Purification and Properties of a Self-associating, 50-kDa Copper-binding Protein from Brindled Mouse Livers*

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The brindled mouse is an animal model of Menkes disease, a fatal, X-linked disease of copper metabolism. A self-associating, 50-kDa copper-binding protein (CuBP) was purified from brindled mouse hepatic cytosols, and some of its properties were determined. When 64Cu-labeled whole hepatic cytosols were fractionated on Superose, statistically significantly less than normal 64Cu binding was detected in both the fraction which contained the tetramer plus dimer (≈26% less) and the fraction containing the monomer of CuBP (≈37% less). CuBP was purified from brindled mouse hepatic cytosols by successive Mono Q, eluting decreased levels of the partially purified protein in earlier studies (16). The data reported here are consistent with significant roles of this protein in normal copper metabolism and the brindled mouse defect.

The brindled mouse model (1) of Menkes disease (2, 3) is a potentially useful tool for identifying copper-binding proteins with specific functions in cellular copper metabolism (4). Like Menkes disease, the brindled mouse defect is an X-linked, inherited disease of copper metabolism (1-4). Although Menkes disease is fatal and untreatable, brindled mice survive and grow to adulthood if given a single dose of copper at days 7-9 of age (5). Thus, the brindled mouse is amenable to studies of copper metabolism, and copper proteins whose structures, amounts, or copper binding may be directly or indirectly affected by the defect can be examined. The basic defect in the brindled mouse does not seem to be in membrane copper transport; the kinetic parameters are normal for both uptake and efflux with brindled mouse hepatocytes (6), fibroblasts (7), or Menkes lymphoblasts (8). Therefore, the defect most likely involves an intracellular copper-transport or copper-trafficking protein (7, 9, 10). One possible function of this protein is the delivery of copper to the cellular sites where it is incorporated into copper enzymes because the activities of several copper enzymes are decreased in brindled mice and Menkes patients (1, 3, 11, 12). Another possible function of the protein involved in the defect may be to mediate copper efflux because net copper efflux is impaired in Menkes or brindled mouse fibroblasts (7, 13) and Menkes lymphoblasts (14), and decreased copper efflux seems to be associated with the basic defect (7).

We reported in an accompanying paper (15) the identification and purification of a self-associating, 50-kDa copper-binding protein (CuBP)1 from mouse liver. Here we report that purified CuBP from brindled mouse liver has several abnormal properties including instability, reduced copper affinity, and abnormal chromatographic properties. The inherent instability of this protein most likely accounts for detecting decreased levels of the partially purified protein in earlier studies (16). The data reported here are consistent with significant roles of this protein in normal copper metabolism and the brindled mouse defect.

EXPERIMENTAL PROCEDURES

Materials—Columns (Superose-12 HR 10/30, Mono Q HR 5/5, and phenyl-Superose HR 5/5), the HPLC pump (Model 2150), the HPLC controller (Model 2152), and all other HPLC accessories including a titanium prefilter were from Pharmacia LKB Biotechnology Inc. 64Cu(NO3)2 was from the Buffalo Materials Research Center of the State University of New York at Buffalo. The specific activity at the time of shipment was ≈14 mCi/mg copper. EDTA (disodium salt), HEPES, glycine, Tris, pepsin, and molecular weight standards were from Sigma. Silver nitrate (99.9+%) was from Alfa (Danvers, MA). All other chemicals were from Fisher.

Animals—Brindled male mice and their normal male littermates were bred as described previously (6, 7). The brindled males were treated with a single injection of CuCl2 in propylene glycol (10 μg of CuCl2/g) at 7-8 days after birth (6). The survival rate was 91%. Littermates were used as the controls for all experiments involving the purification of CuBP and the properties of the purified protein. Age- and weight-matched C57 BL/6 mice from West Seneca Laboratory (West Seneca, NY) were used as the controls in the other experiments described.

Preparation of Cytosols—Hepatic cytosols from normal and brindled mice were prepared as previously described (15, 16). Each frozen sample (=1 ml) was thawed and used only once because the protein from the brindled mice was found to be unstable to thawing and refreezing.

Purification of CuBP—Successive Mono Q, eluting Superose, and phenyl-Superose HPLC were used to purify CuBP from both normal and brindled mice as described previously (15). SDS-PAGE

1 The abbreviations used are: CuBP, copper-binding protein; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; MT, metallothionein.

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with β-mercaptoethanol by the Laemmli method (17) was used to characterize the product of each step using a 10% separating gel. The gels were stained by the AgN03 method described by Wray et al. (18). The molecular weight standards were: β-galactosidase (116,000), phosphorylase b (97,400), bovine serum albumin (66,000), ovalbumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsin (24,000), soybean trypsin inhibitor (20,100), and lysozyme (14,300).

