Vol. 181, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1991 Pages 409-415

A NEWLY IDENTIFIED IRON-BINDING PROTEIN IN RAT LIVER: PURIFICATION AND CHARACTERIZATION

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Received October 21, 1991

A novel iron-binding protein from rat liver homogenates was purified 1,800-fold with a 5.7 % yield, to apparent homogeneity. The molecular weight of the protein was estimated to be 16,000, by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The purified protein exhibited 0.43 mol of iron binding per mol of protein with a dissociation constant (Kd) of 3.5×10^{-6} M. Al^{3+} inhibited the iron-binding and the binding was also slightly inhibited by Ni^{2+}. Other divalent metal ions such as Cu^{2+}, Zn^{2+} and Mn^{2+} were without effect. Immunoblot analysis of the iron-binding protein revealed that the protein is located mainly in microsomes. This newly identified iron-binding protein may be involved in intracellular transport of iron.

Transferrin and ferritin are the major iron-binding proteins in vertebrates. Interest in these proteins has recently increased, as there is evidence that the uptake of iron from transferrin occurs by a unique type of transferrin receptor-mediated endocytosis (1-3). While the overall cellular transferrin cycle is well understood, ligands which may be involved in the intracellular transport of iron after release from transferrin have yet to be identified. There are few definitive descriptions of iron-containing intermediates between transferrin-iron and intracellular iron-containing components including heme, ferritin and non-heme iron proteins and only little is known of phosphorous compounds, such as ATP and AMP which have been received the most attention as candidates for intracellular iron carriers (4-6). Therefore, we addressed questions of whether intracellular iron-binding proteins are present in the liver, an organ vitally important for iron metabolism.

The technique of metal-blotting using radioactive metal ions as probes (7-10) and immobilized metal ion affinity chromatography (11-13)

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography.

0006-291X/91 $1.50

provide pertinent tools for detecting metal-binding proteins and protein fractionation.

We have now characterized and purified a newly identified iron-binding protein with a molecular mass of 16 kDa from rat liver, using the iron-blotting technique with radioactive iron. Specificity of the metal binding and intracellular localization of the protein were also given attention.

MATERIALS AND METHODS

Materials Iron-59, ferric chloride (444-1,140 MBq/mg), was obtained from New England Nuclear, diluted with 10 mM sodium citrate solution to 1 μCi/μl and stored at -10 °C until use. Chelating Sepharose was purchased from Pharmacia, and Cu^{2+}- or Fe^{3+}-bound gels were prepared according to the instruction from the manufacturer. DEAE Sephadex A-50 was purchased from Pharmacia and Blue Cellulofine from Seikagaku Kogyo. An Immobilon P membrane was purchased from Millipore. All other chemicals were of analytical grade.
Purification of Iron-binding Protein from Rat Liver Homogenates Male Wistar rats weighing 250-300 g were maintained on a normal rat diet (Oriental Yeast), killed and the livers were perfused with 1.15 % KCl and homogenized in 4 volumes of 10 mM potassium phosphate buffer, pH 6.8, containing 2 mM EDTA and 0.1 mM PMSF with a Teflon homogenizer. Homogenates were centrifuged at 12,000 x g and the resulting supernatant was frozen at -30 °C. After thawing, the solution was incubated at 70 °C for 10 min and centrifuged at 12,000 x g for 10 min after cooling on ice. To the supernatant, solid ammonium sulfate was added to 60 % saturation. After stirring for 30 min, the mixture was centrifuged at 12,000 x g for 10 min. To the supernatant was added solid ammonium sulfate to 90 % saturation, then the preparation was stirred for 60 min and was then centrifuged at 12,000 x g for 10 min. The precipitate was dissolved in 10 mM potassium phosphate buffer, pH 6.8, containing 2 mM EDTA and 100 mM NaCl, and dialyzed against two changes of the same buffer. The solution was applied to a DEAE Sephadex column (2 x 10 cm) equilibrated with 10 mM potassium phosphate buffer, pH 6.8, containing 2 mM EDTA and 100 mM NaCl. The column was washed with 3 column volumes of the same buffer, followed by 10 mM potassium phosphate buffer, pH 6.8, containing 200 mM NaCl until little protein was detected in the eluant, then the proteins were eluted with 10 mM potassium phosphate buffer, pH 6.8, containing 300 mM NaCl. The fractions containing the iron-binding protein were collected and dialyzed against 25 mM sodium acetate, pH 6.8. The obtained solution was applied to a Blue Cellulofine column (1.5 x 4 cm) equilibrated with 25 mM sodium acetate, pH 6.8. The passed-through fractions were pooled and directly applied to a Cu^{2+}-Chelating Sepharose column (1.5 x 3 cm) equilibrated with 25 mM sodium acetate, pH 6.8. The passed-through fractions were then applied to a Fe^{3+}-Chelating Sepharose column (1.5 x 3 cm). After washing the column with 100 ml of 25 mM sodium acetate, pH 6.8, the protein was eluted with 0.25 M histidine, pH 6.2, and the eluant was dialyzed against 10 mM $NaHCO_3$, pH 8.1. Protein concentration was estimated by the method of Lowry et al.(14).
Iron-binding Assay The proteins were analyzed by SDS-polyacrylamide gel electrophoresis using a 15 % gel, then transferred electrophoretically (10 V for 2 h) from the gel onto an Immobilon P membrane. The resulting membrane was incubated in 100 mM sodium acetate buffer, pH 6.5, containing 50 mM NaCl (buffer A) for 1 h to renaturate the proteins. The membrane was then incubated with ^{59}Fe-citrate (0.5-5 μCi) in buffer A for 1 h at room temperature, washed 3 times with buffer A and air-dried. Radioactivities bound to the proteins were quantified by a Fujix Bio-image Analyzer BAS2000 (Fuji Photo Film) (15).
Preparation of Antibody against Iron-binding Protein and Immunoblotting Purified iron-binding protein (100 μg) was mixed with a complete Freund's

