Iron Exchange between Ferritin and Transferrin in Vitro†

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ABSTRACT: The transfer of iron between horse spleen [^{55}Fe]-ferritin and human apotransferrin or [^{59}Fe]transferrin in homogeneous solution was investigated. Transfer between the two proteins in the presence of citrate, ATP, or ascorbate occurs in both direction, but the net flow is always from ferritin to transferrin. Ferritin which is ca. 1/3 to 1/2 saturated with iron appears to be most reactive. Chemically prepared apoferritin does not accept iron from diferric transferrin. Citrate-mediated transfer of iron from ferritin to apotransferrin is first order with respect to ferritin, zero order with respect to transferrin, and has a complex dependence upon citrate concentration. Direct transfer of iron from native or reconstituted ferritin to apotransferrin in the absence of any identifiable mediating agent was observed to occur at about half the rate attained in the presence of 1 mM citrate. No transfer of iron between the two proteins occurs across a dialysis membrane in the absence of a mediating agent. No binding of transferrin and ferritin to each other was demonstrable. One possible explanation for these observations is that iron from the core of ferritin is in equilibrium with iron near the outer surface of the protein, where the metal would be available to transferrin.

More than two-thirds of the iron in a healthy adult is found in hemoglobin and myoglobin. Almost all of the remainder is found in the storage forms, ferritin and hemosiderin. The myriad of metalloenzymes accounts for only a tiny fraction of the total body iron (Lynch et al., 1974). Responsibility for shuttling iron between stores and sites of biosynthesis is vested in the protein transferrin.

Of the two storage forms, ferritin is far better characterized (Crichton, 1973; Harrison et al., 1974a). It is comprised of 24 subunits arranged to create a hollow spherical protein shell with an inner diameter of 70–80 Å, an outer diameter of 120–130 Å, and a molecular weight near 450 000 (Hoare et al., 1975). The shell is pierced by six channels through which iron can pass. The inside of the shell may contain an amount of iron ranging from zero to about 4300 atoms of iron (Fishbach & Anderegg, 1965) in the form of a ferric hydroxy oxide with some associated phosphate (Michaelis et al., 1943; Granick & Hahn, 1944). Transferrin (Aisen & Brown, 1975; Chasteen, 1977) consists of a single peptide chain of molecular weight near 81 000, on which are disposed two very similar metal binding sites.

The mechanism by which iron travels between ferritin and transferrin in vivo is unknown. Since transferrin is found in the plasma and ferritin is almost completely localized within cells, it is not known if the two proteins ever come in contact with each other. Storage iron released within the cell may find its way into transferrin only after crossing the cell membrane. However, there is some evidence that transferrin enters developing red blood cells in order to deliver iron for hemoglobin synthesis (Hemmaplardh & Morgan, 1977; Sullivan et al., 1976). It is, therefore, also possible that transferrin and ferritin may interact directly.

The present work was undertaken to characterize the interaction of these two proteins in vitro. The only previous study of this kind was reported by Miller & Perkins (1969) who examined the chelate-mediated transfer of iron across a dialysis membrane from human [^{59}Fe]transferrin to horse spleen ferritin. In the present work we have been able to characterize iron transfer in both directions between the two proteins unseparated by a membrane.

Experimental Procedure

Glassware was acid rinsed and buffers used for reactions were extracted with dithizone in carbon tetrachloride to remove traces of iron. Solutions were boiled to remove carbon tetrachloride.

Materials. Cellulose dialysis tubing was obtained from VWR Scientific Co. Chemicals were reagent grade. A-grade ATP was obtained from Calbiochem and stored at −20 °C. Rabbit anti-horse ferritin antibody was obtained as a lyophilized powder from Miles Laboratories and reconstituted with water to a concentration of 28 mg of antibody/mL. Crystalline bovine serum albumin was purchased from Schwarz/Mann. The mesylate salt of desferrioxamine B ("Desferal") was a product of CIBA Pharmaceutical Co.

Transferrin was prepared from fresh human serum (Harris, 1977) or outdated human plasma. In the latter case, the serum was first dialyzed against 0.1 M NaCl/0.01 M phosphate, pH 7.5, before ammonium sulfate precipitation. All transferrin used in this work exhibited a ratio of absorbances A_{470}/A_{410} = 1.40–1.41 in the diferric state. Chelate free apoprotein was prepared as described previously (Harris et al., 1974). Chelate free (Bates & Schlabach, 1973) [^{59}Fe$_1$]transferrin was prepared using [^{59}Fe]nitrilotriacetate (Harris & Aisen, 1975) and finally dialyzed against 0.02 M KCl/0.01 M Hepes,[1] pH 7.4. [^{59}Fe$_2$]transferrin was prepared in a similar manner, but was not rendered free of all chelate using $NaClO_4$ (Bates & Schlabach, 1973). It was freed of excess [^{59}Fe]nitrilotriacetate by passage through Sephadex G-25 (Harris & Aisen, 1975). Iron analyses were conducted as previously described (Harris & Aisen, 1975). The molecular weight of transferrin was assumed to be 81 400 (MacGillivray et al., 1977). [^{125}I]Transferrin calculated to contain an average of one iodine atom per molecule was prepared from diferric transferrin by the method of Azari & Feeney (1961).

Purification of Commercial Ferritin. Unless otherwise

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[1] Abbreviations used: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TIBC, total iron binding capacity.

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stated, all experiments were done with Pentex horse spleen ferritin (6× crystallized, cadmium removed) obtained from Miles Laboratories. The material was further purified by passage of 1-mL aliquots (~92 mg of solid) through an ascending 2.6 × 60 cm column of Sepharose 6B eluted with 0.1 M Tris, pH 7.0, at a flow rate of 20 mL/h. The center of the ferritin monomer peak (Niitsu & Listowsky, 1973) was retained and the remainder discarded. To concentrate the eluate, the combined product of two columns was adsorbed on a 1.5 × 20 cm column of Cellex-D (Bio-Rad) equilibrated with 0.1 M Tris, pH 7.0. The protein was then eluted in a narrow band with 0.5 M NaCl/0.08 M Tris, pH 7.0. In our early experiments we used Pentex 2× crystallized ferritin with no further purification. Results were qualitatively similar to those obtained with the more highly purified material. Studies of the pH dependence of ^{55}Fe transfer from ferritin to apotransferrin and the dialysis experiments were done with the 2× crystallized protein not further purified. All calculations with ferritin assume a molecular weight of 450 000.