Superose and Mono Q HPLC—The copper binding properties of whole hepatic cytosols and CuBP after partial or complete purification were examined by Superose columns. The Superose column was equilibrated with at least two column volumes (48 ml) of HEPES buffer (0.05 M HEPES, 0.1 M NaCl, pH 7.4). Samples were thawed and filtered, and 64Cu was added to a final volume of 250 μl. After 5 min at room temperature, 200-μl samples were injected through a titanium loop. Fraction collecting began 16 min after the sample was applied. The flow rate was 0.4 ml/min, and 220 μl fractions were collected into test tubes containing 10 μl of a phenylmethylsulfonyl fluoride (1.0 mg/ml) and leupeptin (10 μg/ml) solution to inhibit proteolysis. Protein absorbance was monitored at 280 nm by a single path monitor (Pharmacia, UV-1) using a 10-mm, 7.9-μl flow cell. Column fractions were counted for 64Cu, and stored at −20°C for electrophoresis. The following molecular weight markers were used to calibrate the column: ferritin (445,000), aldolase (161,000), ovalbumin (45,000), and myoglobin (17,000).

The concentrations of copper that were added to the whole hepatic cytosols were based on prior experiments with intact mouse hepatocytes (19). After hepatocytes were incubated for 5 min with 2 or 10 μM 64Cu, the cytosols contained 0.65 μM or 3.25 μM 64Cu, respectively. Indistinguishable 64Cu-binding column profiles were obtained with 64Cu-labeled cytosols from cells which were incubated with 64Cu or with cytosols isolated from cells to which equivalent amounts of copper were added (19). The properties of CuBP after partial purification by successive Mono Q, rechromatography on Mono Q, and Superose steps were also determined. The experimental conditions for the Mono Q steps were as described previously (15, 16).

Protein and 64Cu Assays—Protein concentrations were determined by the BCA method with bovine serum albumin as the standard as described by the supplier (Pierce). 64Cu was determined with an LKB γ counter (model 1282), and corrections for decay were by a program supplied with the counter.

Statistics—A two-tailed t-test was performed with a statistical program.

RESULTS

Copper Binding in Whole Hepatic Cytosols from Brindled Mice—Radiolabeled copper (0.65 μM 64Cu(Ill)) was added to the cytosols, and the 64Cu-labeled cytosols were fractionated on Superose (Fig. 1). No significant, reproducible differences between control and brindled mouse samples were detected in the protein elution profiles (absorbance at 280 nm). Three major 64Cu-binding fractions were detected with both the normal and brindled mouse cytosols. These are labeled the I5, H5, and MT (metallothionein) fractions in Fig. 1. The tetramer and dimer forms of CuBP elute in the I5 fraction, and the monomer elutes in the H5 fraction (15). Less 64Cu was detected in both the I5 and H5 fractions from the brindled mice (Fig. 1 and Table 1). The average decrease in the I5 fraction was ≈26% and in the H5 fraction was ≈57% with samples from five different animal sets (Table 1). The MT fraction showed a corresponding increase in copper binding (Table 1); i.e., the increase in 64Cu in the MT fraction (1753 pg) about equaled the decrease in 64Cu bound to proteins in the I5 plus H5 fractions (1635 pg, Table 1). This result is consistent with normal hepatic MT levels in the brindled mice (20, 21), i.e., the increase in MT-bound 64Cu is consistent with an increase in available 64Cu for MT binding due to decreased binding in the I5 and H5 fractions rather than any abnormal property or amount of MT. Also, consistent with this interpretation, the sum of 64Cu bound to MT plus the I5 and H5 fractions from the brindled mice (6078 pg) was similar to the sum from normal mice (5958 pg). It should be noted that while some variability was detected in the relative amounts of 64Cu bound in the I5 and H5 fractions from normal mice, the variability was greater with samples from the brindled mice as indicated by the higher percent standard deviations with the brindled mouse cytosol data (Table 1). As discussed further below, the variability that was detected may be due to variable self-association of the CuBP in the brindled mouse cytosols. The variability was not correlated with the amount of 64Cu in the MT fraction with either normal or brindled mice samples.

Copper Concentration Dependence of Superose Profiles—When the 64Cu concentration in the hepatic cytosol was increased to 3.25 μM, smaller differences were detected between the I5 and H5 fractions from normal and brindled mice (Fig. 2A). The I5 fraction was =12% less of normal and the H5 fraction was =19% less of normal when the data from five different animal sets were averaged. At 20 μM 64Cu, no significant differences were detected between the I5 and H5 fractions from the normal and brindled mice (Fig. 2B) or by the averages from five animal sets. This was most likely due to saturation of the 64Cu-binding protein(s) involved and increased binding to additional proteins with lower copper affinities. At lower copper concentrations (0.2 μM), similar
differences were detected in the Superose fractions from normal and brindled mice as were detected at 9.65 μM 64Cu (data not shown). Also, at 0.2 μM 64Cu, a prominent shoulder on the trailing edge of the II₈s fraction was detected with brindled mouse cytosols which was also often detected as a shoulder on the II₈s fraction from normal mice at 0.65 μM (tubes 38–40).