adjuvant and injected into rabbits subcutaneously. After the third two-week interval injection of the above mixture, blood was obtained from the ear veins. Immunoglobulin was prepared as described (16). Immunoblotting was performed as described previously (16), using the antibody specific for the iron-binding protein as the primary antibody.

Subcellular Fractionation of Rat Liver Subcellular fractionation of rat liver homogenates was performed by differential centrifugation (17). The activities of cytochrome c oxidase and NADPH-cytochrome c reductase were measured, according to the methods of Griffiths and Wharton (18), and Takesue and Omura (19), respectively. The iron-binding protein was detected by immunoblotting and determined by scanning using an Advantec model DMU-33C densitometer.

Gel Filtration The purified protein (25 μg of protein) was incubated with ^{59}Fe-citrate (10,000 cpm) in 100 mM sodium acetate buffer, pH 6.5, containing 50 mM NaCl for 1 h at room temperature. The labeled protein was analyzed by HPLC with a TSK gel G2000SW column (0.75 x 30 cm, Tosoh Co.). The buffer system used was 100 mM sodium acetate, pH 6.5, containing 50 mM NaCl.

RESULTS

Characterization

To detect and characterize iron-binding protein, the homogenates of rat liver were applied to a Fe^{3+}-Chelating Sepharose column, washed and then proteins were eluted with 10 mM EDTA. Eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis and transferred onto an Immobilon P membrane. The iron-binding activity of the proteins was assessed by radioactive iron-blotting. A major iron-binding protein corresponding to a molecular mass of 16 kDa was present and other 35-, 29- and 22-kDa proteins, which exhibited weak iron-binding activities, were also observed. We then purified the 16-kDa iron-binding protein.

Purification of Iron-binding Protein.

Figure 1 shows the pattern of a Coomassie brilliant blue staining and iron-blotting of each step during the purification procedure. After the DEAE Sephadex column chromatography, the band of iron-binding corresponding to the 16-kDa protein was evident. The final preparation of the protein was apparently homogeneous. The protein was purified about 1,800-fold with a 5.7 % yield.

When estimated by gel filtration with a TSK gel G2000SW, the iron-binding protein with radioactive iron appeared as a single peak and had a molecular weight of about 16,000 (Fig. 2). The visible spectrum showed that the protein had no significant absorbance related to prosthetic groups.

Kinetics of the Iron-binding Activity

Figure 3 shows a representative double reciprocal plot (20) for the interaction of iron and the iron-binding protein. The apparent dissociation constant (Kd) calculated from Fig. 3 was 3.5×10^{-6} M, and the maximum iron/protein binding ratio was 0.43. The presence of a 100-fold ferric ion (5 μM) completely inhibited the radioactive iron-binding to the protein.