Purification of Ferritin from Horse Spleens. Horse spleens were obtained on the same day the animal died and were kept at 4 °C for 1 day until use. All operations except heating were done at 0–4 °C. Spleen was ground in a meat grinder and homogenized for 30 s in a Waring blender with 1.5 g of water/g of spleen. The mixture was warmed to 70 °C over a period of ~6 min in a preheated vessel. It was maintained at 70 °C for 10 min and then cooled rapidly on ice. Following centrifugation and filtration through Whatman no. 1 filter paper to remove debris, ferritin was precipitated by addition of 300 g of ammonium sulfate per L while maintaining the pH at 7.0 at 0 °C. The precipitate was isolated by centrifugation and dissolved in a minimal volume of water. Following centrifugation to remove insoluble material, and dialysis against 0.1 M Tris, pH 7.0, the supernatant solution was adsorbed onto a column of Bio-Rad Cellex-D anion-exchange cellulose equilibrated with 0.1 M Tris, pH 7.0. When the entire column was red, the adsorbed material was eluted with 0.5 M KCl. After dialysis against 0.1 M Tris, pH 7.0, the red solution was centrifuged at 23 000*g* for 1 h and the supernatant recentrifuged at 103 000*g* for 3 h in a 5.5-cm long tube to pack the ferritin. Pellets were dissolved in 0.1 M Tris, pH 7.0, and passed through an ascending column of Sepharose 6B (2.6 × 60 cm) eluted with the same buffer. The center of the single, symmetrical protein peak was retained and dialyzed against 0.1 M KCl. We refer to this product as native ferritin.

Apoferritin was prepared by dialysis against three changes of 0.1 M thioglycolic acid–0.1 M sodium acetate at its own pH (4.4), followed by dialysis against three changes of 0.1 M KCl and two changes of 0.1 M imidazole, pH 7.0 (Hoy et al., 1974). The absorbance of a solution containing 10 mg of protein/mL at 280 nm in a 1-cm cell was estimated to be 8.5, based on a protein determination by the method of Lowry et al. (1951) using bovine serum albumin as standard.

[^{55}Fe]Ferritin (Hoy et al., 1974). To a solution of 68.3 μmol of ferrous ammonium sulfate in 0.5 mL of H_2O were added 75 μCi (0.11 μmol) of $^{55}FeCl_3$ (New England Nuclear) and 5 μL of solution containing 0.07 μmol of freshly dissolved ascorbic acid. After 5 min at room temperature, a solution containing 22 mg of apoferritin in 1.0 mL of 0.1 M imidazole, pH 7.0, was added. This was followed by rapid addition of 0.4 mL of 0.5 M Hepes, pH 7.5, and 1.0 mL of a solution prepared from 1.0 g of $Na_2S_2O_3 \cdot 5H_2O$ plus 0.17 g of KIO_3 in 20 mL of H_2O. The rapidly darkening solution was left at 4 °C for 1 h before exhaustive dialysis against 0.1 M KCl.

Density Gradient Centrifugation of [^{55}Fe]Ferritin. A 48% (wt/wt) CsCl solution containing 6 mg of [^{55}Fe]ferritin with an average of 1240 atoms of Fe per molecule was spun at 40 000 rpm (140 000*g*) in a 5.5-cm long, 5.2-mL tube using the SW 50.1 rotor of a Beckman L2-65B ultracentrifuge for 71 h to produce a gradient ranging from 1.35 to 1.71 g/mL. Ten fractions of approximately equal volume was collected by peristaltic pumping, leaving the red button behind. After dialysis against two changes of 0.1 M KCl, protein was analyzed by the method of Lowry et al. (1951) using bovine serum albumin as a standard, and iron was determinated as described below. The resulting fractions contained the following ratios of atoms of iron per ferritin molecule (with protein concentrations relative to fraction 5 in parentheses): 3000 atoms/molecule (0.12); 2250 (0.27); 2230 (0.46); 1950 (0.69); 1520 (1.00); 1270 (0.99); 1180 (0.67); 880 (0.56); 640 (0.53); 250 (1.81). The last fraction should contain a substantial quantity of apoferritin. It can be seen that the most probable iron loading is close to the average iron loading, but the distribution is quite broad (cf. Stefanini et al., 1976).

Ferritin Iron Analysis. Solutions to be analyzed contained ferritin plus water with a total volume of 100 μL. After 15 min in the presence of 100 μL of 12 M HCl to allow dissolution of the ferritin iron core, 100 μL of ascorbic acid (25 g/L, freshly prepared) was added. Following addition of 0.30 mL of saturated aqueous sodium acetate and 0.20 mL of ferrozine (disodium 3-(2-pyridyl)-5,6-bis(4-phenylsulfonate)-1,2,4-triazine, 5 g/L, from Sigma Chemical Co.), the absorbance at 562 nm was measured after ≥10 min. Standards were prepared from iron wire dissolved in HCl. Comparison with a sample treated with trichloroacetic acid showed that the amount of protein present in the samples did not produce any measurable light scattering due to protein precipitation.

