IRON TRANSPORT FROM SEPHAROSE-BOUND TRANSFERRIN

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SUMMARY. To ascertain whether transferrin need enter the reticulocyte to deliver its iron after the association of transferrin with the cell membrane, {^{125}I,^{59}Fe-}labeled transferrin was covalently bound to Sepharose beads. Iron uptake from Sepharose-bound transferrin into rabbit reticulocytes was about 9% that from free transferrin while heme synthesis was more efficient at nearly 19%. Similar results were obtained with murine transferrin and murine reticulocytes.

These results indicate that the entrance of transferrin inside the cell is not an obligatory step in the process of iron uptake in rabbit and murine reticulocytes.

The first step in the interaction of reticulocytes with transferrin is the association of transferrin with the cell surface. There is considerable divergence of opinion regarding the subsequent mechanism by which iron is transported in the cell. One postulate, as presented by Jandl and Katz (1) is that transferrin interacts with the reticulocyte surface and delivers iron to the cell membrane without entering the cell. Speyer and Fielding (2) have proposed that iron is accepted on the membrane, passes through a series of membrane proteins in a sequential fashion and is then carried to the mitochondria by a cytoplasmic protein. Workman and Bates (3) have identified a small molecular weight protein in the cytosol capable of mobilizing iron from reticulocyte plasma membranes. A second hypothesis suggests that the normal pathway of iron uptake involves the entrance of transferrin into the cell by micropinocytosis (4,5), followed by the interaction of Fe-transferrin with the mitochondria (6). This hypothesis is supported by the electron microscopic studies of Morgan and Appleton (4) demonstrating that 35% of ^{125}I-labeled transferrin cell-associated grains are located over the cytosol of rabbit reticulocytes. Sullivan et al (5) have used ferritin labeled rabbit anti-rat transferrin

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antibody to demonstrate pinocytotic vesicles containing transferrin within rat reticulocytes. Moreover, Koller et al (6) have recently demonstrated that transferrin can interact directly with rat liver mitochondria, releasing its iron in an energy-dependent process.

To ascertain whether transferrin need enter the reticulocyte to deliver its iron, we covalently bound {^{125}I, ^{59}Fe-}labeled transferrin to Sepharose beads and used this immobilized transferrin to study iron uptake and iron incorporation into heme in rabbit and murine reticulocytes.

MATERIALS AND METHODS.

Rabbit and murine transferrins were purified and labeled with ^{125}I by the lactoperoxidase method as previously described (7). Rabbit transferrin was derived either from Research Plus Laboratories (Danville, N.J.) or from heparinized plasma of New Zealand female rabbits. Mouse transferrin was purified from heparinized plasma of CD_1 virgin female mice (Charles River Laboratories, Wilmington, MA). ^{125}I-labeled transferrin was labeled with ^{59}Fe by the method of Bates and Schlabach (8). After extensive dialysis the iron saturation was measured by the characteristic absorbance at 470 nm (8). An aliquot of double labeled transferrin was covalently linked to CNBr-activated Sepharose 4B (Pharmacia, Upsala, Sweden) by the method of Cuatrecasas et al (9) using 10 mg transferrin per gram of CNBr-activated-Sepharose. The procedure was modified to avoid washing the covalently bound transferrin with low pH buffers since this might release iron from the transferrin. Hence the washing procedure consisted of many alternating washes of the resin with 0.1 M borate-1M NaCl (pH 8.0) and 5 mM phosphate-0.15 M NaCl (pH 7.2) at 37°C. The Sepharose-bound transferrin was then loaded into a small column and washed continously with 5 mM phosphate-0.15 M NaCl (pH 7.2) for 18 hours at room temperature. The efficiency of the coupling of transferrin to Sepharose was 75%, with the bound transferrin retaining all of its original iron.

Rabbit reticulocytes were produced either by frequent phlebotomy or by the daily injection of acetylphenylhydrazine, 10 mg per kg body weight for 3 days followed by two days at a dose of 5 mg per kg. Reticulocyte percentages were 75% and 55% respectively on the third and fourth days after the last injection of acetylphenylhydrazine and cells were harvested on either day. Mouse reticulocytes were produced by the schedule previously described (10); reticulocyte counts of 30-35% were normally obtained.