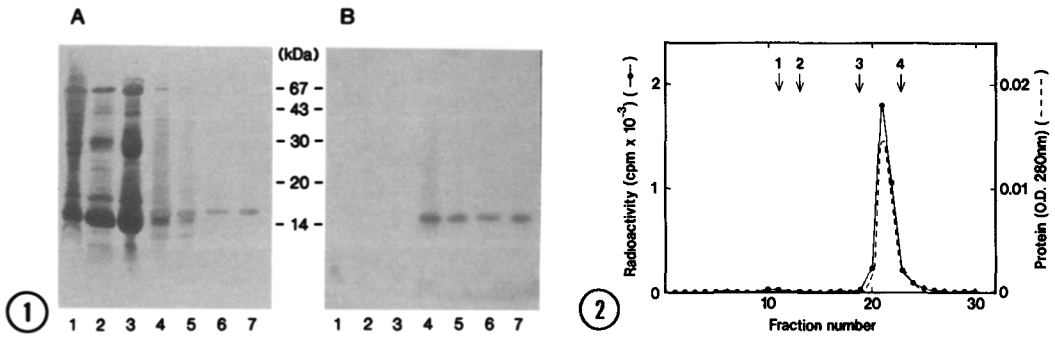

Fig. 1. Purification of the iron-binding protein from rat liver homogenates. (A) proteins were analyzed by SDS-polyacrylamide electrophoresis, using a 15 % gel and stained with a Coomassie brilliant blue R-250. (B) Iron-blotting was carried out as described under MATERIALS AND METHODS. The fractions were: lane 1, 12,000 x g supernatant (80 μg of protein); lane 2, heat treatment at 70 °C (40 μg of protein); lane 3, ammonium sulfate fractionation (60-90 %)(80 μg of protein); lane 4, DEAE Sephadex (22 μg of protein); lane 5, Blue Cellulofine (16 μg of protein); lane 6, Cu^{2+}-Chelating Sepharose (12 μg of protein) and lane 7, Fe^{3+}-Chelating Sepharose (6 μg of protein). Marker proteins used were bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and lactalbumin (14.4 kDa).

Fig. 2. Gel filtration of the ^{59}Fe-labeled iron-binding protein. The purified iron-binding protein was incubated with ^{59}Fe-citrate as described under MATERIALS AND METHODS, and applied to a TSK gel G2000SW (0.75 x 30 cm). Elution was performed with 100 mM sodium acetate, pH 6.5 at a flow rate of 1 ml/min. Radioactivity and absorbance at 280 nm were determined. Marker proteins used are shown by arrows: 1) albumin (67,000), 2) ovalbumin (43,000), 3) trypsin inhibitor (20,100), and 4) cytochrome c (12,000).

Al^{3+} inhibited the iron-binding, in a concentration-dependent manner. Ni^{2+} slightly reduced the binding (Fig. 4). Other divalent metal ions including Zn^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+} were without effect.

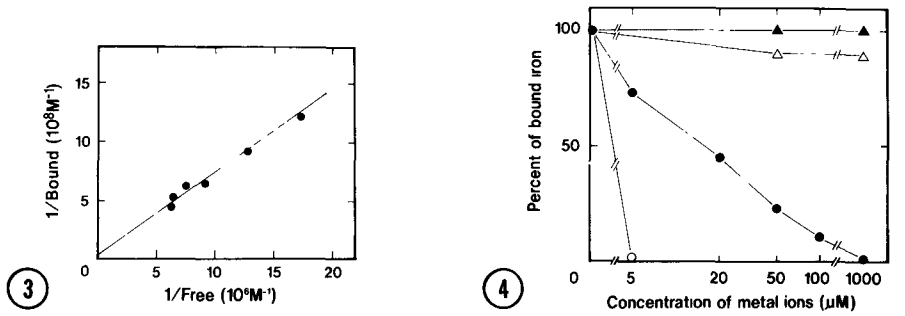

Fig. 3. Representative double reciprocal plot of the iron-binding protein. Iron-binding assay was performed using 2 μg of the purified iron-binding protein under different concentrations of ferric ion (10^{-8}-10^{-6} M). The reciprocal of the bound iron was plotted against that of unbound concentration. Data are the means of duplicate experiments.

