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Purification of a Novel Glycosylated Ferritin From Horse Heart

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Abstract We have previously shown that mRNA coding for ferritin L subunit is present on both cytosolic ribosomes and endoplasmic reticulum-bound ribosomes in rat heart tissue [Campbell et al. (1989) Arch Biochem Biophys 273:89–98]; from this we infer that heart tissue is capable of making a secreted ferritin. We now report the purification from horse heart, of a ferritin that specifically binds to Conconavalin A–Sepharose and is immunologically cross-reactive with antibodies raised against both horse cellular ferritin and horse serum ferritin. Where cellular ferritin is 10 nm in diameter and contains primarily 21-kDa subunits (as determined by gel exclusion chromatography and electron microscopy), the glycosylated heart ferritin is smaller with diameters of 3–5 nm. Antisera raised against serum ferritin cross-reacted with the glycosylated heart ferritin did but did not show significant cross-reactivity with cellular ferritin thus indicating that serum ferritin and glycosylated heart ferritin have antigenic determinants which may not be present on cellular ferritin. The glycosylated ferritin also differs from cellular ferritin in subunit composition, with subunits of 66, 60.5, 53.5, 43.5, and 29.5 kDa, as shown by SDS–PAGE and Western blot analysis. Interestingly, ferritin purified from horse serum contains subunits of similar size.

Key words: serum ferritin, heart ferritin, glycosylated ferritin, horse serum, mRNA coding

Ferritin is found in most mammalian cells, where it detoxifies and sequesters iron [Munroe and Linder, 1978]. Cellular ferritin (MW ~450,000) is composed of a shell of 24 subunits of two sizes H (21,099 Da) and L (19,766 Da) surrounding a core of hydrous ferric oxide FeO(OH). The protein can contain up to 4,500 iron atoms. Subunits associate via hydrophobic interactions, forming both hydrophobic channels (lined with conserved amino acids such as leu 165) and hydrophilic channels (lined with conserved amino acids such as asp 127 or glu 130) [Thiel, 1987]. Molecules of free cytosolic Fe^{2+} are thought to enter via the hydrophilic channels and to become oxidized to Fe^{3+} as they enter the (FeO(OH)) core [Levi et al., 1988]. Tissue-specific expression of ferritin is regulated primarily at the level of translation [Munro, 1990]. Ferritin mRNA has a 5′ untranslated region that has a binding site for an iron-responsive translational repressor; this has recently been identified as aconitase [Klausner et al., 1993]. Increases in intracellular iron levels cause the repressor to dissociate and/or become degraded, which allows initiation of translation [Klausner et al., 1993]. Inflammation induces ferritin synthesis as well [Campbell et al., 1989; Madani and Linder, 1992].

Most ferritin is intracellular, but small amounts are also present in blood plasma. These levels are specifically increased in many diseases [Seligman et al., 1987; Munro, 1990] including cancer [Bomford and Munro, 1992]. Serum ferritin has a lower molecular weight (~140,000 Da) and iron content than that of cellular ferritin, and it contains some higher molecular weight subunits [Zhou et al., 1993; Linder et al., 1989]. In addition, serum ferritin has been shown to contain a glycosylated subunit [Cragg et al., 1981] which is cross-reactive with anti-ferritin L chain antibodies [Santambrogio et al., 1987].

Abbreviations used: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Con A, concanavalin A; PMSF, phenylmethanesulfonylfluoride; ER, endoplasmic reticulum; MW, molecular weight; Da, dalton; EM, electron microscopy.

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Little else is known about the primary structure of serum ferritin, its regulation, its site of synthesis, its precise function in the blood, or the mechanism by which its concentrations increase in disease.

Heart tissue from rat, horse and human has been shown to contain a ~140-kDa (7S) ferritin that is not present in spleen or liver [Linder et al., 1989; Goode et al., in preparation]. Recently, the heart has been discovered to have an endocrine function; the hormone atrial natriuretic factor [ANF] is specifically synthesized, glycosylated, and secreted by the heart [Rosenzweig, 1991]. This combination of findings prompted consideration of the possibility that the heart might be a source of serum ferritin. In previously published work [Campbell et al., 1989b], cytosolic ribosomes from rat heart tissue were quantitatively separated from endoplasmic reticulum-bound polyribosomes. The mRNA hybridizing with ferritin L-subunit was present on both ER-bound and cytsolic ribosomes, whereas ANF mRNA was present only on ER-bound ribosomes. As secreted proteins are synthesized mostly on ER-bound polyribosomes [Pfeffer and Rothman, 1987], these data indicate that heart cells are could potentially secrete a ferritin into the blood. We now report that a glycosylated ferritin has been purified from horse heart with subunits that differ in molecular weight from cellular ferritin but are similar to those of serum ferritin.

MATERIALS AND METHODS

Polyacrylamide Gel Electrophoresis

SDS–PAGE was performed on 8 × 8 cm, 0.75-mm or 1.5-mm-thick minigels in an apparatus that holds two gels (Hoefer Scientific, San Francisco, CA). Conditions for optimal separation of the ferritin subunits (19,000–23,000 Da and 53,000–63,000 Da) were developed. Separating gels were 15% acrylamide, pH 8.8 (30:0.8, acrylamide:bis-acrylamide) and stacking gels were pH 6.8, 4% acrylamide as previously described [Laemli, 1979]. Protein (1–10 μg) was solubilized in an equal volume of 0.125 M Tris–HCl buffer, pH 6.8, containing 4% SDS, 20% glycerol, and 10% β-mercaptoethanol, then boiled for 10 min. Low-molecular-weight standards (BioRad, Richmond, CA) were run simultaneously. Electrophoresis was carried out at 40 mA constant current (20 mA/gel) for 40–50 min, with constant ice water recirculation, in a pH 8.3 reservoir buffer containing 0.02 M Tris, 0.192 M glycine, and 0.1% SDS. Minigels (0.75 mm) were either silver stained according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO) or stained in 0.25% Coomassie Blue R-250, containing 40% methanol and 7% acetic acid, for 30 min with shaking. Gels were destained in 7% acetic acid and 5% methanol for 1–20 h at 25°C. Molecular weights were assigned to ferritin subunits based on the migration Rf position of standards relative to tracking dye.

Western Blot Analysis

Proteins were separated by PAGE followed by electrotransfer (BioRad TE 22 mini-transfor unit) onto nitrocellulose at 250 mA for 40 min at 15°C in 25 mM Tris/HCl buffer containing 0.14 M glycine and 20% methanol (Towbin buffer) as previously described [Towbin et al., 1979]. Nitrocellulose sheets were blocked for 1 h in 20 mM Tris buffer, pH 7.5, containing 500 mM NaCl and 3% gelatin (TBS). Blocked filters were then washed in TBS buffer containing 0.05% Tween-20 (TTBS) for 10 min with agitation. Filters were incubated overnight in TTBS containing a 1:75 dilution of α-horse serum ferritin antiserum (described below) or a 1:100 dilution of α-horse cellular ferritin antiserum (Sigma Chemical Co.). Filters were washed two times in TTBS for 10 min with agitation and incubated for 1 h in TTBS buffer containing a 1:3,000 dilution of alkaline phosphatase-conjugated goat-antirabbit antibody (Sigma Chemical Co.). This conjugate solution was decanted and filters were washed two times in TTBS for 5 min. The filter was washed in TBS for 5 min to remove residual Tween-20. A 30 mg aliquot of p-nitroblue tetrazolium chloride (NBT) or 15 mg of 5-bromo-4-chloro-3-indoyl phosphate p-toluidine salt (BCIP) was first dissolved in 1 ml 70% N,N-dimethylformamide, then added to 100 ml of 0.1 M $NaHCO_3$ pH 9.8 carbonate buffer containing 1.0 mM $MgCl_2$. The final concentrations were 0.3 mg/ml BCIP and 0.15 mg/ml BCIP. Color was developed by immersing filters in this solution for 30 min. The color development was stopped by immersing filters in water.