Liquid Scintillation Counting: Demonstration of ^{55}Fe Quenching by Ferritin. Four identical 50-μL samples of [^{55}Fe]ferritin containing 1500 iron atoms per molecule were placed in scintillation counting vials. Two were treated with 50 μL of 12 M HCl and left for 2.5 h at 23 °C. The others were left at 23 °C without HCl. To each were then added 200 μL of H_2O, 50 μL of 20 mM EDTA, and 150 μL of saturated aqueous sodium acetate. Finally, 50 μL of 12 M HCl was added to the two samples not previously acidified. Both sets of samples were therefore at pH 4.2 with the same concentrations of reagents, but one set had been digested in HCl and the other had not. Following addition of 10 mL of Aquasol (New England Nuclear), all samples were counted with a Beckman LS-250 counter. The acid-digested samples produced an average of about 40% more counts than the undigested samples. All samples were colorless and none quenched external ^{137}Cs, as measured by the channels ratio method. It therefore appears that the quenching of the low energy emission from ^{55}Fe occurs within the ferritin core. In similar experiments, it was found that ^{55}Fe is not quenched when bound to transferrin.

The following method was used to count all samples in the present work: To each sample in a total aqueous volume of 0.80 mL (generally 0.05 M KCl, 0.05 M Hepes, pH 7.5) was added 100 μL of 12 M HCl. After ≥3 h at 23 °C were added 50 μL of 20 MM EDTA, 300 μL of saturated aqueous sodium acetate, and 10 mL of Aquasol (New England Nuclear) or Scintiverse (Fischer Scientific Co.) for counting.

Procedure for the Study of Reactions of [^{55}Fe]Ferritin and Apotransferrin. Chelate and fresh ascorbic acid solutions were brought to pH 7.5 before addition to each reaction. Unless otherwise noted, all reactions contained 0.10 M $NaNO_3$/0.02 M Hepes at a pH near 7.5 and were incubated at 37 °C in 0.40-mL volumes in test tubes sealed with four layers of parafilm. At the desired time, 50-μL aliquots were taken as

TABLE I: Transfer of Iron across a Dialysis Membrane.[a]

initial contents		incubation time at 37 °C (h)	final iron distribution			
			side A		side B	
side A	side B		% ^{55}Fe	% ^{59}Fe	% ^{55}Fe	% ^{59}Fe
[^{55}Fe]ferritin (0.28 mg/mL)	apotransferrin (2.8 mg/mL)	74	99.9		0.07	
[^{55}Fe]ferritin (0.28 mg/mL)	buffer	74	100.0		0.007	
buffer	[$^{59}Fe_2$]transferrin (2.8 mg/mL)	72		0.05		99.9
[^{55}Fe]ferritin (0.14 mg/mL), 1.0 mM citrate	[$^{59}Fe_2$]transferrin (1.4 mg/mL), 1.0 mM citrate	169	97	20	2.7	80
[^{55}Fe]ferritin (0.14 mg/mL), 1.0 mM citrate	1.0 mM citrate	169	98		1.6	
1.0 mM citrate	[$^{59}Fe_2$]transferrin (1.4 mg/mL), 1.0 mM citrate	169		1.2		99

[a] The concentration of ferritin, which contained an average of 1500 iron atoms per molecule, refers to the quantity of protein, not protein plus iron. The percent of each isotope refers to the percent of the total of that isotope in the experiment.

scintillation counting standards, and 300-μL aliquots were passed through a 0.7 × 2.5 cm column of Bio-Rad AG1-X4 anion-exchange resin eluted with 0.05 M KCl/0.05 M Hepes, pH 7.5. About 5 mL of eluate was collected and duplicate 0.80-mL aliquots were used for scintillation counting with acid digestion as described above. Control experiments showed that no measurable transferrin was retained by the anion-exchange column and only 0.2–0.6% of the ferritin was eluted, with 0.2% being a more typical result. No attempt was made to correct the results for ferritin being eluted with transferrin. If the KCl was left out of the eluate, only about 1/4 of the transferrin was eluted. In the absence of ferritin, all transferrin is eluted by 0.05 M Hepes, pH 7.5. Control experiments also showed no measurable elution of ferric citrate or ferric-ATP from the column by 0.05 M KCl/0.05 M Hepes, pH 7.6. One percent of ferrous ascorbate came through the column under these conditions. Heating apotransferrin with 10 mM ascorbate at 37 °C for 1 day did not alter the behavior of the protein upon polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Wu & Bruening, 1971). Nor did ascorbate release iron from diferric transferrin at pH 7.5. We found that heating ferritin to 37 °C for 1 week led to no decomposition discernible by electrophoresis in 5% polyacrylamide gels (Brewer & Ashworth, 1969). Neither did ferritin heated for 4 days at 37 °C pass through the Bio-Rad AG1-X4 resin used to separate ferritin from transferrin.

In some experiments, native ferritin without radioactive iron was used. In these cases, iron in the eluate from the Bio-Rad AG1-X4 column was analyzed as follows: 5 mL of eluate was treated for 30 min with 100 μL of solution prepared from 0.50 g of ascorbic acid, 1.0 g of 12 M HCl, and 4 mL of water. Following addition of 150 μL of 70% aqueous trichloroacetic acid and centrifugation of the precipitate, 4 mL of the supernatant solution was treated with 0.50 mL of 0.1% ferrozine in saturated aqueous sodium acetate. The absorbance was read at 562 nm after 10 min. Alternatively, iron in these solutions could be analyzed using a graphite oven in a Perkin-Elmer 306 atomic absorption spectrometer. Samples containing ~0.1 ppm Fe in 10 μL were dried at 125 °C for 20 s, ashed at 1200 °C for 60 s, and atomized at 2700 °C for 10 s. Deuterium correction was found to be unnecessary.

