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MOBILIZATION OF IRON FROM THE PLASMA MEMBRANE OF THE MURINE RETICULOCYTE

THE ROLE OF FERRITIN

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Summary

1. Plasma membranes prepared by pre-incubation of mouse reticulocytes with ^{125}I,^{59}Fe-labeled murine transferrin were able to release ^{59}Fe in preference to ^{125}I when incubated in the presence of murine reticulocyte cytosol, demonstrating that the latter mobilized iron which had been dissociated from transferrin.

2. ^{59}Fe in the cytosol was associated with at least two components in addition to hemoglobin, a high molecular weight component, identified as ferritin by specific immunoprecipitation, and an as yet unidentified, low molecular weight component of approx 17 000.

3. Ferritin itself, in the absence of added cytosol, was able to mobilize ^{59}Fe from ^{59}Fe-labeled reticulocyte plasma membranes.

4. Lysates of reticulocytes synthesized ^{59}Fe-labeled heme when incubated with ^{59}Fe-labeled ferritin.

5. These findings reflect a pathway of iron uptake and incorporation into heme in which ferritin plays an active role.

Introduction

Although considerable advances have been made in understanding the interaction of transferrin with the reticulocyte plasma membrane, the mechanisms by which iron passes from membrane to mitochondria are still obscure.

Several investigators have postulated that transferrin enters the cell during iron delivery [1—3] but this is not an obligatory step in the iron uptake process. Jandl and Katz [4] suggested that transferrin interacts with surface receptors on the reticulocyte membrane and transfers its iron at these sites. Our

recent observation [5] that transferrin bound to Sepharose beads is capable of delivering its iron to reticulocytes supports this conclusion. Hence, mechanisms must be defined for iron transport across the reticulocyte membrane through the cytosol and to the mitochondria where the iron is incorporated into heme.

In the present studies we have examined the ability of murine reticulocyte cytosol to mobilize ^{59}Fe from ^{59}Fe-labeled reticulocyte plasma membranes, with particular attention to the role of ferritin in the iron transport process.

Materials and Methods

Transferrin purification and labeling. Transferrin was purified as described earlier [6] with the modification that 20—30 ml of mouse plasma previously dialyzed against 0.15 M sodium phosphate (pH 5.0) were loaded onto a 7 × 80 cm DEAE-Sephadex A-50 column that had been equilibrated with 0.15 M sodium phosphate (pH 5.0). The second purification step was gel filtration on a Sephacryl S-200 column (2.5 × 90 cm) equilibrated with 0.1 M $NaClO_4$/10 mM Tris · HCl (pH 7.4). Transferrin was charged with ^{59}Fe at an iron saturation of about 60% by the nitrilotriacetic method [7]. When required, transferrin was first labeled with ^{125}I by the lactoperoxidase technique [8].

Preparation of reticulocytes. Reticulocytes were induced in mice in response to phenylhydrazine · HCl as an initial stimulus [9]. The mice were subsequently bled by intraorbital bleeding on days 1, 3 and 5 after the phenylhydrazine injections were completed and blood was used from days 3 and 5. With this schedule a reticulocytosis of 40—60% was obtained while any residual damage of phenylhydrazine on reticulocyte plasma membranes was eliminated because of the generation of new crops of cells.

Preparation of ^{59}Fe-labeled reticulocyte plasma membranes, cytosol, and mitochondria. The procedure was similar to that described by Workman and Bates [10]: a reticulocyte-rich suspension was incubated with ^{59}Fe-labeled transferrin or ^{59}Fe,^{125}I-labeled transferrin, for 20 min at 37° C. In some experiments, reticulocytes were incubated with 10 mM isonicotinic acid hydrazide for 15 min before the addition of ^{59}Fe-labeled transferrin. The cells were washed and then lysed with 20 volumes of 20 mosM Tris · HCl (pH 7.6) and membranes prepared by the method of Dodge et al. [11]. The membranes were washed 5—6 times with the lysis buffer. Special care was taken to eliminate the more dense greenish material which pelleted to the bottom of the tube after each centrifugation. The resulting membranes were white or slightly pink with a 2-fold enrichment of ^{59}Fe to ^{125}I radioactivity compared with the original transferrin. The membranes were kept at −80° C in 20 mosM Tris · HCl (pH 7.6), and used within 2 weeks.