Purification of Ferritins From Horse Heart

Heart ferritins were isolated from 500 g of frozen heart tissue from normal horses and horses with chronic illness (obtained from Mr. Dennis Madden, Colorado State University Veterinary Teaching Hospital, Ft. Collins, CO; Pa-

thology number 91NO706). Heart tissue was thawed immediately before use and homogenized in 5 volumes of 0.03 N NaCl containing 0.02% sodium azide and 0.2 mM PMSF with three 10-s bursts in a Waring blender. The homogenate was heated, with stirring, to 70°C for 10 min, cooled on ice for 15 min, and centrifuged at 9,000*g* for 10 min to remove denatured protein (ferritin is stable under these conditions and remains in the supernatant). The supernatant from the heat treatment was acidified by the addition of 0.1 vol of 0.2 N sodium acetate buffer, pH 4.8. then centrifuged at 9,000*g* for 10 min. The supernatant from the acid treatment was titrated to pH 6.5 by the addition of 0.2 M K_2HPO_4 and heart ferritins were precipitated by the addition of ammonium sulfate at a ratio of 3.06 g/10 ml, yielding a final concentration of 50% saturation at 4°C. After 24 h at 15°C, the ammonium sulfate pellet was collected by centrifugation at 9,000*g* for 20 min. The pellet was dissolved in 5 ml of a 20 mM phosphate buffer, pH 7.0, containing 0.9% NaCl, 0.2 mM PMSF, and 0.02% NaN_3, (AcA 34 buffer), then dialyzed in three changes of the same for 24 h. This ferritin-enriched solution was applied to a 500-ml column containing Ultragel AcA 34 (LKB, Gaithersburg, MD) equilibrated with AcA 34 buffer. Five-ml fractions were collected at 25°C and monitored at 280 nm. The AcA column was calibrated with standard proteins (thyroglobulin, 690 kDa; horse cellular ferritin, 450 kDa; aldolase, 160 kDa; and albumin, 66 kDa). Fractions containing ferritin were identified by elution volume and PAGE. Further purification of ferritin was achieved by immunoaffinity chromatography (see below). The typical yield of glycosylated heart ferritin (purified from Pool II) was 4–6 mg from 500 g of heart tissue.

Purification of Serum Ferritin

Ferritin was purified from 4 liters of horse serum that was obtained from Colorado State University Veterinary Teaching Hospital. Serum was heated to 70°C for 10 min, then placed on ice to precipitate nonferritin proteins. Serum was then acidified to pH 4.8 and centrifuged at 9,000*g* for 10 min. The heat treatment was repeated once. The final supernatant was neutralized to pH 7 and ammonium sulfate precipitated as above. The ammonium sulfate pellet was dissolved in 10 ml of 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M $NaCl_2$, and 0.02% NaN_3 (Ab column buffer), followed by dialysis against 700 ml of the same overnight with three changes of buffer. Serum ferritin was purified by immunoaffinity chromatography (see next paragraph). The typical yield of serum ferritin was approximately 7.4 mg from 2.5 liter of whole blood, or 3 ng/ml.

Antiferritin Immunoaffinity Chromatography

Cellular ferritin antiserum was obtained from Sigma Chemical Co. The antigen used for this preparation was horse spleen ferritin (cellular ferritin). Contamination of this antigen with a significant amount of serum ferritin was highly unlikely due to the low concentration of serum ferritin in blood (1 ng/ml). The typical yield of cellular ferritin was 600 mg/kg spleen (Wall G: personal communication). If the spleen contained 10% contaminating blood, this would correspond to only 0.4 mg of serum ferritin. This antiserum was therefore considered to have be raised specifically against cellular ferritin. Serum ferritin antiserum was prepared as described below. Antisera were covalently attached to Sepharose (2 ml packed volume) by CNBr activation as described in the manufacturer's instructions (ImmunoPure® Antigen/Antibody Immobilization kit, Pierce Chemical Co., Rockville, IL). Ten- to 50-ml volumes of ferritin solutions were applied to the immunoaffinity column, recirculated once, then washed with 20 ml of Ab column buffer (see previously described). One milliliter fractions were collected and monitored spectrophotometrically at 280 nm. Ferritins were specifically released from the column in fractions 3 and 4 following the addition of 0.1 M glycine, pH 2.8. The column was regenerated by washing with 20 ml of Ab buffer and storing it in Ab buffer containing 0.05% NaN_3.

Preparation of Antihorse Serum Ferritin Antiserum

Five-lb New Zealand White rabbits were kept at the University of Colorado Health Science Center Animal Resource Center (Denver, CO) and antiserum prepared according to protocol number 13514792(07)1C. Serum ferritin (0.8 ml, 0.3 mg/ml) that had been purified using a cellular ferritin immunoaffinity column was combined with 0.2 ml of adjuvant (TiterMax, Hunter, Inc., Norcross, GA). On day 1 the rabbit was given four 0.25-ml subcutaneous injections (in the shoulders and haunches) of antigen/adjuvant. This was repeated on day 21 using 0.03

mg/ml serum ferritin. Two 0.1-ml subcutaneous injections of 0.03 mg/ml serum ferritin solution (without adjuvant) were given on days 28 and 29. On day 30, one 0.5-ml intraperitoneal injection of 0.03 mg/ml serum ferritin was given. On day 36, approximately 30 ml of blood was collected from the ear artery. Serum was separated by centrifugation at 2,000 rpm in a clinical centrifuge. Antiserum was shown by rocket gel electrophoresis and ouchterlony immunoprecipitation to be cross-reactive with purified serum ferritin and only slightly cross-reactive with cellular ferritin (data not shown) [Campbell et al., 1992].

Concanavalin A Chromatography

Glycoproteins with high mannose or complex-type carbohydrate side chains specifically bind to the lectin Concanavalin A (Con A). Ferritin samples were diluted in 30 ml of a 0.02 M Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3, and 0.2 mM PMSF (Con A buffer), as previously described [Campbell and Rome, 1983]. This solution was placed over a 25 ml column containing Con A–Sepharose (Sigma Chemical Co.) equilibrated in Con A buffer, and 1 ml fractions were collected. The column was washed with 15 ml of Con A buffer, followed by 10 ml of Con A buffer containing 0.1 M methyl-α-D-glucoside followed by 10 ml of Con A buffer with 0.5 M methyl-α-D-glucoside. Glycosylated proteins were specifically eluted from the column after the addition of 25 ml of Con A buffer containing 0.5 M methyl-α-D-mannopyranoside. Protein elution was monitored by absorbance at 280 nm and SDS–PAGE.