Procedure for the Study of Reactions of [^{55}Fe]Ferritin and [^{59}Fe]Transferrin. Reactions were carried out as described above, but with a volume of 1 mL. At the desired time, duplicate 50-μL aliquots were taken as scintillation counting standards. A 300-μL aliquot was passed through an anion exchange column and analyzed for transferrin iron content as described above. Three 100-μL aliquots were placed in separate test tubes prepared from 6-mm pyrex glass tubing drawn to a point at the bottom. Each of these 100-μL samples was immediately treated with 8 μL of rabbit anti-horse-ferritin antibody solution and allowed to stand 30 min at 23 °C. Following centrifugation and removal of the supernatant liquid, each antibody precipitate was washed 3× with 200-μL aliquots of 0.05 M KCl/0.05 M Hepes, pH 7.5. Each was then treated with 100 μL of 12 M HCl for 10 min to completely solubilize the material and digest the ferritin cores. The HCl solution was transferred to a scintillation counting vial and the test tube was washed four times with 200-μL portions of water. The HCl and washings were then treated with EDTA, acetate, and scintillation cocktail as described above. ^{55}Fe and ^{59}Fe counting references for these experiments were prepared from [^{55}Fe]ferritin and [^{59}Fe]-transferrin. Equations for the simultaneous determination of the two isotopes have been given previously (Harris & Aisen, 1975). In a control experiment, a mixture of [^{55}Fe]ferritin and [$^{59}Fe_2$]transferrin was separated using the antibody precipitation. The ^{55}Fe recovery was 103.5% and the ^{59}Fe retention was only 0.17%. The range of ^{55}Fe counts in the triplicate analyses was about 2% of the total counts.

Results

Labeling of Ferritin with Radioiron. To prepare [^{55}Fe]-ferritin we first sought to add 100 atoms of radioactive iron per molecule to commercial horse spleen ferritin containing an average of 1600 atoms of Fe per molecule. This was accomplished by oxidizing ^{55}Fe(II) with IO_3^- in the presence of $S_2O_3^{2-}$ (Hoy et al., 1974). A sample of labeled protein which had been at 4 °C for 2 weeks was then incubated with [$^{59}Fe_2$]transferrin and the iron distribution measured at the end of the experiment. It became clear that the ^{55}Fe added to the ferritin could not have equilibrated with ^{56}Fe already present because the amount of ^{55}Fe bound to the transferrin was far too high. The labeled iron must have been preferentially lost by the ferritin. This contrasts with the finding of Hoy et al. (1974) who observed randomization of 1300 atoms of radioactive iron with 1300 atoms of ^{56}Fe initially present, after 72 h at room temperature.[2] To obtain uniformly labeled protein, we resorted to adding ^{55}Fe to chemically reduced apoferritin.

[2] The source of the discrepancy between our observations and those of Hoy et al. (1974) might be that they reconstituted ferritin at room temperature and we performed this procedure at 4 °C. We learned of the effect of temperature on ferritin reconstitution after completing this work and are exploring this effect further.

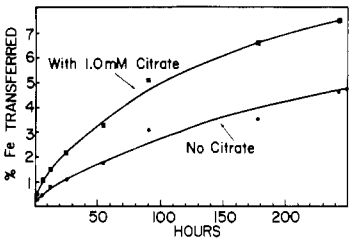

FIGURE 1: Uptake of iron by apotransferrin (5.19 mg/mL) from reconstituted ferritin (99 μg of protein/mL; 1310 Fe/molecule). This ferritin was reconstituted as described in the Experimental Procedure section, but it was prepared and dialyzed at 22 °C instead of 4 °C. The maximum iron uptake for the upper curve represents 23% of the TIBC. The curves are extrapolated back to 0.2% Fe transferred at $t = 0$ because this is the average amount of [^{55}Fe]ferritin which is eluted with transferrin.

TABLE II: Effect of Transferrin Concentration on Iron Uptake from [^{55}Fe]Ferritin.[a]

transferrin concn (mg/mL)	μg of ^{55}Fe transferred to transferrin		
	1 mM citrate 98.5 h	1 mM citrate 98 h	no citrate 96 h
1.15		0.562	0.371
1.53		0.539	
1.91	0.539	0.586	0.381
3.82	0.561	0.570	0.443
7.65	0.645	0.485	0.501
11.5	0.719	0.556	0.348

[a] Ferritin (0.16 mg protein/mL) contained an average of 1240 atoms of iron per molecule.

Analytical Techniques. Attempts to quantitatively separate transferrin and ferritin and measure their iron content were thwarted when ~10–50% of the radioactivity associated with ferritin was not eluted from columns of Sephadex G-150, G-200, Bio-Rad AG1-X4 anion exchange resin, or Bio-Gel P-300. The latter gel was particularly frustrating because on some occasions no counts were retained by the column but on most occasions ~5–30% of the counts were lost. Most of the iron retained by this column could be eluted with ascorbic acid.

We ultimately settled on separate methods for isolating each protein from a mixture. Transferrin passed cleanly through an anion-exchange resin (Bio-Rad AG1-X4) which retained ferritin and ferric chelate complexes. Ferritin could be quantitatively precipitated by rabbit anti-horse ferritin antibody. As described in the Experimental Procedure section, it was necessary to dissolve the ferritin core with HCl in order to prevent quenching of ^{55}Fe by the core itself during scintillation counting.

All of the ferritin protein determinations used in this work were done by the method of Lowry et al. (1951) using bovine serum albumin as standard. Stauffer & Greenham (1976) have reported that horse spleen ferritin gives less color than an equal mass of albumin. If their factor applies, then all values of Fe/protein reported by us should be divided by 1.38. Our values have not been corrected.

Transfer of Iron across a Dialysis Membrane. Miller & Perkins (1969) have previously shown that there is no metal transfer across a membrane from diferric transferrin to ferritin in the absence of a chelate or reducing agent. Table I shows that in the absence of a chelate very little iron passes between [^{55}Fe]ferritin and apotransferrin separated by a dialysis membrane with a nominal pore size 24 Å. In the presence of 1 mM citrate, substantial quantities of iron were exchanged between ferritin and diferric transferrin. However, when

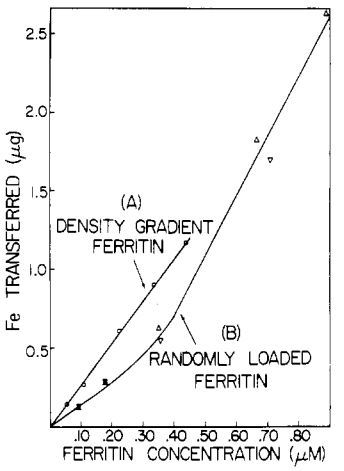

FIGURE 2: (A) Uptake of iron after 100 h at 37 °C by apotransferrin (9.18 mg/mL) from a density gradient [^{55}Fe]ferritin fraction containing 1520 Fe/molecule. (B) Uptake of iron by apotransferrin (11.5 mg/mL) from [^{55}Fe]ferritin containing an average of 1240 iron atoms per molecule. The two triangles correspond to two independent sets of experiments run for 97 h. The maximum iron taken up by transferrin represents 42% of the TIBC. Reactions contained 1.0 mM citrate.

correction is made for the amount of iron transferred by chelate alone, it appears that the iron lost by transferrin is replaced by metal from ferritin such that the transferrin remains saturated. All other experiments in this paper were done with the two proteins mixed together in one test tube.