To prepare cytosol, washed reticulocyte suspensions were lysed with two times the packed volume of cells in 20 mosM Tris · HCl (pH 7.6). The lysate was centrifuged at 25 000 × g for 20 min and the supernatant carefully collected and stored at −80° C. Loss of release activity was not observed in cytosols stored up to 1 month. In some experiments cytosol was fractionated by centrifugation through Centriflow membranes, molecular weight cut-off 50 000 (Amicon Company, Lexington, Mass.).

Mitochondria were prepared from reticulocytes by the method of Guggen-

heim et al. [12]. The composition of proteins contained in the mitochondria was compared to that in reticulocyte plasma membrane by fractionation according to molecular weight under dissociating conditions on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [13]. The electropherograms were stained with Coomassie Blue and densitometric tracings performed at 550 mm.

Mobilization of ^{59}Fe from ^{59}Fe-labeled membranes. The ability of cytosol to mobilize ^{59}Fe from ^{59}Fe-labeled membranes was assayed by a modification of the method of Workman and Bates [10]. Aliquots of cytosol and labeled membranes, each equivalent to $5 \cdot 10^8$ cells unless otherwise stated, were incubated in a final volume of 0.5 ml 20 mosM Tris · HCl (pH 7.6) at 37°C. At the indicated times the assay was terminated by rapid cooling and centrifugation at 25 000 × *g* for 15 min. The pelleted membranes were washed once with 20 mosM Tris · HCl (pH 7.6) and the supernatants pooled. Radioactivity in membrane and cytosol was determined in a Nuclear-Chicago well-type gamma counter. In double label experiments ^{125}I counts were corrected for cross-over of ^{59}Fe counts. Results are expressed as the percent of the total membrane counts released to the cytosol.

Purification of ferritin and preparation of rabbit anti-ferritin antibodies. Mice were pretreated with 1 mg iron dextran (Imferon, Lakeside Labs, Milwaukee, Wisc.) daily for 3 days and killed 24 h after the last injection. Ferritin was prepared from the livers according to the method of Linder and Munro [14] modified by performing the final gel filtration step on a Biogel A-1.5 column (2.5 × 90 cm). Murine spleen ferritin was isolated in a similar fashion from spleens of mice with splenic erythroid hyperplasia in response to phenylhydrazine. Antibodies against mouse liver ferritin were raised in New Zealand white rabbits by the intramuscular injection of 1 mg ferritin protein in 50% Freund's complete adjuvant weekly for 4 weeks. Rabbit sera were chromatographed on DEAE-Sephadex A-50 and the immunoglobulin G (IgG) fraction eluted with 0.15 M sodium phosphate (pH 5.0). IgG antibody specific for ferritin was isolated by affinity chromatography by the method of Lee and Richter [15]. The resulting antibody was able to precipitate at least 80% of ferritin protein.

Ferritin was labeled with ^{59}Fe according to the method of Miller and Perkins [16]. 0.5 mg mouse spleen ferritin protein (20% (w/w) iron saturated) was dialyzed at 4°C for 1 week against 1.5 ml of a solution containing 10 mM ascorbic acid, 2 mM AMP, 0.1 M Tris · HCl (pH 7.4), 0.2 mCi $^{59}FeCl$ (10 mCi/ml, New England Nuclear Corp.). 0.02% NaN_3 was added as a bacteriostatic agent. The ferritin solution was then dialyzed for 4 h against 20 mosM Tris · HCl (pH 7.6) and gel filtered in a Biogel A-1.5 column (1 × 34 cm) equilibrated with the above buffer.