Flame Atomic Absorption

The iron content of ferritin samples was measured using a Perkin Elmer Model 5000 flame atomic absorption spectrometer set at a wavelength of 248.3 nm. The flame was set at a mixture of 50% air and 50% acetylene as described [Olson and Hamlin, 1969]. A calibration curve was established with solutions containing 1–4 ppm analytical grade ferric ammonium sulphate. Samples of cellular, serum, or glycosylated heart ferritin were diluted to a concentration of 0.01 mg/ml with water and aspirated into the flame. Five measurements were taken and averaged and iron content determined relative to the standard curve.

RESULTS

Separation of Ferritins From Normal Heart Tissue by AcA Chromatography

A ferritin-enriched fraction (composed of ammonium sulfate-precipitated proteins resistent to heating to 70°C and acidification to pH 4.0) was applied to a 500-ml column containing AcA 40 Ultragel. Five-ml fractions were collected, and absorbance was monitored at 280 nm. The void volume was approximately 85 ml, followed by the elution of nonferritin proteins (as determined by PAGE) in fractions 18 to 22 (Fig. 1A). Fractions 26–30 were combined as "Pool I," and fractions 31–34 were pooled as "AcA Pool II." The subunit composition of AcA pool I differed considerably from that of AcA pool II (Fig. 1B). Of particular interest in AcA pool II was the presence of a band triplet at 66, 60.5, and 53.5 kDa and a band at 29.5 kDa. AcA Pool I contained a prominent band at 22 kDa that corresponded in size to a ferritin H subunit [Munroe and Linder, 1975] and a 42-kDa band that corresponds in size to the H-subunit dimers previously described [McKenzee et al., 1989; Mertz et al., 1983].

In a separate experiment from that described in Figure 1, ferritin-enriched fractions were purified from 500 g of heart tissue taken from a normal horse and a tumor-bearing horse. These fractions were applied to AcA columns and fractions eluted as described above. The protein content of AcA pool II, purified from the tumor-bearing horse (Fig. 2, solid squares), was dramatically increased (~ 83%) relative to AcA pool II from the normal horse heart tissue (Fig. 2, open squares). AcA Pool I protein levels were also specifically elevated, but to a lesser degree. Hearts taken from horses which died from pneomonia, colic and viral infection also showed a similar increase (data not shown).

Electron Microscopy of Negatively Stained AcA Pool I and Pool II Preparations

Cellular ferritin can easily be visualized by electron microscopy because of its size (10 nm) and its electron-dense iron core. Cellular ferritin was present in electron micrographs of AcA Pool I (Fig. 3A, open arrows) along with some smaller ferritin molecules (Fig. 3A, closed arrows). Simultaneous elemental analysis by Edax energy dispersion spectroscopy indicated that these proteins contained iron (data not shown). Conversely, AcA Pool II contained mostly smaller,

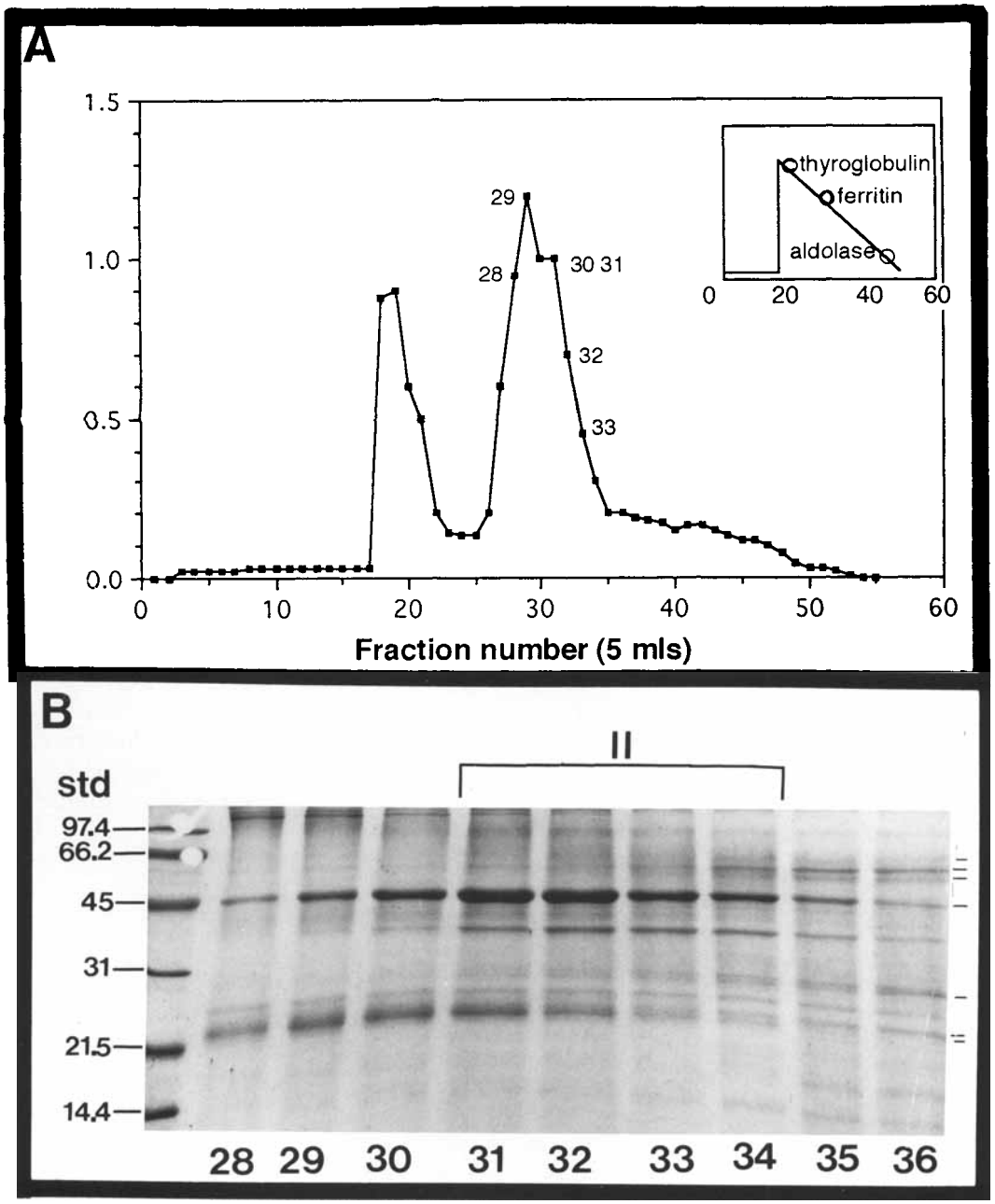

Fig. 1. Gel exclusion chromatography and SDS–PAGE of fractions. **A:** Horse heart tissue was homogenized and subjected to heat treatment and acid treatment followed by ammonium sulfate precipitation, as described in Materials and Methods. The ammonium sulfate-precipitated proteins (7 mls) were applied to a 500-ml column containing AcA 34 gel exclusion matrix. The column was run for ~18 h using a pump set at 0.5 ml/min. Eluate was monitored at 280 nm (ordinate) and 5 ml fractions were collected. Inset, elution of protein standards. **B:** For SDS–PAGE, aliquots of fractions 28–36 were boiled for 10 min in buffer containing 1% SDS and β-mercaptoethanol. Electrophoresis was carried out with a 15% acrylamide (30:0.8 acrylamide:bis) 0.75 mm mini gel at 20 mA/gel for 45 min with ice water recirculation. Gels were stained with Coomassie Blue and dried. Low MW standards ($\times 10^3$) are shown. Fractions 26–30 were pooled as AcA Pool I and fractions 31–36 pooled as AcA Pool II.