Kinetics of Iron Release by [^{55}Fe]Ferritin. Figure 1 shows the rate of iron transfer from reconstituted ferritin to transferrin in the presence or absence of citrate. Although the ferritin used for this experiment was reconstituted at 22 °C, the same citrate curve was found with ferritin reconstituted at 4 °C. We did this experiment to see if iron transfer in the absence of chelate might cease after a certain amount of iron was removed from the ferritin. Such would be the case if there were a small amount of iron on the outer surface of the protein, not in equilibrium with internal iron. We found no evidence that iron release stops. In another experiment using the same ferritin and no citrate, 9.0% of the iron was found in transferrin after 16.2 days of incubation.

Ferritin Concentration Dependence. When increasing concentrations of ferritin randomly labeled with an average of 1240 iron atoms per molecule were incubated with apotransferrin and citrate, the complex uptake curve shown in Figure 2B resulted. However, when a relatively homogeneous population of ferritin molecules from a density gradient centrifugation was used, the reaction followed first-order kinetics (Figure 2A). For the remainder of this paper, homogeneous ferritin from a centrifugation will be referred to as "density gradient" ferritin.

Transferrin Concentration Dependence. [^{55}Fe]Ferritin was incubated with varying amounts of apotransferrin. In the presence or absence of 1 mM citrate, iron is transferred at a rate which appears to be independent of transferrin concentration (Table II).

Citrate Concentration Dependence. As indicated in Table II, no chelate is necessary for apotransferrin to receive iron from ferritin. However, chelates such as citrate increase the rate of this reaction and the complex dependence upon citrate concentration is illustrated in Figure 3.

pH Dependence. The results in Figure 4 show that the rate of iron transfer from ferritin to apotransferrin is somewhat pH dependent, with a minimum near pH 8. The increased reactivity at lower pH could be due to the greater solubility of

TABLE III: Effect of Citrate, ATP, and Ascorbate on Iron Exchange between [^{59}Fe]Transferrin and [^{55}Fe]Ferritin.[a]

entry	mediating agents	starting transferrin (Fe per molecule)	iron distribution at the end of the experiment: transferrin μg of ^{55}Fe	transferrin μg of ^{55}Fe	transferrin % ^{55}Fe	transferrin % ^{59}Fe	transferrin % TIBC	ferritin % ^{55}Fe	ferritin % ^{59}Fe	chelate % ^{55}Fe	chelate % ^{59}Fe
1	none	1	0.70	1.02	3.3	99.6	91.2	97.2	0.3	−0.5	0.0
2	imidazole	1	0.85	1.02	3.3	103.0	95.7	not measured			
3	imidazole	2	0.05	1.85	0.18	97.8	98.6	not measured			
4	citrate	2	0.47	1.30	2.1	68.6	91.7	95.5	15.6	2.4	15.8
5	ATP	2	0.54	1.26	2.4	66.6	93.3	92.9	26.5	4.3	6.9
6	citrate/ascorbate	2	0.98	0.86	4.4	45.4	95.3	44.8	20.0	50.8	65.4
7	ATP/ascorbate	2	1.02	0.75	4.6	39.9	91.7	36.0	15.5	59.4	44.6
8	citrate	1	0.86	0.94	3.9	92.1	95.2	94.3	4.9	1.8	3.0
9	ATP	1	0.79	0.99	3.6	97.3	94.2	93.1	3.7	3.3	−1.0
10	ascorbate	1	0.98	0.83	4.6	81.7	96.1	53.6	8.3	41.8	10.0
11	citrate/ascorbate	1	1.30	0.54	5.9	53.0	97.4	37.0	13.8	57.1	33.2
12	ATP/ascorbate	1	1.36	0.44	6.1	43.4	95.2	26.7	10.2	67.2	46.4

[a] Mediating agents were each present at a concentration of 1.0 mM. Each reaction was incubated for 71 h at 37 °C except for the imidazole runs which lasted 72 h. "Monoferric" transferrin starting material contained 1.08 iron atoms per molecule and "diferric" transferrin contained 1.93 iron atoms per molecule. Transferrin concentrations were 1.38–1.40 mg/mL in the reactions. Ferritin concentrations were 0.14 mg protein/mL. Different ferritin preparations contained an average of 1240–1500 atoms of iron per molecule, so the initial ferritin Fe content of solutions ranged from 21.6 to 26.1 μg Fe/mL. The iron in the chelate was taken as the difference between the total iron present and that identified in transferrin and ferritin. Percentages in this table refer to percentage of the total isotope in the reaction.

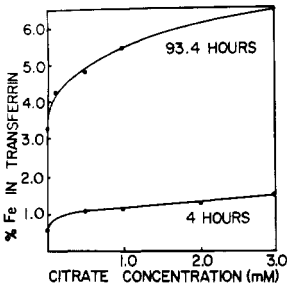

FIGURE 3: Uptake of iron by apotransferrin (7.65 mg/mL in upper curve, 10.9 mg/mL in lower curve) from [^{55}Fe]ferritin (0.14 mg of protein/mL; 1240 Fe/molecule) in the presence of citrate. The maximum iron uptake in these experiments corresponds to 13% of the TIBC.