Partial Purification of CuBP from the Brindled Mouse—In principle, decreased 64Cu binding in both the Iₛ and II₈s fractions from the brindled mice could be due to decreased copper binding by multiple proteins in these fractions or to decreased copper binding to the different oligomeric forms of CuBP. To address these possibilities, hepatic cytosols from the brindled mice were partially purified by two successive Mono Q steps followed by Superose. Both the 64Cu-binding profiles and SDS-PAGE patterns with the brindled mouse cytosols were similar to what was observed with the hepatic cytosols from normal mice at 0.65 μM (tubes 38–40).

FIG. 2. Copper concentration dependence of Superose copper-binding fractions. Cytosols containing 3.25 μM 64Cu (A) and 20 μM 64Cu (B) from normal (○) and brindled (●) mice were fractionated on Superose as described in Fig. 1.

FIG. 3. Copper binding by partially purified CuBP from normal and brindled mice. Mono Q fractions from three separate cytosol samples from the normal or brindled mouse (each containing ~13 mg of total protein) were collected in the same tubes using a stepwise salt gradient (15). CuBP eluted in nine fractions as detected by SDS-PAGE. These fractions were pooled and rechromatographed on Mono Q. The fractions containing CuBP from the second Mono Q column were pooled, labeled with 2 μM 64Cu(II), and fractionated on Superose as described in Fig. 1. A, 64Cu in fractions from normal mouse (●—●) and brindled mouse (○—○). A, B, and C are the peak elution positions predicted for the tetramer, dimer, and monomer forms, respectively, of CuBP according to the elution volume of the standards shown at the top. B, SDS-PAGE of each 0.22-ml Superose fraction shown in A and standard(s).

to at least the dimer and monomer forms of CuBP.

The similarities in the 64Cu-binding profiles from Superose columns of the partially purified CuBP and the profiles with the whole cytosols (15) are consistent with the decreases in the Iₛ and II₈s fractions in Fig. 1 being due to decreased binding to the CuBP. Interestingly, at the lowest protein concentration tested, i.e., at the highest copper to protein ratio, no 64Cu binding peak corresponding to the putative dimer of CuBP was detected in the brindled mouse sample, and a new 64Cu-binding fraction was detected in the fractions which contained high concentrations of a ~38-kDa protein or subunit (Fig. 4). This result may be due to low binding by the dimer of CuBP at these protein concentrations and corresponding increased binding by the 38-kDa protein which was still present at a high concentration.

Purification of CuBP from the Brindled Mouse—The CuBP from brindled mouse hepatic cytosols was purified to homogeneity by the same protocol that was used to purify the protein from the normal mouse (15): successive Mono Q, chelating Superose, and phenyl-Superose HPLC. The protein from the brindled mouse behaved somewhat differently in some of the purification steps. One significant difference was in the overall yield of CuBP from the chelating Superose
column which was ≈20% less from the brindled mouse than from the control. This seemed to be due to instability during handling. The most significant chromatographic difference was that the CuBP from the brindled mouse eluted anomalously from the phenyl-Superose column (Fig. 5). While CuBP from the normal mouse began to elute at 15 min and peaked at 20 min after initiating the H2O step, the protein from the brindled mouse began to elute immediately after initiating the H2O step and peaked at 2.5 and 5 min (Fig. 5). Moreover, the samples from normal mice showed a major central peak with small shoulders on the trailing and leading edges while the brindled mouse sample showed relatively more protein in the first fraction (Fig. 5). Since these peaks most likely represent the three oligomeric forms of CuBP, the protein from the brindled mouse may have had different percentages of the oligomeric forms at this stage of the purification. It should be noted that the elution profiles of CuBP from the phenyl-Superose with samples from normal mice were highly reproducible, i.e. it is unlikely that the abnormal elution of the brindled mouse protein was due to the specific sample that was used for Fig. 5. Interestingly, the CuBP elutes in two fractions from chelating Superose (15) and while Fraction II eluted anomalously from phenyl-Superose, Fraction I eluted normally (data not shown). This could be due to differences in the relative amounts of tetramer, dimer, and monomer in these fractions. However, once the phenyl-Superose-purified CuBP was concentrated in water and applied to Superose, the properties of the protein obtained from the two chelating Superose fractions were indistinguishable. SDS-PAGE of the pooled and concentrated phenyl-Superose fractions from the brindled mouse showed a single, 50-kDa band.