Results

Cytosol mobilization of ^{59}Fe from ^{59}Fe-labeled reticulocyte plasma membranes

The ability of mouse reticulocyte cytosol to mobilize ^{59}Fe from ^{59}Fe-labeled plasma membranes is depicted in Fig. 1. These experiments were performed with amounts of cytosol and membranes derived from equivalent numbers of reticulocytes. Cytosol produces rapid release of ^{59}Fe for about 10 min with a

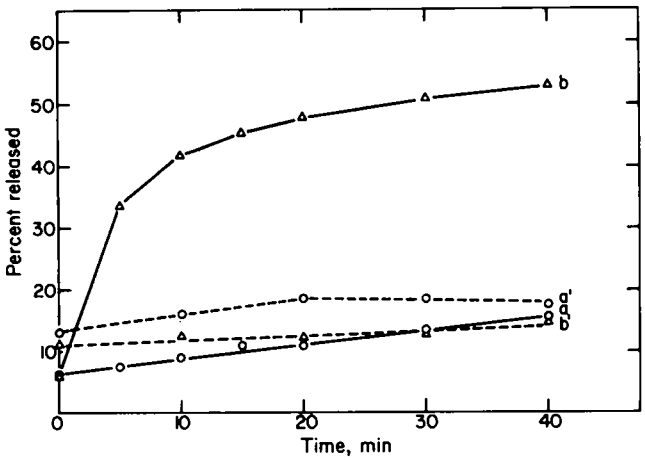

Fig. 1. Time course of ^{59}Fe and ^{125}I release from ^{59}Fe,^{125}I-labeled membranes. Mouse reticulocyte membranes were labeled by interaction of reticulocytes with ^{59}Fe,^{125}I-labeled transferrin and the membranes were then incubated with reticulocyte cytosol as described in Materials and Methods. The ordinate shows the percent of the ^{59}Fe or ^{125}I radioactivity originally present in the membranes that was released into the cytosol as a function of time of incubation. Presented are the means of three experiments: a and a', ^{125}I released in the presence of cytosol or buffer, respectively; b and b', ^{59}Fe released in the presence of cytosol or buffer, respectively. The ratio of radioactivity of $^{59}Fe/^{125}I$ was 1.05 in the original transferrin, 2.03 in the plasma membranes, and 3.50 in the cytosol after 40 min incubation. In this and subsequent figures the points at zero time refer to cytosol radioactivity immediately after reaction with membranes followed by the unavoidable delay of the washing procedures.

slower release thereafter, so that 50% of membrane ^{59}Fe is mobilized by 40 min. Buffer alone is capable of releasing about 13% of ^{59}Fe during the same period of time. In contrast, cytosol does not promote significant release of ^{125}I, indicating that the mobilization of ^{59}Fe does not represent release of membrane-bound transferrin.

The effect of altering the ratio of cytosol to membrane on ^{59}Fe mobilization

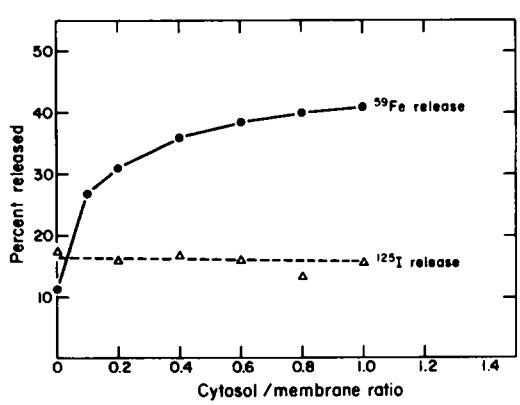

Fig. 2. Concentration curve of cytosol ^{59}Fe-mobilizing activity. The release reaction as described in Materials and Methods was performed for 15 min with a constant amount of ^{59}Fe,^{125}I-labeled mouse membranes (equivalent to $5 \cdot 10^8$ cells) and aliquots of cytosol derived from cell numbers ranging from $0.5 \cdot 10^8$ to $5.0 \cdot 10^8$. The ratio of cells used to prepare the cytosol to the number used to prepare the membranes is given on the abscissa. Presented are the means of two experiments. The points at a ratio of 0 refer to incubations of labeled membranes with buffer.