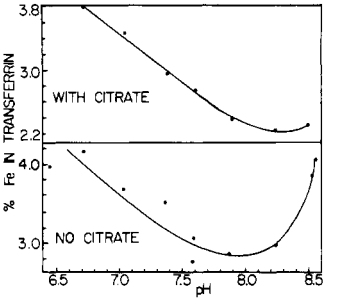

FIGURE 4: pH dependence of iron transfer from ferritin to transferrin. All samples contained [^{55}Fe]ferritin (0.14 mg of protein/mL; 1500 Fe/molecule) and apotransferrin (1.4 mg/mL) in 0.05 M $NaNO_3$/0.04 M Hepes/0.002 M imidazole whose pH was adjusted to near the desired value before protein addition. The exact pH was measured at the end of the experiment. For the lower experiment, the incubation time was 71 h. Samples for the upper experiment contained, in addition, 1.2 mM citrate and were incubated for 18 h. Control experiments showed that, at the lowest pH used, no measurable [^{55}Fe]ferritin came through the Bio-Rad AG1-X4 column used for analysis. The maximum transferrin saturation at the end of either experiment represents 54% of the TIBC.

Fe(III) at low pH. This would not explain the apparent increase in reactivity above pH 8. In contrast, Miller & Perkins (1969) observed a maximum rate of transfer from [$^{59}Fe_2$]-transferrin to ferritin near pH 7.3.

Reactions between [^{55}Fe]Ferritin and [^{59}Fe]Transferrin. Table III shows the results of reactions between [^{55}Fe]ferritin and monoferric or diferric [^{59}Fe]transferrin. Entries 1 and 2 show that, in the absence of a good iron chelate, monoferric transferrin becomes saturated with ^{55}Fe from ferritin but virtually none of the ^{59}Fe from transferrin goes to ferritin. Entry 3 shows that in the absence of a good mediator very little exchange of iron takes place between ferritin and diferric transferrin. In entries 4–7, very substantial iron exchange occurs between ferritin and diferric transferrin in the presence of chelate (citrate or ATP) or chelate plus reducing agent (ascorbate). As shown by Miller & Perkins (1969), we believe that ATP serves only as a chelate in these experiments, and not as an energy source. Entries 8–12 suggest that monoferric transferrin holds its iron more tenaciously than diferric transferrin, at least in the presence of chelates. The presence of ascorbate leads to significant iron removal from monoferric transferrin. In all reactions, the transferrin ends up saturated with iron, and in most cases the chelate contains a substantial quantity of iron. Contrary to the preliminary evidence of Miller & Perkins (1969), uptake of iron by transferrin does not stop when the protein becomes half saturated.

Dependence on Ferritin Iron Content. A series of experiments was undertaken to learn the effect of ferritin iron content on the reactions of apotransferrin or $^{59}Fe_2$ transferrin with [^{55}Fe]ferritin. In Figures 5A and 5B it can be seen that, when apotransferrin was incubated with density gradient ferritin at either constant ferritin protein concentration or constant initial ferritin iron concentration, ferritin with ~2000 Fe/molecule is the most reactive. (If the correction of Stauffer & Greenham (1976) is applied to our protein analyses, the maximum occurs near 1400 Fe/molecule.) Figure 5C shows the results of ATP-mediated exchange of iron between density gradient [^{55}Fe]ferritin and [$^{59}Fe_2$]transferrin. Although considerable exchange occurs, the transferrin remains saturated and the ferritin suffers a small net loss of iron to chelate. When [$^{59}Fe_2$]transferrin (1.94 mg/mL) was incubated with chemically reduced apoferritin (0.14 mg/mL) for 71 h at 37 °C in the presence of 1 mM citrate, no detectable iron (<0.2 atom per ferritin molecule) was transferred to ferritin. Similar results were reported by Miller & Perkins (1969). We therefore find that at no value of ferritin iron content is there a net flux of iron from transferrin to ferritin.

Comparison of Native and Reconstituted Ferritin. We used

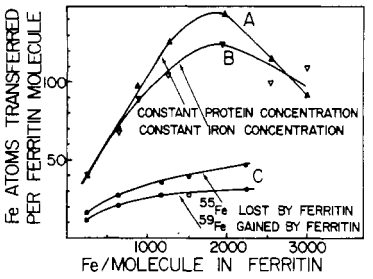

FIGURE 5: (A) Iron transfer from density gradient [^{55}Fe]ferritin to apotransferrin (3.06 mg/mL) in the presence of 1.0 mM citrate. Reactions were run at a constant ferritin protein concentration (50 μg/mL) for 202.5 h. (B) Reactions were performed as in A for 185 h, but at a constant ferritin iron concentration (7.28 μg/mL). The maximum iron uptake by transferrin in A or B corresponds to 28% of the TIBC and the maximum fraction of iron lost by ferritin is 16%. (C) Iron exchange between density gradient [^{55}Fe]ferritin (0.14 mg of protein/mL) and [$^{59}Fe_2$]transferrin (1.4 mg/mL) in the presence of 1.0 mM ATP after 72 h. The ^{55}Fe lost by ferritin is the amount found in transferrin.

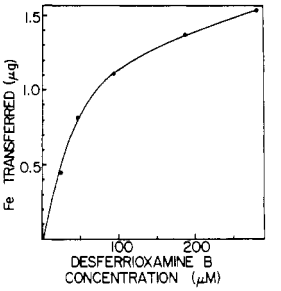

FIGURE 6: Uptake of iron after 11.7 h at 37 °C by desferrioxamine B from [^{55}Fe]ferritin (0.356 μM) containing an average of 1240 Fe/molecule. Solutions were analyzed by passing them through the same anion exchange columns used for transferrin reactions. The reaction volume was 0.40 mL and the maximum iron uptake observed in this experiment corresponds to 16% of the total iron present.

ferritin reconstituted from chemically reduced apoprotein plus Fe(II) in order to get material uniformly labeled with radioiron. Macara et al. (1972) have presented evidence from gel electrophoresis, electron microscopy, and x-ray diffraction that the iron in such a preparation is actually inside the protein. However, we have observed that ferritin reconstituted at 4 °C gives up iron two to eight times faster than native ferritin to nitrilotriacetate or thioglycolate (M. L. Bertrand & D. C. Harris, submitted for publication). In view of this, we felt that it was possible that our reconstituted ferritin might be more reactive toward transferrin than would be native ferritin.

