascorbate. B, the data of Curve I in A was analyzed by Scatchard plot. [CuCl₂], total concentration of CuCl₂; r, average number of copper ions bound to S100b protein; C, concentration of free copper ion.

**Fig. 2. Copper binding of bovine S100b protein studied by an ultrafiltration technique. A: I, the concentration of free copper ion in 20 mM Tris-HCl (pH 7.5) containing S100b protein (0.87 μM) and CuCl₂ (1.25–10 μM) was determined by ultrafiltration as detailed under "Experimental Procedures." II, the copper binding experiment was carried out on mixtures of S100b protein and CuCl₂ including 50 μM L-ascorbate. B, the data of Curve I in A was analyzed by Scatchard plot.**

**Fig. 3. Inhibition of copper ion-catalyzed oxidation of L-ascorbate by bovine S100b protein. Oxidation of L-ascorbate (50 μM) with (II) or without (I) 0.95 μM S100b protein was measured spectrophotometrically at 265 nm at 20°C in 20 mM Tris-HCl buffer (pH 7.5) containing varying concentrations of CuCl₂.**

The rate of L-ascorbate oxidation. These results indicate that the binding constant of S100b protein for copper ions is far larger than that for Ca²⁺, if Cu²⁺ binds to the binding sites for Ca²⁺, and that Zn²⁺ and Cu²⁺ probably share at least a part of the binding sites.

**Inhibition of Copper-induced Hemolysis by S100b Protein—We examined whether S100b protein can inhibit copper-induced hemolysis of mouse erythrocytes (Fig. 4). When erythrocytes were incubated at 37°C in phosphate-buffered saline containing 30 μM CuCl₂, the degree of hemolysis after 2.5-h incubation was 50%. Addition of 1.1 μM S100b protein caused about 50% inhibition of the hemolysis, and almost total inhibition was achieved by addition of 2.3 μM S100b protein. The degree of hemolysis with 40 μM CuCl₂ reached about 90% after 2-h incubation, and S100b protein caused about 50% inhibition of the hemolysis at a concentration of 2.3 μM, while it had almost no inhibitory effect at a concentration of 1.1 μM. These results clearly indicate that S100b protein inhibits the hemolysis by sequestering copper ions.

**Copper Resistance of E. coli Cells by Expression of Recombinant Rat S100b Protein—**A cDNA for rat S100β subunit was generated by reverse transcriptase-polymerase chain reaction from rat cerebellum mRNA and inserted into the bacterial expression vector pGEX-5X-3 such that the S100β subunit could be expressed as a fusion protein with glutathione S-transferase. Cells of E. coli BL21(DE3) pLysS were transformed with the resulting expression plasmid and checked for production of the fusion protein. It was evident that a substantial amount of the fusion protein was produced by induction with isopropylthio-β-D-galactopyranoside (Fig. 5). The test for copper resistance of E. coli cells expressing S100b protein was carried out as follows. The cells possessing pGEX-5X-3/S100β were induced to produce the fusion protein, treated with 1.0 mM CuCl₂, and then further treated with varying concentrations of H₂O₂. E. coli cells transformed with pGEX-5X-3 were used as a control for comparison; they produced the enzyme in an amount comparable to that of the fusion protein produced by the cells transformed with pGEX-5X-3/S100β (Fig. 5). The pretreatment with copper ions alone gave only a slight difference in the cell viability.
between the test and control cells. However, the cells expressing S100b protein were markedly resistant to the subsequent treatment with H$_2$O$_2$ at all the concentrations tested (1–4 mM) (Fig. 6).

**DISCUSSION**

The results presented in this study clearly demonstrated that S100b protein, an EF-hand calcium-binding protein, can prevent the oxidation of l-ascorbate catalyzed by copper ions. This prevention is due to the sequestration of free copper ions: among such processes involving copper ions. Among such processes are l-ascorbate oxidation catalyzed by copper salt (20) and the hemolysis induced by copper salt alone (12) or by copper salt plus l-ascorbate (21).