is shown in Fig. 2. A fixed amount of ^{59}Fe-labeled membranes was incubated with aliquots of cytosol corresponding to varying numbers of reticulocytes. There was considerable mobilization of ^{59}Fe at a cytosol : membrane ratio as low as 0.1 and more than 90% of the mobilized iron was released at a ratio of 0.6. In contrast, release of ^{125}I was not influenced by cytosol concentration.

If the observed mobilization represents non-specific binding of ^{59}Fe by chelators, such as ATP and GTP, then excess non-labeled iron should saturate the chelators and suppress ^{59}Fe mobilization from the membrane. When cytosol was preincubated for 5 min with 0.4 mM $FeCl_3$ or $FeSO_4$ and then interacted with ^{59}Fe-labeled membranes no suppression of ^{59}Fe release was seen (data not shown).

Lack of mitochondrial contamination of plasma membranes

To assay for possible contamination of plasma membranes with mitochondria as a contributor to the mobilized iron, the degree of contamination of plasma membrane proteins by mitochondrial proteins was assayed by polyacrylamide gel electrophoresis under dissociating conditions [13]. Fig. 3 shows the densitometric tracings of electropherograms of reticulocyte plasma membranes (a) and mitochondria (b) prepared as described in Materials and Methods. The plasma membrane protein composition is similar to that described [23] and small

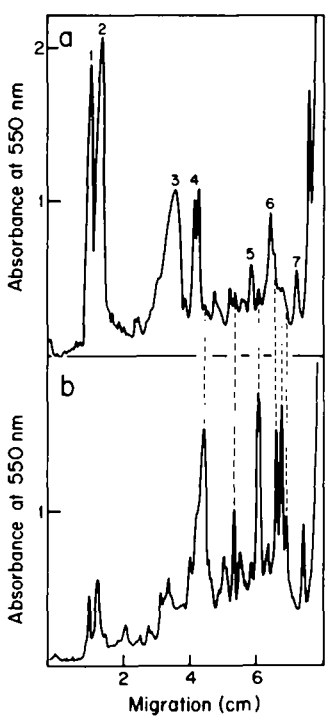

Fig. 3. Polyacrylamide gel electrophoresis of plasma membranes and mitochondrial preparations. Reticulocyte plasma membranes (a) and mitochondria (b) were prepared as described in Materials and Methods. Similar amounts of proteins were applied to a 7.5% SDS-polyacrylamide slab gel [13]. Proteins were stained with Coomassie Blue and gels scanned at 550 nm. The major bands of plasma membrane were numbered following the system of Fairbanks et al. [23].

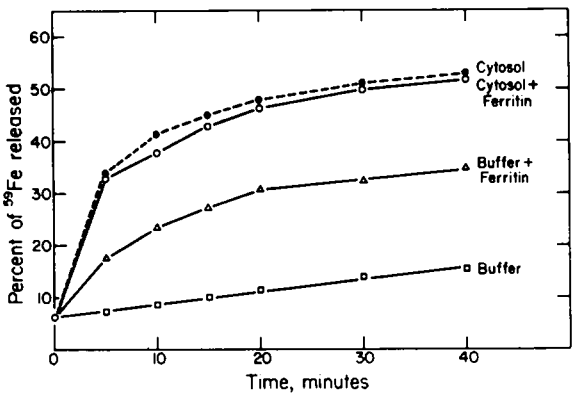

Fig. 4. The effect of ferritin on ^{59}Fe release. ^{59}Fe-labeled mouse membranes were incubated either with cytosol (a), cytosol plus 5 μg ferritin protein (b), buffer plus 5 μg ferritin protein (c) or buffer (d) for the indicated times. Presented are the means of three experiments.