About 1 x 10^8 red blood cells in a final volume of 200 µl of phosphate buffered saline (pH 7.2) were incubated either with transferrin in solution or Sepharose-transferrin at final concentrations of both of 10 µM, at 37°C with vigorous shaking in a giratory waterbath (New Brunswick Scientific). In all incubations the homologous transferrin and reticulocytes were used. At the indicated time the cells were diluted with phosphate buffered saline and were separated from the sepharose beads by filtration through 15 µ mesh Nitex cloth (Tobler, Ernst and Traber, Elsford, NY) secured to the bottom of 1 cc disposable syringes. Sepharose-bound transferrin and soluble transferrin-incubated cells were then washed three times with a large excess of cold phosphate-buffered saline. Radioactivity in one aliquot of cells was determined in a Nuclear Chicago

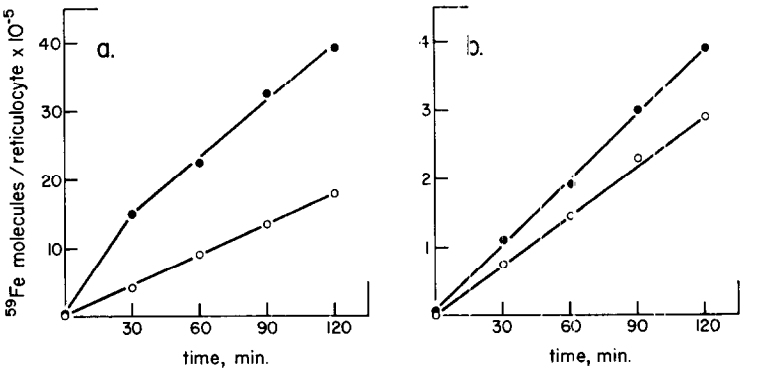

Figure 1 Iron uptake from free (a) and Sepharose-bound (b) transferrin by rabbit reticulocytes. a: rabbit reticulocytes were incubated with 10 μM transferrin 70% saturated with ^{59}FE for the indicated times. ●——●, ^{59}Fe incorporated into the cell. o——o ^{59}Fe incorporated into heme. b: rabbit reticulocytes were incubated with 10 μM Sepharose-bound transferrin 70% saturated with ^{59}Fe for the indicated times. ●——●, ^{59}Fe incorporated into the cell. o——o ^{59}Fe incorporated into heme.

gamma counter with correction for the crossover of ^{59}Fe radioactivity into the ^{125}I channel. Other aliquots were lysed with water and the heme extracted and counted (11).

RESULTS.

The uptake of iron by rabbit reticulocytes from {^{125}I, ^{59}Fe-}labeled transferrin not bound to Sepharose and the incorporation of this iron into heme are depicted in Figure 1a. Both processes were linear for at least 120 minutes. The results of a series of six experiments are shown in Table I. ^{59}Fe incorporation into heme was about 37% of the total iron uptake by the reticulocytes. The results of parallel experiments with the same reticulocytes and the same transferrin bound to Sepharose are shown in Figure 1b and Table I. Again, uptake of iron and iron incorporation into heme were linear. Iron uptake in these experiments was 9% of that observed with free transferrin. In contrast to the iron delivered from free transferrin, twice the iron delivered from Sepharose-transferrin was incorporated into heme. Hence, although the rate of iron uptake from Sepharose-transferrin was only 9% of that from free

Table I: Iron Uptake and Incorporation Into Heme from Free and Sepharose-Bound Transferrin Rabbit Reticulocytes

	Iron Uptake (molecules Fe^{59}/min/cell)	Heme Synthesis (molecules Fe^{59}/min/cell)	$\frac{\text{Heme Synthesis}}{\text{Iron Uptake}}$ x100
Free Transferrin	24,005 ± 6716	8890 ± 2617	37%
Bound Transferrin	2135 ± 686	1648 ± 560	77%
Bound/Free x100	8.9%	18.5%	

Rabbit reticulocytes were incubated with free transferrin or Sepharose-bound transferrin both 60 - 70% saturated with ^{59}Fe as described in the Methods and iron uptake and heme synthesis determined. The results are the means ± SEM of 6 experiments.