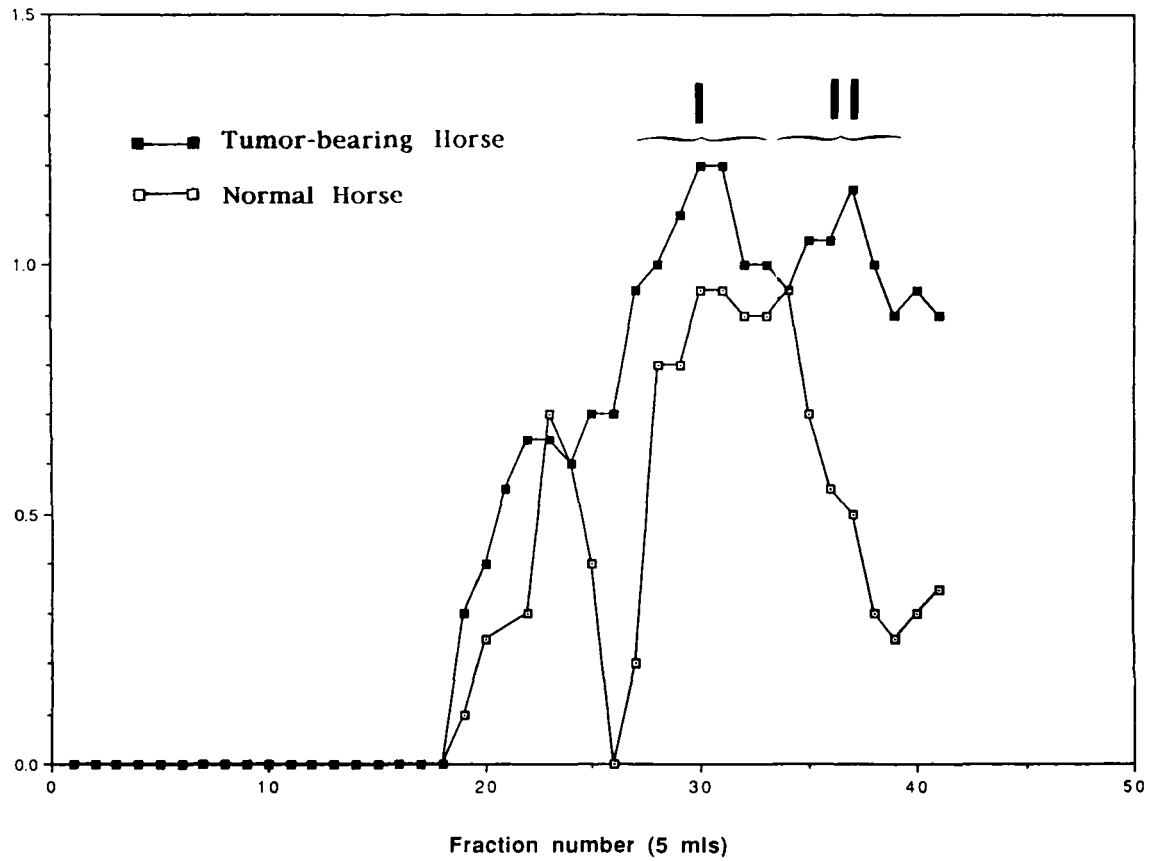

Fig. 2. Gel exclusion chromatography of samples from the normal and the tumor-bearing horse. Ferritin was purified, in parallel, from hearts taken from a tumor-bearing horse and a normal horse as described in Materials and Methods. Ammonium sulfate pellets were resuspended in buffer, dialyzed and applied to an AcA column as described in legend to Figure 1. ■, normal heart; □, heart from tumor-bearing horse.

3–5 nm, iron-containing proteins (Fig. 3B). Similar structures were found in electron micrographs of purified serum ferritin (Fig. 3B, inset). At higher magnification, the smaller ferritin had the same donut-like shape as cellular ferritin (data not shown) [Linder M: personal communication].

Immunoaffinity Purification of Ferritins

Ferritin was purified from horse serum (Fig. 4A, open squares) or the AcA Pool II (Fig. 4A, closed diamonds) by heat and acid treatment followed by immunoaffinity chromatography using immobilized cellular ferritin antiserum, as described in Materials and Methods. Ferritin was specifically eluted in fractions 22 and 23 following the addition of 0.1 M glycine, pH 2.8 (Fig. 4A, arrow). Figure 4B shows the PAGE corresponding to these fractions. Fraction 24 from the serum ferritin purification was placed next to fraction 24 from the AcA pool II ferritin purification for comparison (Fig. 4B, lanes 3 and 4, respectively). The major subunits of both ferritins had molecular weights of 66, 60.5, 53.5, 43.5, and 29.5 kDa. Serum ferritin had an additional band at 80 kD that may represent contaminating transferrin, which is present in the blood at concentrations of approximately 3 mg/ml. There were no 22 kDa "H" subunits present. Serum ferritin and cellular ferritin were found to be cross-reactive with cellular ferritin antisera by both rocket gel electrophoresis and ouchterlony immunoprecipitation (data not shown) [Campbell et al., 1992] and Western blot analysis (Fig. 6).

Immunoaffinity-purified serum ferritin was injected, as antigen, into a rabbit (see Materials and Methods) to produce antihorse serum ferritin antiserum. A serum ferritin immunoaffinity column was then prepared and used to purify ferritin from AcA Pool I (Fig. 5A) or AcA Pool II (Fig. 5B). Ferritin purified from Pool I of a normal horse heart (Fig. 5A, open squares, fractions 21–23) or Pool II (Fig. 5B, open squares, fractions 21–23) contained subunits which were identical to those shown in Figure 4 (data not shown). Interestingly, this column did not bind