amounts of some of these proteins (e.g. bands 1, 2, and 3) are found in the mitochondrial preparation. However, six mitochondrial proteins migrating at 4.4, 5.3, 6.1, 6.6, 6.8 and 6.9 cm, respectively (Fig. 2b, dashed lines) are present to only a minor degree in the plasma membranes, accounting for less than 5% of the total surface area of the stained membrane proteins. The percentage of ^{59}Fe extractable as heme [17] also was markedly different between membrane and mitochondria being 10.6 ± 2.0% in the former and 59.9 ± 9.7% in the latter (means ± S.E. of three experiments). Moreover, pretreatment of reticulocytes with isonicotinic acid increased by three times the iron incorporated into the membranes, extractable heme was reduced to a level of 4%, but the isonicotinic acid-pretreated membranes showed no difference in their iron release properties as compared to non-pretreated membranes. On the basis of these results we conclude that mitochondria do not represent a major contamination of the plasma membranes preparations.

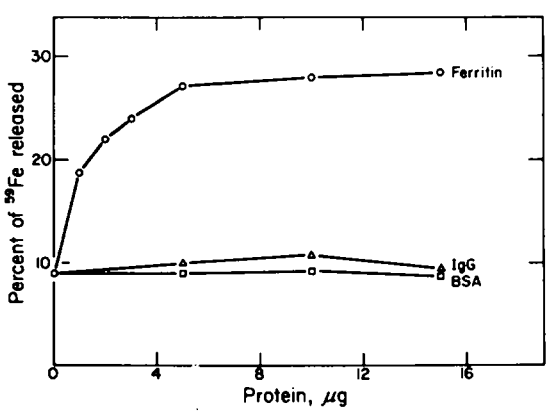

Fig. 5. Effect of ferritin concentration on ^{59}Fe-mobilizing activity. ^{59}Fe-labeled mouse reticulocyte membranes were incubated for 15 min with 1—15 μg mouse spleen ferritin protein, mouse IgG, or bovine serum albumin (BSA). Presented are the means of three experiments.

The effect of ferritin on ^{59}Fe mobilization

Previous studies have suggested that ferritin may be an intermediary in the passage of iron from the reticulocyte plasma membrane to the mitochondria [18]. Hence it was of interest to investigate a possible role for ferritin in the release process. Neither the extent nor the rate of ^{59}Fe release was affected by the addition of mouse liver ferritin to cytosol (Fig. 4). However, ferritin in the absence of cytosol was capable of mobilizing about 30% of the membrane ^{59}Fe after 20 min of incubation. The activity of ferritin in mobilizing iron from ^{59}Fe-labeled membranes is shown in Fig. 5. Maximum mobilization was achieved with 5 μg ferritin protein (about 0.02 μM) in the assay mixture. Control experiments with IgG and bovine serum albumin failed to demonstrate any iron mobilization.

Molecular weight of cytosol ^{59}Fe-mobilizing activity

To determine whether a high molecular weight moiety, e.g. ferritin, was directly involved in ^{59}Fe release activity of the cytosol, the following series of experiments was performed. The release reaction was first conducted with mouse reticulocytes with the ^{59}Fe-labeled membranes confined within a dialysis bag having a molecular weight cut-off of about 12 000 (Spectapore membranes, Fisher Scientific Co.). After 3 h only 8% of ^{59}Fe was released through the dialysis membrane to the cytosol. Under the same experimental conditions rabbit reticulocyte cytosol was capable of releasing 25% of ^{59}Fe from ^{59}Fe-labeled rabbit reticulocyte membranes; this finding is in accord with the results of Workman and Bates [10] who reported a molecular weight of 5000 for an iron carrier protein in rabbit reticulocyte cytosol.