To test this possibility, samples of native or reconstituted ferritins of similar iron content were incubated with transferrin at 37 °C in the absence of any mediating agents. In three experiments with ferritin from two different spleens, the native ferritin was 20–30% *more* reactive than reconstituted ferritin. In one experiment using 1 mM citrate as mediating agent, the native protein was 25% more reactive than reconstituted protein. It appears, therefore, that, while reconstituted ferritin is not closely comparable to native protein in "fast" reactions with nitrilotriacetate or thioglycolate, the two proteins are nearly equally reactive in "slow" reactions with transferrin or citrate.

Failure of Transferrin to Bind to Ferritin. A solution containing [^{125}I]apotransferrin (0.9 mg/mL) and ferritin with about 1800 Fe/molecule (13 mg of protein/mL) in 0.1 M $NaNO_3$/0.02 M Hepes, pH 7.5, was centrifuged at 110 000*g* for 3 h at 20 °C to pellet 95% of the ferritin. The supernatant showed no loss of counts and the pellet contained no more radioactivity than could be accounted for by its volume. Similar results were obtained with monoferric transferrin labeled with ^{59}Fe instead of ^{125}I. We conclude that there is no strong interaction between the two proteins. The association constant for complex formation between the two proteins must be less than 10^3 M^{-1}.

Reaction of Ferritin with Desferrioxamine B. [^{55}Fe]Ferritin was incubated with varying concentrations of the strong ferric chelator, desferrioxamine B. As shown in Figure 6, the amount of iron uptake by desferrioxamine depends on the chelate concentration, and is much more rapid than iron uptake by transferrin. For example, at a concentration of 100 μM, desferrioxamine takes 1.1 μg of iron from ferritin in 12 h. At the same concentration, transferrin takes about 0.5 μg in 96 h.

Discussion

Iron Exchange between Transferrin and Ferritin. Our results present a picture of unidirectional net transfer of iron from ferritin to transferrin at all levels of ferritin iron saturation. The back reaction is significant, but only as a means of iron exchange. It is noteworthy that the flow of iron from reticuloendothelial stores to transferrin in vivo appears to be a unidirectional process, with iron going only from cells to transferrin (Lynch et al., 1974).

Although the rate of iron transfer is increased in the presence of chelating and reducing agents, transfer is observed in the absence of any identifiable mediating agent. The fact that no transfer of iron across a dialysis membrane is observed in the absence of mediating agents suggests that there is not any significant quantity of free chelate (such as thioglycolate or its oxidized product) remaining with the ferritin from its previous handling. We cannot exclude the possibility that some chelate is tightly bound to the ferritin. However, native ferritin which had not been exposed to any reducing or chelating agents (except Tris buffer) was able to donate iron directly to transferrin. We cannot rule out the possibility that the protein shells of some ferritin molecules are broken and that transferrin gets iron from these molecules only; but we do not detect any damaged protein by gel electrophoresis. We have observed iron transfers as large as 10% which would represent a great deal of damaged protein.

The smallest diameter of transferrin is estimated to be ~50 Å (Rosseneu-Motreff et al., 1971) and the diameter of the six channels through the ferritin protein shell ranges from 9 to 17 Å (Harrison et al., 1975; Hoare et al., 1975). Since it is therefore unlikely that transferrin can gain direct access to the ferritin core, we suggest that ferritin is capable of making iron available for release at or near the protein surface. This is consistent with a model of the type proposed by Crichton & Roman (1977) in which iron from the ferritin core is in equilibrium with iron bound to the protein. Since the process we observed is zero order in transferrin, the slow step cannot involve iron binding by transferrin. The rate-determining step could be mobilization of iron from the ferritin core to the ferritin surface. If transferrin then picks up the surface iron, it does so without tightly binding to the ferritin. The action of chelating agents in promoting iron release by ferritin does depend on chelate concentration, as found previously (Pape et al., 1968). It is possible that small chelates penetrate deeper into the ferritin channels than can transferrin. In regions accessible to these smaller molecules, but not to a molecule as large as transferrin, the chelates might actively participate in iron mobilization. This suggestion is consistent with the differences in rates of iron mobilization between chelates and transferrin, and is also consistent with the observation of large

differences between different chelates such as nitrilotriacetate and citrate (Pape et al., 1968).

Throughout this work we have been bothered by the possibility that the iron transferred from ferritin to transferrin might represent iron bound on the outer surface of the protein, not in equilibrium with internal iron. We cannot rule out this possibility, but the evidence suggests that this is not the case. While it is very possible that artificial reconstitution could result in Fe(III) precipitated on the surface of the protein, it is hard to see why native ferritin should contain a substantial amount of external iron. Yet native ferritin was able to denote slightly *more* iron than reconstituted ferritin in the absence (or presence) of citrate. It also seems unlikely to us that a single molecule of ferritin would contain 150–200 atoms of external iron, which was the typical amount taken up by transferrin from native ferritin. When we became aware of the importance of temperature on ferritin reconstitution, we reconstituted one batch at room temperature instead of 4 °C. The rate of iron transfer from this ferritin to transferrin in the absence of mediators was similar to that observed with native protein or samples reconstituted at 4 °C. If these samples contain external iron, they all contain a large amount of it.