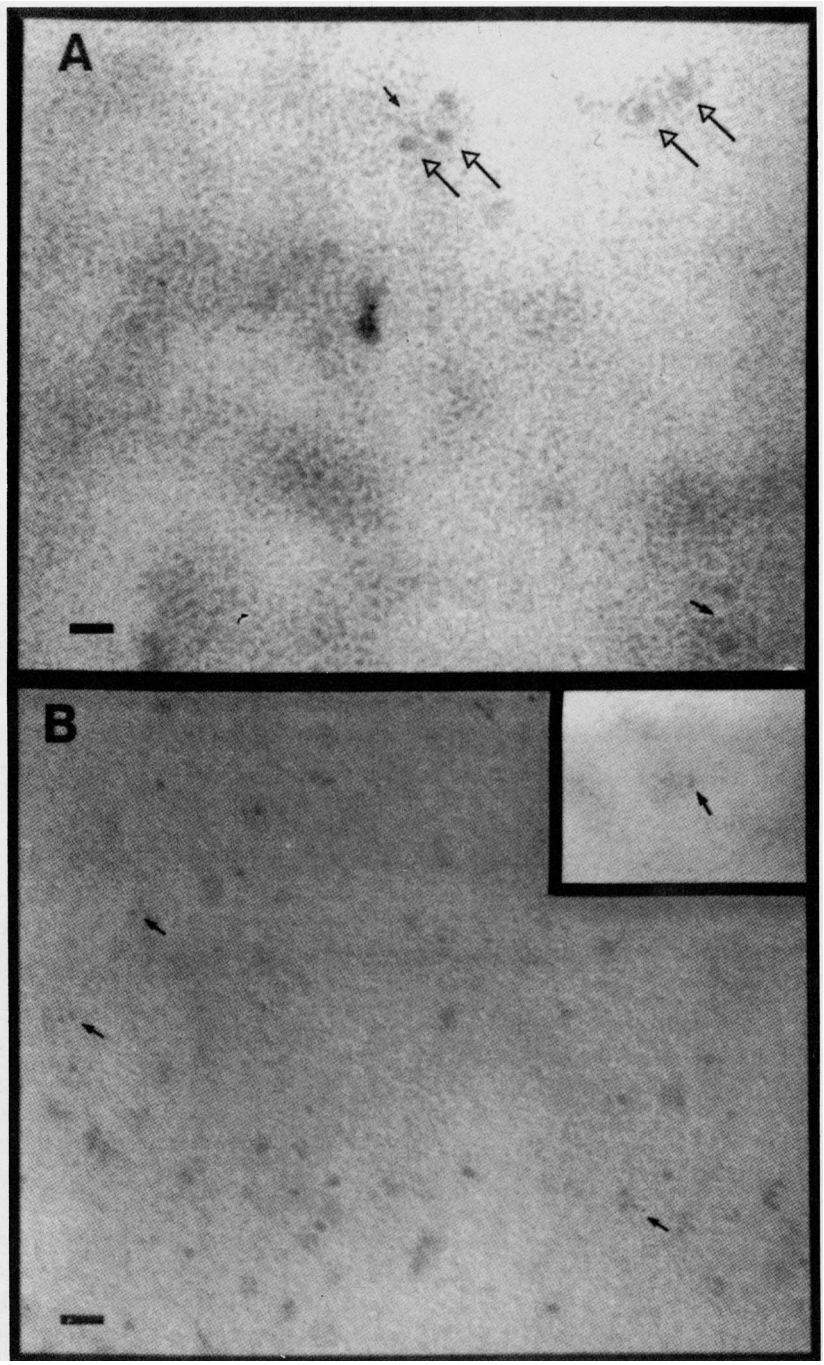

Fig. 3. Electron microscopy of negatively stained AcA Pool I ferritin and AcA pool II ferritin. Aliquots of AcA Pool I (**A**), AcA Pool II (**B**), or serum ferritin (B, inset) were applied to a carbon-coated grid and negatively stained with 2% uranyl acetate, as described [Campbell and Rome, 1983]. The samples were viewed at 100 kV on a Joel model JEM-1200 EX transmission electron microscope. Cellular ferritin (open arrows) and smaller ferritins (closed arrows) were present in A. Only the smaller ferritins were present in B. Bar = 20 nm; ×550,000.

heart cellular ferritin, which would be expected to be present in the AcA Pool I (data not shown). This column was also used to purify ferritin from the AcA Pools I and II (see Fig. 2) of a tumor-bearing (Fig. 5, closed squares). The concentration of ferritin purified from AcA Pool I (Fig. 5A, closed squares, fractions 22–23) was similar for the normal and tumor-bearing horse heart. The relative concentration of ferritin purified from AcA Pool II was higher in the tumor-bearing horse heart (Fig. 5B, fractions 22–23). Similar increases in ferritin purified from Pool