Mouse reticulocyte cytosol was also fractionated through Amicon centriflow membranes of molecular weight cut-off of approx. 50 000. Fig. 6 shows the

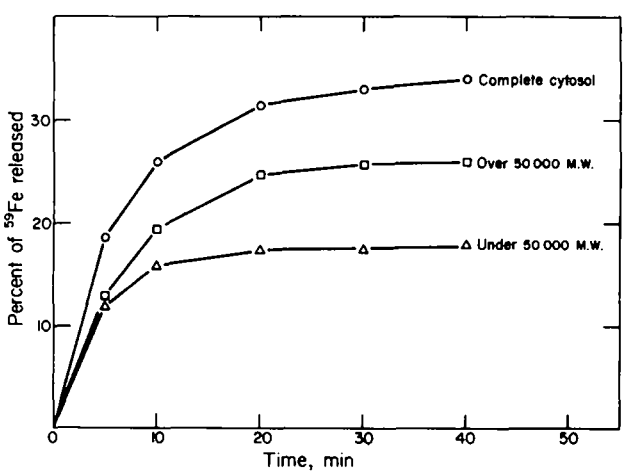

Fig. 6. The effect of different molecular weight fractions of cytosol on ^{59}Fe-labeled membranes. Mouse reticulocyte cytosol was centrifuged through Centriflow membranes resulting in a retentate and a filtrate with molecular weights greater than and less than 50 000, respectively. Retentate, filtrate, and equal amounts of retentate and filtrate together were assayed for ability to release ^{59}Fe from ^{59}Fe-labeled membranes. Presented are the means for four experiments of the percent ^{59}Fe released minus the buffer control value.

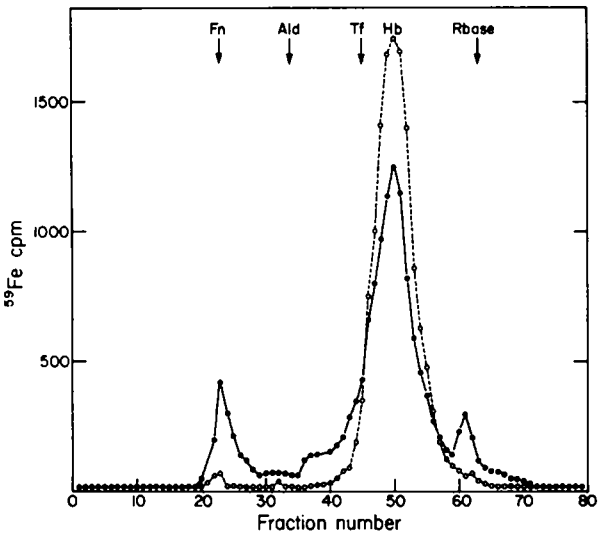

Fig. 7 Gel filtration of ^{59}Fe-labeled reticulocyte cytosol. Mouse reticulocytes were preincubated for 30 min at 37°C in a medium containing phosphate-buffered saline (pH 7.4), 1% bovine serum albumin and 1 mM glucose in the presence (●——●) or the absence of (○- - - - -○) of 10 mM isoicotinic acid. ^{59}Fe-labeled transferrin was then added to a final concentration of 10 μM and the incubation continued for 30 min. Cells were lysed with 20 mosM Tris · HCl (pH 7.6) and centrifuged at 25 000 × *g* for 20 min. Supernatants were collected and radioactivity counted. Similar amounts of ^{59}Fe counts were loaded onto Biogel A-1.5 columns (1 × 34 cm) equilibrated and eluted with 20 mM phosphate (pH 7.0). Fractions of 0.56 ml were collected and radioactivity measured. Elution of protein standards ferritin (Fn, 450 000), aldolase (Ald, 158 000), transferrin (Tf, 78 000), hemoglobin (Hb, 62 000), and ribonuclease A (Rbase, 13 700) are indicated in the figure.

findings when the two fractions (greater than and less than 50 000 daltons) were used to mobilize ^{59}Fe. While both fractions have mobilizing activity, the higher molecular weight fraction is more active, accounting for 70% of the total iron mobilized. About 80% of the ^{59}Fe radioactivity in the retentate was identified as heme and nearly 20% as ferritin by cyclohexanone extraction [17] and rabbit specific anti-mouse liver ferritin antibodies, respectively. Neither ferritin nor heme were detected in the filtrate.