In other work (M. L. Bertrand & D. C. Harris, submitted for publication) we have observed an initial "burst" of iron liberation from ferritin by the reducing agent, thioglycolate. Approximately 5–10% of the iron from native or reconstituted ferritins was released in ≤50 s under conditions where the remainder was released at a rate of approximately 30% per h. An effect about one-tenth of this magnitude was found when bipyridyl was the releasing agent (Crichton & Roman, 1977) and 12–120 atoms of rapidly released iron per molecule have been observed with 1,10-phenanthroline (Harrison et al., 1974b; P. M. Harrison, personal communication). Since the iron taken up by transferrin in the present experiments was generally 3–6% of the ferritin iron, it is likely that the iron comes from the same "loosely bound" pool as that responsible for the "burst" observed in the kinetics experiments cited above. The nature of this iron is unknown.

It is of interest that May & Fish (1977) have interpreted experiments with ^{14}C-labeled compounds to indicate that diffusion of small molecules such as glucose into and out of ferritin is much too slow to account for observed rates of iron mobilization from the ferritin core by chelates or reducing agents. They suggested that the protein must be involved in translocation of iron into and out of ferritin. (By contrast, Stuhrmann et al. (1975) have interpreted neutron scattering results to indicate that glucose diffuses into ferritin in <10 s.) As found previously by Harrison et al. (1974b), we observed that the availability of iron from ferritin is dependent on the iron content of the core, with a maximum availability observed for protein approximately $\frac{1}{3}$ to $\frac{1}{2}$ saturated with iron (Figure 5). If iron is available at the protein surface, the data seem to require a synthesis of iron uptake and release mechanisms involving iron binding and transport by the protein and mechanisms proposed by Harrison et al. (1974b) involving nucleation sites on the iron core crystallites.

Comparison of Desferrioxamine B and Transferrin. Desferrioxamine B is a potent drug used in chelation therapy of iron-overloaded patients (Anderson & Hiller, 1977). Its strength as an iron chelate is such that it will take iron from ferritin or transferrin at physiologic pH (Wöhler, 1963; Pollack et al., 1977). Since hexadentate ferrioxamine B has a diameter of about 13 Å (Harrison et al., 1975), we thought that desferrioxamine might not be able to penetrate the ferritin channels and perhaps desferrioxamine would exhibit the same kinetic features as transferrin. Figure 6 shows that this idea was clearly wrong. The reaction is markedly dependent on desferrioxamine concentration and is very much faster than the reaction with transferrin. This contrasts with the transferrin concentration independence in Table II. The curvature in Figure 6 might represent saturation of the ferritin by desferrioxamine or it could represent iron mobilization from two different pools of different reactivity. The behavior of desferrioxamine suggests that this molecule plays an active role in the removal of iron from ferritin.

Acknowledgments

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Hemocyanin from the Australian Freshwater Crayfish *Cherax destructor*. Characterization of a Dimeric Subunit and Its Involvement in the Formation of the 25S Component†

Peter D. Jeffrey,* Denis C. Shaw, and G. Barbara Treacy

ABSTRACT: The molecular weight of a dimeric subunit, M_3', isolated from *Cherax destructor* hemocyanin has been measured by sedimentation equilibrium to be 144 000. Peptide mapping and end-group analysis together with gel electrophoresis show that the dimer consists of two very similar or identical monomers, cross-linked by disulfide bridges. Dissociation of the 25S component of the hemocyanin shows that it contains the dimer and two previously identified monomers, M_1 and M_2. Its molecular weight is 900 000 by sedimentation equilibrium, and reconstitution studies show that the dimer is essential for its formation. Analysis of the results of polyacrylamide disc gel electrophoresis experiments with the 25S component indicates that it consists of a population of 11 compositional isomers. These all contain one dimeric subunit and ten monomeric subunits, the latter being present in all the combinations of M_1 and M_2.

Evidence for the existence in *Cherax destructor* hemocyanin of a subunit of about twice the molecular weight generally accepted for the monomer of arthropod hemocyanins has been presented previously (Murray and Jeffrey, 1974). It was reported that dissociation of aggregated forms in whole serum at high pH, or in sodium dodecyl sulfate, gave rise to three different subunits. These were detected by polyacrylamide disc gel electrophoresis and were denoted M_1, M_2, and M_3'. The molecular weights of M_1 and M_2 were estimated by gel electrophoresis to be in the range 70 000–80 000, a more accurate value of 74 700 being measured by sedimentation equilibrium for M_1, which could be isolated in pure form. The molecular weight of M_3' was found by gel electrophoresis to be 132 000 in the absence, and 190 000 in the presence, of sodium dodecyl sulfate. Further dissociation of M_3' could be effected by the inclusion of dithiothreitol in the incubation with sodium dodecyl sulfate to produce a subunit, denoted M_3, of molecular weight about 84 000. It was concluded that M_3' was likely to be a dimer of this subunit which, as a subsequent study showed (Jeffrey et al., 1976), differed in amino acid composition from M_1 and M_2.

The dimeric subunit M_3' was observed following dissociation of the 25S component of *C. destructor* hemocyanin; it was not observed when the hexameric 17S component was dissociated (Murray and Jeffrey, 1974). Further, reconstitution studies showed (Jeffrey et al., 1976) that the hexameric component was the largest aggregate produced when only the monomer M_1 or M_2 was present. These findings suggested that M_3' was necessary for the formation of the higher aggregates, notably the 25S component. We present here the results of our studies on some properties of M_3' and its role in the formation and composition of the 25S component, which accounts for about 50% of the hemocyanin present in the native serum of *C. destructor*.

Experimental Section

Preparation of Hemocyanin Components. Serum was prepared from *C. destructor* hemolymph as described previously (Murray and Jeffrey, 1974) and stored under toluene at 5 °C. All of the hemocyanin components to be discussed were isolated from serum pooled from several animals.

The monomer, M_1, was separated on Sephadex G-200 as described before (Jeffrey et al., 1976) or by column electrophoresis on polyacrylamide gel using the LKB 7900 Uniphor apparatus. In the latter method, a 10-cm column of 5% polyacrylamide gel in 0.025 M glycine at pH 10.1 containing 0.1 mM EGTA[1] was used. The reservoir contained 1.5 L of the

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[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

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