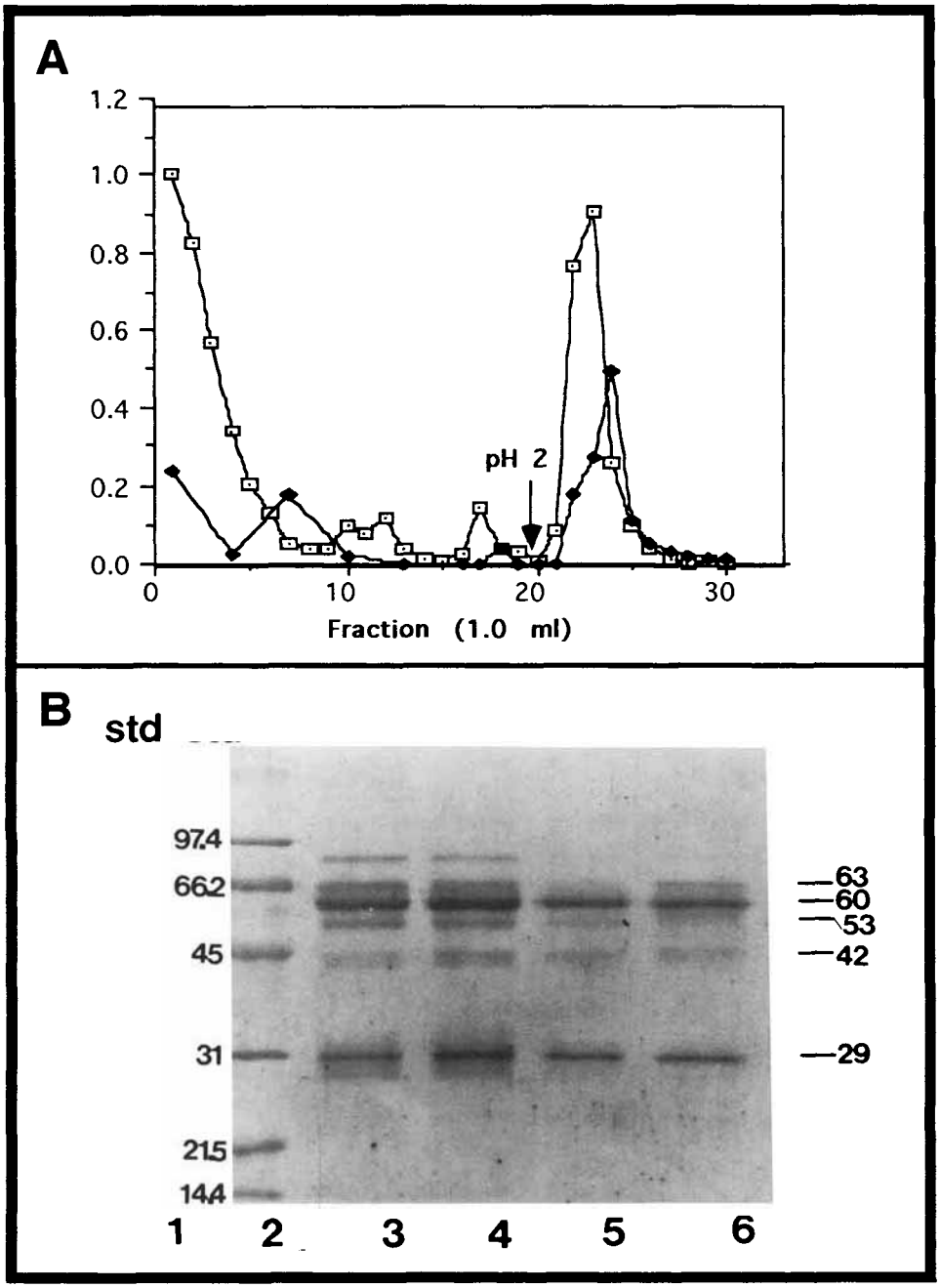

Fig. 4. Purification of ferritin from AcA pool II and serum using a cellular ferritin immunoaffinity column. **A:** Ferritin was purified by immunoaffinity chromatography from AcA Pool II (solid diamand) or serum (open square) as described in Materials and Methods. Ferritin was specifically eluted from this column following the addition of 0.1 M glycine, pH 2.8 (**arrow**). **B:** Fractions were compared by SDS–PAGE: low-molecular-weight standards $\times 10^{3}$ (**lane 1**); serum ferritin fraction 23 (**lane 2**), and 24 (**lane 3**); AcA Pool II ferritin fraction 24 (**lane 4**), fraction 23 (**lane 5**), and fraction 22 (**lane 6**).

II were seen in heart tissue from horses which died from pneomonia and viral infection (data not shown) and may not therefore be a specific response to cancer but rather disease in general.

Western Blot Analysis

Four horse ferritins (from spleen, AcA Pool I, AcA Pool II, and serum) were separated by SDS–PAGE and electrotransferred onto nitrocellulose in Towbin buffer [Towbin et al., 1979]. Duplicate filters were incubated with serum ferritin antiserum (Fig. 6A) or cellular ferritin antiserum (Fig. 6B). Both antisera reacted with 66-, 60.5-, 53.5-, 43.5-, and 29.5-kDa subunits of serum ferritin and ferritin purified from AcA Pool II (Fig. 6, lanes 4 and 3, respectively). In

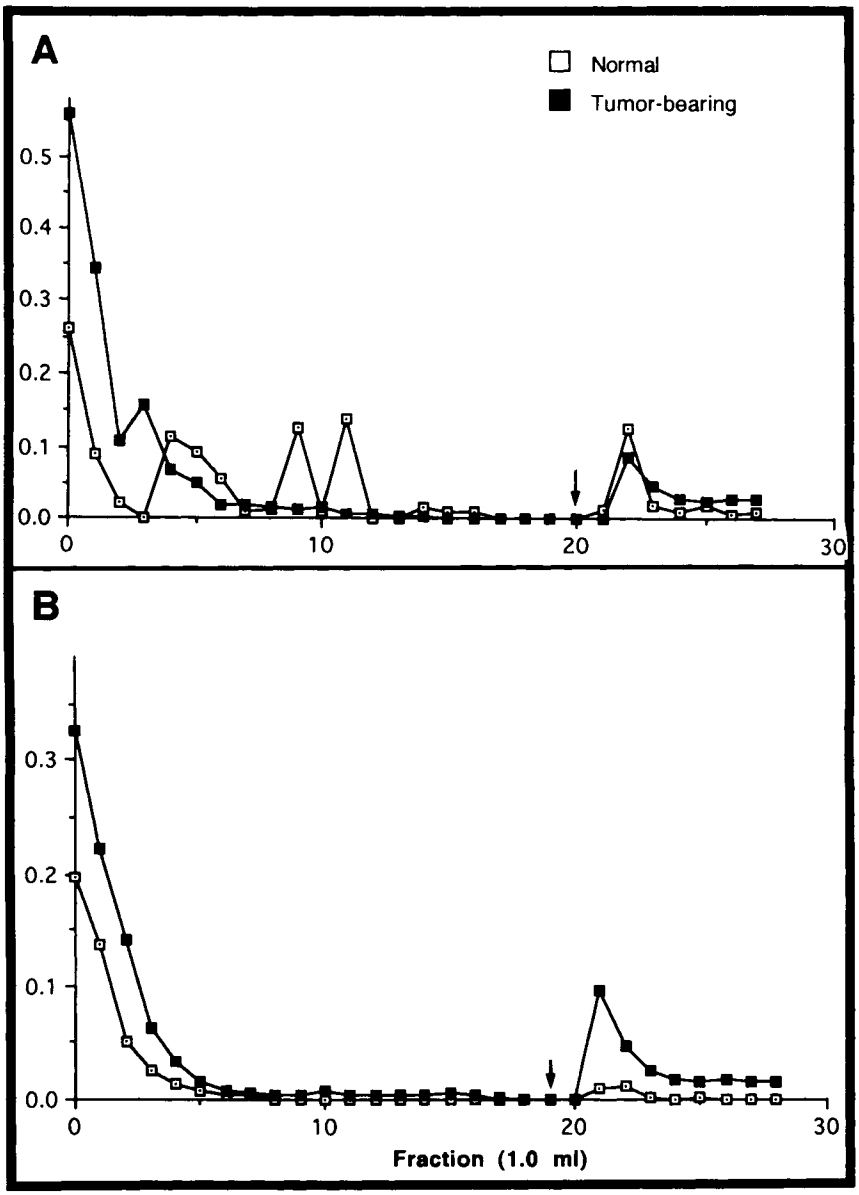

Fig. 5. Purification of horse heart ferritin using a serum ferritin immunoaffinity column. Horse serum ferritin which had been purified on a cellular ferritin immunoaffinity column (see Fig. 4) was used as antigen to prepare antiserum in a rabbit. An immunoaffinity column with immobilized serum ferritin antiserum was prepared and used to purify heart ferritin from the normal (□) or tumor-bearing horse (■) as described in Materials and Methods. **A:** AcA Pool I; **B:** AcA Pool II.

addition, the cellular ferritin antiserum, but not the serum ferritin antiserum, reacted with a cellular ferritin control (Fig. 6, lane 1) and a 22 kDa subunit present in AcA Pool I and II (Fig. 6 lanes 2 and 3, respectively).