To further identify ^{59}Fe-labeled cytosol components and to identify their molecular weights, reticulocytes were incubated with ^{59}Fe-labeled transferrin, cytosol prepared and fractionated by gel filtration. In these studies (Fig. 7, dashed line) ^{59}Fe radioactivity corresponding to hemoglobin and ferritin was present as determined by use of unlabeled carriers. If the reticulocytes were first incubated with isonicotinic acid to inhibit heme synthesis prior to incubation with ^{59}Fe-labeled transferrin, the ^{59}Fe-labeled ferritin peak became more apparent and a peak of radioactivity of 17 000 daltons (as determined by calibration of the column with standard proteins) was now present (Fig. 6, solid line). The identity of ^{59}Fe-labeled ferritin, which accounted for about 20% of the total radioactivity, was again confirmed by immunoprecipitation. The radioactivity in the hemoglobin region was identified as heme by extraction with cyclohexanone [17].

Ferritin as an iron donor for heme synthesis

To place ferritin directly on the route of iron passage from membrane to

TABLE I

INCORPORATION OF ^{59}Fe FROM FERRITIN INTO HEME

To 50 μl of packed mouse reticulocytes the following reagents were added: 200 μl ^{59}Fe-labeled ferritin (13.6 μg of protein, 2.72 μg of iron, 64 455 cpm of ^{59}Fe) in 20 mosM Tris · HCl (pH 7.6), 20 μl of 20 mM glucose 6-phosphate, 5 μl creatine phosphate kinase and 5 μl M creatine phosphate. The assay mixture was incubated at 37°C and heme extracted by cyclohexanone [18]. Presented are the means ± S.E. for ^{59}Fe incorporated into heme for three experiments.

Time of incubation (min)	^{59}Fe incorporated into heme (cpm)	^{59}Fe incorporated into heme (%)
0	54 ± 24	0.09
60	2348 ± 211	3.64
120	3689 ± 64	5.72

mitochondria it is necessary to demonstrate not only that ferritin accepts iron from membranes but that the ferritin iron can be donated for heme synthesis. In vitro ^{59}Fe-labeled ferritin was incubated with a reticulocyte lysate enriched with an ATP-generating system (see legend of Table I). After 2 h, 5.7% of the ^{59}Fe from ferritin was found in heme. Although the percentage mobilization of ^{59}Fe from ferritin is small, the specific activity of the ^{59}Fe in ferritin has been lowered by the non-radioactive iron already present in the ferritin molecule. Assuming equal accessibility of radioactive and non-radioactive ferritin iron for heme synthesis, the rate of iron incorporation into heme is approx. 50 000 molecules per min per reticulocyte, similar to the rate of uptake of ^{59}Fe by intact mouse reticulocytes [5].

Discussion

These studies demonstrate (1) that mouse reticulocyte cytosol is capable of mobilizing ^{59}Fe from ^{59}Fe-labeled reticulocyte plasma membranes; (2) that the cytosol-releasing activity may be found in two molecular weight compartments (greater than and less than 50 000 daltons); and (3) that the high molecular weight activity is ferritin. The last conclusion is supported by the finding that ferritin is capable of both mobilizing membrane ^{59}Fe and donating ^{59}Fe for heme synthesis.