Culotta et al. (22) have recently reported that the copper toxicity in yeast lacking metallothionein was suppressed by overexpression of copper/zinc superoxide dismutase. In this study, the function of superoxide dismutase was ascribed not to its superoxide scavenging activity but to its role in copper sequestration. We intended to examine whether the copper toxicity can be reduced by expressing S100b protein extraneously in *E. coli* cells, and actually found that the cells manipulated to express rat S100b protein (Fig. 6) were resistant, to a great degree, to copper-induced oxidative damage in comparison with the control cells not expressing S100b protein. Copper ions would be reduced by reducing substances in cells, and the resulting Cu$^{1+}$ would be oxidized with H$_2$O$_2$, producing highly reactive OH$^-$ radical. This redox activity of copper ions may be suppressed by their binding to S100b protein. Even if the

**FIG. 4. Inhibition of copper-induced hemolysis by bovine S100b protein.** Hemolysis of mouse erythrocytes (1.5% hematocrit) in phosphate-buffered saline was induced by CuCl$_2$ at concentrations of 30 μM (A) and 40 μM (B). The concentrations of S100b protein added were nil (○), 1.1 μM (●), and 2.3 μM (×).

**FIG. 5. Production of rat S100b subunit as a fusion protein with glutathione S-transferase in E. coli cells.** Cells of *E. coli* BL21(DE3) pLysS, transformed with pGEX-5X-3/S100b, were cultured in the presence (+) or absence (−) of 1 mM isopropylthio-β-D-galactopyranoside at 30 °C for 2 h to produce the fusion protein. The cells were disrupted by sonication, and the proteins (20 μg) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The major band on each lane marked by + represents the inducibly expressed protein.

The enhancement of copper binding by l-ascorbate may be interpreted to suggest that Cu$^{1+}$ formed by the reduction of Cu$^{2+}$ by l-ascorbate has a higher affinity to S100b protein and binds additionally to two sites, but the elucidation of the underlying mechanism of this phenomenon awaits further investigation.

Since addition of copper salt to an erythrocyte suspension is known to cause hemolysis (12), we tested whether S100b protein can prevent this phenomenon and found that it was really the case (Fig. 4). S100b protein at a concentration of 2.3 μM was able to almost totally inhibit the hemolysis of mouse erythrocytes (1.5% hematocrit) induced by 30 μM CuCl$_2$. Assuming the above copper binding ratio of four for S100b protein (without l-ascorbate), the S100b protein present in the reaction system could sequester only 31% of the copper ions, yet it could almost completely inhibit the hemolysis. Under these conditions, the remaining concentration of CuCl$_2$ (21 μM) after sequestration by S100b protein appears to be not enough for induction of hemolysis. In fact, 20 μM CuCl$_2$ was almost ineffective to induce hemolysis (data not shown). In relation to the suppression of copper-induced cell damage by S100b protein, it should be noted that serum albumin, known to tightly bind copper ions (19), likewise exerts inhibitory effects on various oxidative processes involving copper ions. Among such processes are l-ascorbate oxidation catalyzed by copper salt (20) and the hemolysis induced by copper salt alone (12) or by copper salt plus l-ascorbate (21).
copper ions bound to S100b protein were otherwise prone to react with H2O2 and damage the protein itself, loss of the extraneous protein would not be harmful.

S100b protein is relatively abundant in the brain, amounting to approximately 2% of the soluble protein of its certain regions (23); therefore, it is possible that S100b protein as a copper-binding protein plays a role in copper hemeostasis as well as it prevents the copper-induced oxidative damage in the brain. In this regard, a recent finding should be noted that βA4 amyloid precursor protein reduces Cu2+ to Cu1+ and possibly contributes to neurodegeneration in Alzheimer’s disease through generation of reactive oxygen species (24). It is also reported that the copper level of the cerebrospinal fluid is significantly elevated in patients of Parkinson’s disease (25). Therefore, it seems likely that S100b protein intervenes the pathogenic processes of these diseases.

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