**DISCUSSION**

CuBP from the brindled mouse appears to have several abnormal properties. The apparent copper affinity of CuBP from the brindled mouse is less than normal, and the brindled...
The copper binding in both of the Superose fractions which are not an artifact of the purification procedure. These results suggest that the tetramer has a higher affinity than the monomer (data not shown). Also, the competitive effect of EDTA on $^{65}$Cu binding to the monomer was greater than its effect on binding to the dimer or tetramer.

An abnormal structure of CuBP may be the primary defect in the brindled mouse. Clearly, a decreased affinity for copper, instability, abnormal aggregation, or abnormal conformation of CuBP could significantly decrease the activity of this protein in intracellular copper metabolism and give rise to the biochemical abnormalities that are associated with the disease. However, it is also possible that the abnormalities that were detected with CuBP from the brindled mouse are secondary consequences of the primary defect. For example, CuBP may play an important role in intracellular copper metabolism, but, in vivo, it may bind copper that is donated to it by the protein that is actually responsible for the basic defect in the brindled mouse. If CuBP from the brindled mouse had less than its normal complement of copper, when isolated, its stability, aggregation, and elution from phenyl-Superose could all be affected. Clearly, further analyses of the protein and the gene encoding its sequence are necessary to determine if the abnormal properties of CuBP from the brindled mouse are primary or secondary to the basic defect in the brindled mouse.

We previously reported that the amount of a partially purified 48-kDa protein was less in brindled mouse hepatic and renal cytosols than in the normal mouse (16). The migrations of the 48-kDa and 50-kDa CuBP on SDS-PAGE gels and their elution positions from Mono Q columns indicate that these proteins are identical. Previously, the cytosols that were used for the HPLC columns were thawed and refrozen for further use. Here, aliquots sufficient for single HPLC columns were stored at $-70\,^\circ\text{C}$ and used once. Also, cytosols were isolated and frozen more rapidly than in the initial studies. These precautions markedly reduced the differences from the Mono Q-Mono Q-Superose protocol which also indicated that the tetramer has a higher affinity than the monomer (data not shown). Also, the competitive effect of EDTA on $^{65}$Cu binding to the monomer was greater than its effect on binding to the dimer or tetramer.

The simplest interpretation of the Superose results with the whole hepatic cytosol is that the decreases in $^{65}$Cu in the $I_{S}$ and $I_{I}$ fractions that were detected were due to the decreased affinity of the CuBP rather than to decreased binding to multiple proteins in these fractions. This follows from the fact that the affinities of the tetramer, dimer, and monomer from the brindled mice are each decreased, and that the monomer elutes at or near the peak of the $I_{I}$ fraction from whole hepatic cytosols while the tetramer and dimer elute in the $I_{S}$ fraction (15). Moreover, the fact that similar quantitative differences in copper binding between normal and brindled mice were detected in the Superose fractions of the whole cytosol, the Superose fractions after two Mono Q purification steps, HPLC fractions containing CuBP after various other partial purification protocols involving Mono Q and Superose columns (22), and the fully purified protein indicates that the lower affinity of CuBP purified from the brindled mice was not an artifact of the purification procedure. These results are also consistent with CuBP being a major contributor to the copper binding in both of the Superose fractions which elute before MT with the whole hepatic cytosol. If CuBP were a minor contributor, then decreased binding to the CuBP would not be detected in the Superose profiles. The apparently greater effect of the brindled mouse defect on the $I_{I}$ fraction than the $I_{S}$ fraction with whole hepatic cytosols is consistent with the monomer having a lower affinity than the tetramer in both normal and brindled mice. This is also consistent with the concentration dependence of copper binding to the fractions from the Mono Q-Mono Q-Superose protocol which also indicated that the tetramer has a higher affinity than the monomer (data not shown). Also, the competitive effect of EDTA on $^{65}$Cu binding to the monomer was greater than its effect on binding to the dimer or tetramer.
low in copper in the brindled mice, decreased copper binding by CuBP or decreased ability to deliver copper to the sites where it is retained may contribute to their decreased hepatic copper levels. In addition, this protein may mediate copper efflux from, e.g. fibroblasts, lymphoblasts, and kidney cells (7); preliminary results with antibodies raised to the mouse liver CuBP indicate that mouse kidney and human lymphoblasts also contain CuBP. The liver may either have more of this protein or another protein(s) which can mediate copper efflux which would account for normal efflux from the liver, but not from the kidney and other cell types in the brindled mouse (7). Thus, CuBP may be a general copper trafficking protein with several related functions.

Although it has been known since the early 1970s that copper binds to high molecular weight proteins and MT when it first enters most organs (25–28), CuBP is the first protein which has been identified and purified other than MT that potentially plays a pivotal role in cellular copper metabolism, and an abnormal CuBP may be the basic defect in the brindled mouse and Menkes disease.

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