The nature of iron transport from transferrin binding sites through the membrane and across the cytosol to mitochondria is still incompletely understood. The kinetic studies of Speyer and Fielding [19,20] demonstrated sequential passage of ^{59}Fe through proteins in the human reticulocyte membrane. Previous studies have suggested an active role of ferritin in the process of iron uptake by erythroid cells: ferritin has a short half-life in early erythroid cells [21] and iron kinetic experiments have suggested that ferritin may be an intermediate in hemoglobin formation [18,20]. In rabbit cytosol, however, a low molecular weight protein (5000 daltons) appears to mobilize iron from the plasma membrane and to donate the iron for heme and ferritin synthesis [10]. A low molecular weight protein seemingly related to iron delivery for hemoglobin synthesis has also been reported in the cytosol of dog marrow cells [22]. Our studies expand on these previous results to demonstrate that iron transport in murine reticulocyte cytosol is a complex system involving at least two components: the high molecular weight ferritin and a low molecular weight activity

of 17 000. A recent study also attributes release activity to ferritin and a low molecular weight component [24].

Specificity of iron release in the murine system was documented by use of ^{59}Fe,^{125}I-labeled membranes. The finding that ^{59}Fe was released from the reticulocyte membrane to a greater extent than ^{125}I precludes the possibility that iron release was a mere reversal of transferrin binding to the membrane or represented membrane proteolysis. These data contrast to those of Blackburn and Morgan [24] who in studying a similar system found that ^{125}I-labeled transferrin was released in a time-dependent fashion by reticulocyte cytosol. Similarly, the ^{59}Fe release observed in the present studies does not appear to be merely the exchange of membrane-bound ^{59}Fe-labeled heme with hemoglobin as suggested [24] since in our studies only about 10% of the ^{59}Fe in membranes was identified as heme, compared to the 48% reported by Blackburn and Morgan [24]. The differences in the two studies may reflect the care taken in the present study in the preparation of membranes to eliminate the dense (presumably mitochondrial) material which pellets during centrifugation. As with rabbit reticulocyte cytosol [10], murine cytosol never releases more than 50—60% of membrane ^{59}Fe. Neither cytosol in excess nor the addition of fresh cytosol to the reaction stimulates further iron release (data not shown). The nature of the residual strongly bound iron is unclear although it may represent membrane portions, for example, vesicles, inaccessible to the cytosol components involved in the mobilization process.

By performing the release reaction across a dialysis membrane it was possible to define a difference between the low molecular weight activities of the murine and rabbit systems, of 17 000 and 5000 daltons, respectively. The 17 000 molecular weight component in murine cells was further documented by gel filtration of ^{59}Fe-labeled reticulocyte cytosol. The murine activity was detected by gel filtration of reticulocyte cytosol only when heme synthesis was inhibited by isonicotinic acid. Hence under conditions of active heme synthesis (i.e. in intact reticulocytes) the 17 000 dalton material appears to act as a rapid turnover intermediate in the delivery of iron for heme synthesis.

By itself the mouse low molecular weight activity is not capable of full release of membrane ^{59}Fe but added to the high molecular weight activity of the Centriflow retentate, full activity is restored. The active high molecular weight material appears to be ferritin; either ferritin alone or the entire retentate releases 30% of membrane ^{59}Fe and in both events ferritin becomes labeled.

Since ferritin can donate iron for heme synthesis, the role of ferritin in erythroid cells may be broader than that of iron storage as proposed, for example, by Borová et al. [25]. Our present hypothesis is that ferritin in erythroid cells is capable of mobilizing iron from plasma membrane components and transferring the iron for heme synthesis. However the ^{59}Fe-labeled ferritin used to donate ^{59}Fe to heme was not labeled physiologically. Hence to accurately quantitate the rate at which ferritin can contribute iron to heme synthesis it will be necessary to employ erythroid ferritin labeled under physiologic conditions and to determine the amounts of ferritin iron which are labile or fixed. Also to be defined is whether the 17 000 molecular weight protein mobilizes a separate pool of membrane iron and acts in parallel with the ferritin pathway or whether the two proteins act in series.

Acknowledgments

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