Fig. 4. Effect of metal ions on the iron-binding. Iron-binding assay was carried out using 2 μg of the purified iron-binding protein in the presence of zinc chloride (▲), nickel chloride (△), aluminium chloride (●) or ferric citrate (○). The degree of inhibition was expressed in terms of the percentage of the ^{59}Fe-binding obtained in the absence of metal ions.

Table I. Subcellular distribution of the iron-binding protein

	Iron-binding protein		Specific activity	
	Relative amount*	% of total amount	NADPH-cytochrome c reductase**	Cytochrome c oxidase***
Nuclei and plasma membrane	20	29	0.015	1.04
Mitochondria	1.25	1	0.007	1.03
Microsomes	100	59	0.460	0.15
Cytosol	5	11	0.056	0.04

* The values were expressed by the percentage of the amount of the iron-binding protein per mg of protein in microsomes.
** μmol cytochrome c reduced per mg of protein per min.
*** μmol cytochrome c oxidized per mg of protein per min.

Intracellular Localization of Iron-binding Protein in Rat Liver

Table I shows the intracellular localization of iron-binding protein. Fifty-nine % of the iron-binding protein was found in the microsomal fraction. The relative amount of the iron-binding protein in the nuclei and plasma membrane fraction was 1/5 of that in the microsomal fraction and in the cytosolic fraction it was 1/20. The activity of NADPH-cytochrome c reductase, a marker enzyme of microsomes, was found mainly in the microsomal fraction. Consequently, most of the iron-binding protein was present in microsomes with a small amount in nuclei and plasma membranes. When the microsomes were frozen and thawed, about 40 % of the iron-binding protein was released.

DISCUSSION

This is the first demonstration of an iron-binding protein in the rat liver. Simple methods to identify metal-binding proteins included fractionation of proteins by SDS-polyacrylamide gel electrophoresis, their transfer onto membrane and incubation with radioactive metal ions (7-10). We used ^{59}Fe as a probe for protein blotting to detect and characterize iron-binding protein which was then purified to apparent homogeneity using Cu^{2+}- and Fe^{3+}-Chelating Sepharose column chromatography. Use of Cu^{2+}-Chelating Sepharose was prerequisite to remove materials that nonspecifically stick to the subsequent affinity column. The overall purification ratio was 1,800-fold with a 5.7 % yield. The protein has a molecular mass of 16 kDa, as estimated by SDS-polyacrylamide gel electrophoresis. The molecular weight estimated by gel filtration was about 16,000. Immunoblot analysis showed that the iron-binding protein is present predominantly in microsomes (Table I). Although the molecular mass of the iron-binding protein was similar to that of the light chain of ferritin, the antibody against the iron-binding protein did not

react with either light or heavy chains of rat ferritin, by immunoblotting (data not shown).

The iron-binding protein exhibited 0.43 iron binding per protein with a Kd of 3.5×10^{-6}M. The assay by gel filtration showed that the native iron-binding protein can bind ferric ion (Fig. 2).

Although the affinity for iron-binding is not so high, the binding-ability is highly specific since various divalent metal ions did not disturb the iron-binding. Al^{3+} inhibited the iron-binding of the iron-binding protein. Based on the observation that transferrin can bind not only Fe^{3+} but also Al^{3+} with an extremely high affinity (21), iron-binding protein may have an affinity for trivalent metal ions.

Conrad et al. (22) showed that mobilferrin, corresponding to a molecular mass of 55 kDa, is present specifically in the intestinal mucosa and may be involved in the intestinal absorption of iron. It was also reported that there are several iron-binding proteins associated with tumor cells (23-25) but the physiological roles of these proteins has yet to be determined. The molecular mass, the metal-binding specificity and the intracellular localization distinguished the iron-binding protein presented here from the above iron-binding proteins as well as other well known iron-binding proteins including transferrin, lactoferrin and ferritin. Ultraviolet-visible spectra of the iron-binding protein revealed that it does not retain prosthetic groups such as iron-sulfur, heme and flavins, thus, the protein is not related to cytochromes or ferredoxins.

Although little is known of the mechanisms of intracellular transport of iron, the iron-binding protein presented here may play a role in the transport of iron. Attempts to clarify physiological roles of the protein are under way.

ACKNOWLEDGMENTS. We thank M. Ohara for critical comments. This work was supported in part by grants for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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