Concanavalin A Purification of Heart Ferritin

The AcA Pool II proteins were applied to a Con A affinity column before immunoaffinity chromatography to determine whether or not a glycosylated ferritin was present. As shown in Figure 7A, most of the proteins in AcA Pool II passed through the Con A column in buffer washes and buffer containing 0.1 M glucoside (Fig. 7A, fractions 1–21). Addition of buffer containing 0.5 M glucoside did not dissociate additional proteins. Glycosylated proteins were specifically eluted from the column in fractions 39–45 following the addition of buffer containing 0.5 M mannoside. These fractions were pooled and applied to the cellular ferritin immunoaffinity column (Fig. 7B). Most of the glycoproteins passed through the column (fractions 1–10), but a portion did bind; these were specifi-

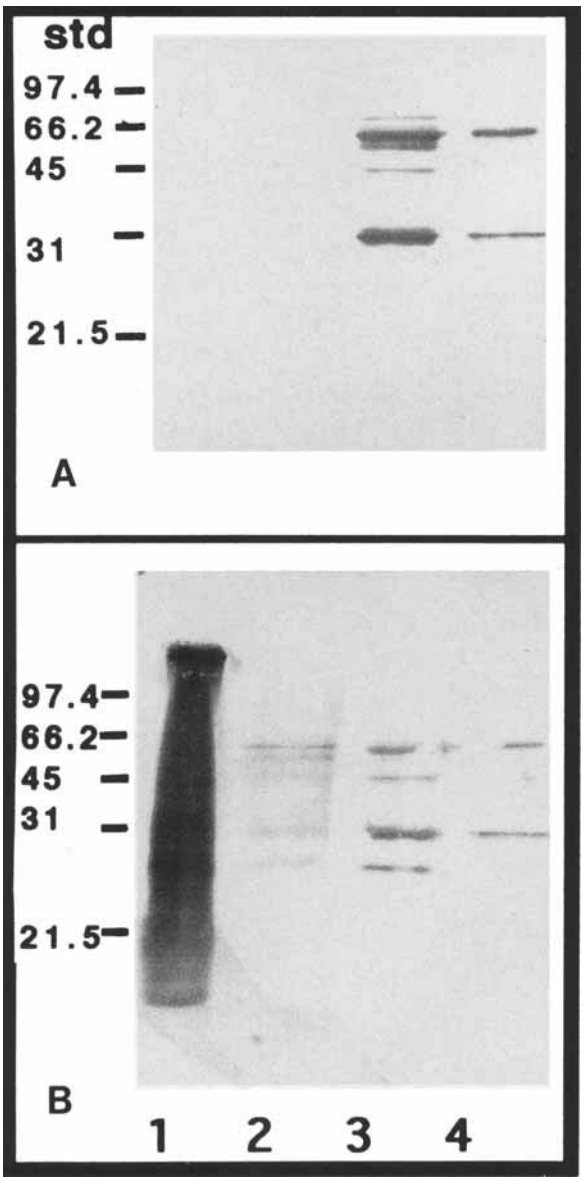

Fig. 6. Western blot analysis of immunoaffinity-purified ferritin. Ferritin was purified from AcA Pool I **(lane 2)**, AcA peak II **(lane 3)**, and serum **(lane 4)** on a cellular ferritin immunoaffinity column and compared with cellular ferritin **(lane 1)** by Western blot analysis (see Materials and Methods). **A:** Western blot using serum ferritin antiserum (prepared as described in Materials and Methods); **B:** Western blot using cellular ferritin antiserum.

cally eluted in pH 2.8 buffer (fractions 22–24). Figure 7C shows the PAGE of fractions 22 and 23 from the AcA Pool II purification (Fig. 7C, lanes 2 and 3, respectively) or the serum ferritin (Fig. 7C, lanes 4 and 5). The subunit composition of all samples was similar to those identified by Western blot analysis (Fig. 6). Similar results were seen when glycosylated ferritin was purified in the reverse order (immunoaffinity purification of serum and AcA Pool II ferritin first, then Con A chromatography; data not shown).

DISCUSSION

The field of heart biochemistry has gained much interest since the discovery of the endocrine function of the heart [Rozenzweig, 1991]. Atrial natriuretic factor (ANF) is a hormone with diuretic and natriuretic action, is specifically synthesized, glycosylated and secreted by the heart. It was previously shown that mRNA coding for ANF was present on ER-bound ribosomes in rat heart, but not on cytosolic ribosomes, as would be expected of a secreted protein [Pfeffer and Rothman, 1987]. Surprisingly, mRNA coding for ferritin was present on both cytosolic ribosomes and ER-bound ribosomes in rat heart tissue, as evidenced by hybridization with ^{32}P-labeled ferritin cDNA [Campbell et al., 1989]. The translation product of this mRNA may therefore be secreted ferritin.

Recent epidemiological data have shown that an elevation in serum ferritin levels is a primary risk factor in cardiovascular disease, although the underlying mechanism is currently unknown [Salonen et al., 1992]. Such increases, which are characteristic of many disease states, may indicate a response by the heart to that disease. For instance, a threefold increase in serum ANF concentrations was recently reported in a patient with lung cancer [Shimizu et al., 1991], a condition also known to elevate serum ferritin levels. Whether secretion of ferritin is a specific or secondary effect of the heart's response to disease remains to be seen.

The present study was designed to purify the putative translation product of ER-bound ferritin mRNA from horse heart; this would presumably be a glycosylated ferritin. Size exclusion fractionation of heat and acid-treated heart homogenates resulted in the separation of two distinct ferritins. Cellular ferritin eluted in fractions 26–30 (AcA Pool I), as expected of a 450-kDa protein (Fig. 1A), and was shown by PAGE (Fig. 1B) to contain H-chain subunits (22 kDa) and 42-kDa dimers that remained intact following boiling in β-mercaptoethanol and SDS [Linder et al., 1981; Munro, et al., 1975; Thiel, 1987; Mertz et al., 1983; McKenzie et al., 1989]. Electron micrographs of AcA Pool I revealed the presence of cellular ferritin molecules, with diameters of 10 nm (Fig. 3A, open arrows). AcA Pool II contained a smaller protein (3–5 nm) similar in size to serum ferritin (Fig. 3B, inset). This protein satisfied many criteria as ferritin: it