Table II. Iron Uptake and Incorporation Into Heme from Free and Sepharose-Bound Transferrin in Murine Reticulocytes

	Iron Uptake (molecules Fe^{59}/min/cell)	Heme Synthesis (molecules Fe^{59}/min/cell)	$\frac{\text{Heme Synthesis}}{\text{Iron Uptake}}$ x100
Free Transferrin	19110 ± 2596	10029 ± 2477	52.5%
Bound Transferrin	1591 ± 303	1044 ± 80	65.6%
Bound/Free x100	8.3%	10.4%	

Murine reticulocytes were incubated with free transferrin or Sepharose-bound transferrin both 60 - 70% saturated with ^{59}Fe as described in the Methods and iron uptake and heme synthesis determined. The results are the means ± SEM of 6 experiments.

transferrin, the rate of heme synthesis was nearly 19% of that observed with free transferrin. Since the transferrin was double labeled it was possible to determine the amount of transferrin liberated from the Sepharose beads during the incubation and hence the iron potentially delivered by free transferrin in these studies. In all experiments free transferrin, as detected by ^{125}I radioactivity accounted for less than 1% of the total transferrin present in the reaction medium after 120 minutes of incubation.

Similar experiments were performed with murine reticulocytes and transferrin. The patterns of iron incorporation were similar to that observed with rabbit reticulocytes. In a series of six experiments (Table II) using mouse transferrin 60-70% saturated with ^{59}Fe the mean of iron uptake and heme synthesis were 8.3% and 10.4% respectively of the corresponding values for free transferrin.

The saturation of Sepharose-bound transferrin was found to affect the rate of iron uptake by rabbit reticulocytes. Free and Sepharose-bound rabbit transferrins were generated with iron saturations of 33% and 60%. The rate of iron uptake from free transferrin increased 1.3 fold as transferrin saturation increased from 33 to 60%. In contrast the rate of iron uptake increased 3.5 fold when the same transferrins were bound to Sepharose.

DISCUSSION.

The experiments reported here clearly demonstrate that all transferrin need not enter the cells to deliver iron. Under conditions in which transferrin is bound to a matrix of Sepharose, the bound transferrin is still able to release iron to the reticulocyte at a rate of about 8-9% that observed for "free" transferrin in solution. This is a minimal estimate of transferrin-iron transport at the surface of the red cell, since the rate at which iron can be delivered from Sepharose-bound transferrin is probably limited for the following reasons: a) the effective concentration of the bound transferrin may be decreased by the interaction and packing of the 40 - 200 μ diameter Sepharose beads, tending to exclude the 6 - 7 μ diameter reticulocytes from portions of the bead surface and to limit the number of interactions of reticulocytes with transferrin; hence effective collisions of cells with bound transferrin in contrast to the mobility of transferrin molecules in solution; b) transferrins may be covalently bound to portions of the beads, e.g., the

interior of the bead, which are inaccessible to the reticulocytes; and c) the mobility of transferrin may be limited by its covalent linkage to the Sepharose matrix, diminishing the efficienty of its interaction with the cell membrane. These considerations suggest that under physiological circumstances the contribution of iron from transferrin which does not enter the reticulocyte may be considerably greater than the 8-9% found in these experiments.

The present findings do not exclude pinocytosis of transferrins as a mechanism of iron uptake by immature red blood cells and it is possible that both mechanisms may function simultaneously.

That the extent of iron saturation of bound transferrin should affect delivery of iron to a much greater degree than similar changes in free transferrin is as yet unexplained. Perhaps the iron site more distal from Sepharose matrix is less constrained and can deliver its iron more readily.

Also of interest is the more complete utilization for heme synthesis of the iron that has entered the cell after incubation with Sepharose-transferrin as compared to free transferrin. It is possible that competition for iron exists between mitochondria and iron storage sites, directing more of the iron toward mitochondrial heme synthesis under conditions in which iron availability is limited. Such competition could occur either at the level of cytoplasmic carriers for iron or within iron-binding proteins of the cell membrane.

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