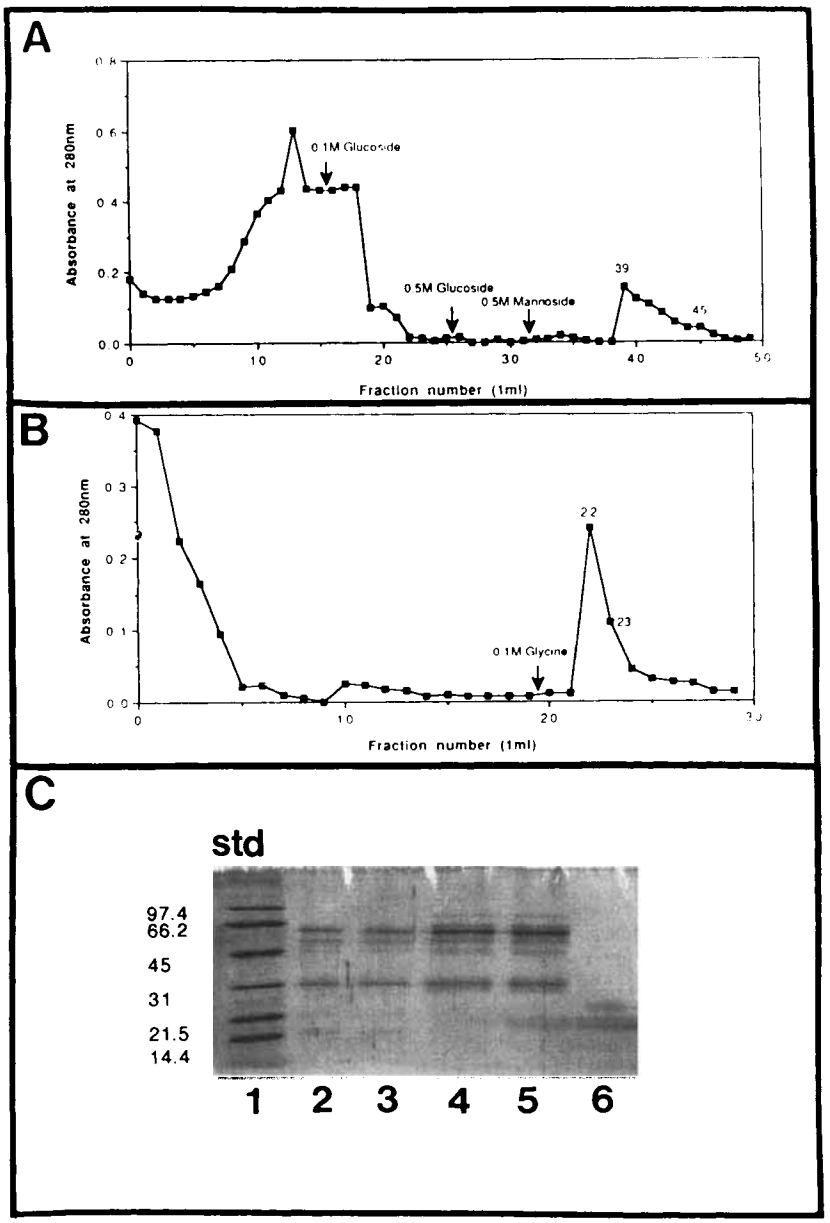

Fig. 7. Concanavalin A-purification of AcA pool II protein followed by immunoaffinity purification of ferritin. **A:** AcA Pool II was applied to a Conconavalin A (Con A) affinity column as described in Materials and Methods. Nonspecifically bound protein was removed in 0.1 M and 0.5 M glucoside (*arrows*). Specifically bound glycoproteins were eluted in buffer containing 0.5 M mannoside (*arrow*). **B:** Fractions 39–45 from the Con A column were pooled and applied to a cellular ferritin immunoaffinity column. Glycosylated heart ferritin was specifically eluted in a pH 2.8 buffer (fractions 23–25). **C:** SDS-PAGE was performed on glycosylated heart ferritin fractions #22 (**lane 2**) and #23 (**lane 3**) and compared with ConA purified serum ferritin (elution profile not shown) fractions #22 (**lane 4**) and #23 (**lane 5**). Lane 1 contains molecular weight markers ($\times 10^3$), and lane 6 is horse cellular ferritin.

was heat and acid stable (Fig. 1), it was cross-reactive with cellular ferritin antiserum (Fig. 6), and it was found to contain iron by electron microscopy/elemental analysis (Fig. 3B) and flame atomic absorption spectroscopy (Table I). This ferritin differed from cellular ferritin in many ways (as summarized in Table I): (1) it was smaller, as determined by its elution position following size exclusion chromatography (Fig. 1) and by its apparent size (3- to 5-nm diameter) on electron micrographs (Fig. 3); (2) it had subunits of higher MW than those of cellular ferritin, as shown on SDS–PAGE (Fig. 4) and Western blot analysis (Fig. 6); (3) it was

TABLE I. Comparison of Ferritin From Horse Spleen, Serum, and Heart

	Cellular ferritin	Serum ferritin	Glycosylated heart ferritin
Subunit (kDa)[a]:	19.925 ± 0.010[b]	80	
		66.0	66.0
		60.5	60.5
		53.5	53.5
		43.5	43.5
		29.5	29.5
Iron (nmole/mg)[c]	284 ± 13	39.4 ± 16 (5)	86.5 ± 16 (5)
Size (EM)[d]	10 nm	3–5 nm	3–5 nm
Serum Ft antiserum[e] cross-reactivity[f]	–	+	+
Cellular Ft antiserum cross-reactivity[f]	+	+	+
Binding to Con A[g]	–	+	+

[a]Determined by SDS-PAGE; data shown are the average of 8 values from 4 separate experiments; standard deviations are < 1%.
[b]Determined by electrospray-mass spectroscopy [Leimer et al., 1993].
[c]Determined by flame atomic absorption spectroscopy (see Materials and Methods; average of 5 measurements).
[d]Approximate diameter as seen on electron micrographs (Fig. 3).
[e]Prepared in rabbit against immunoaffinity-purified serum ferritin; see Materials and Methods.
[f]As determined by Western blot analysis; see Fig. 6.
[g]Specific binding to Concanavalin A-sepharose column; see Fig. 7.

glycosylated, as shown by binding to Con A–Sepharose (Fig. 7); and (4) it had an iron content far less than that of cellular ferritin, but similar to that of serum ferritin (Table I). These data are in agreement with those of Linder et al., who reported the purification of a small 7S heart ferritin with similar subunit molecular weights [Linder et al., 1989], apparent size on electron micrographs (Linder M: personal communication) and evidence of glycosylation [Goode et al., 1993].

Not only is the glycosylated horse heart ferritin described above distinct from cellular ferritin in size and subunit distribution; to our surprise, it has a subunit composition similar to that of serum ferritin (Table I) [Zhou et al., 1993]. The MW of these subunits, however, is based on migration during SDS-PAGE and is therefore an approximation. Experiments are now in progress to determine the precise MW of these subunits by electrospray/mass spectroscopy [Biemann, 1992]. Using a modification of the procedure described by Stachowiak et al. [1988], the molecular weight of cellular ferritin L-subunit was determined as 19.925 ± 0.010 kDa (data not shown) [Leimer et al., 1993], a value within 30 atomic mass units (amu) of the molecular weight calculated from the reported amino acid sequence of cellular L chain [Theil, 1987].

Of particular interest was the antiserum raised against purified serum ferritin. It showed remarkable specificity for serum ferritin and was only minimally cross-reactive with cellular ferritin or the Pool II H subunit on Western blots (Fig. 6). Conversely, the cellular ferritin antiserum reacted with cellular ferritin, serum ferritin, and glycosylated heart ferritin. The question that arises from these data is, why does serum ferritin antiserum preferentially recognize serum ferritin and glycosylated heart ferritin, whereas the cellular ferritin antiserum recognizes both? It is possible that both serum ferritin and the glycosylated heart ferritin are structurally different from cellular ferritin but bear a common antigenic determinant that cross-reacts with cellular ferritin antibodies. For instance, the antiserum raised against serum ferritin might be recognizing a glycosylated domain which is not be present on cellular ferritin. Studies are currently in progress to characterize the structural relationship between serum ferritin, glycosylated heart ferritin and cellular ferritin and determine the nature of the carbohydrate side chains present on the former. In preliminary studies, serum ferritin and glycosylated heart ferritin were each digested with chymotrypsin, with trypsin, and with V8 protease. The resulting peptide fragments were compared by PAGE and found to contain a high degree of homology (data not shown) [Leimer et al., 1993].

The present study has shown that horse heart tissue contains a glycosylated ferritin similar to serum ferritin. Metabolic studies in rats and subcellular fractionation studies are now required to determine whether a precursor-prod-

uct relationship exists between the two. If heart is indeed secreting serum ferritin (as with ANF), then this raises a number of questions regarding the heart's response to, and role in, the various diseases known to elevate serum ferritin levels.

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