MOBILIZATION OF IRON FROM RETICULOCYTE GHOSTS BY CYTOPLASMIC AGENTS[+] [*]

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SUMMARY

The preparation of ghosts from rabbit reticulocytes previously incubated with ^{59}Fe-transferrin, allows the study of membrane iron mobilization by cytoplasmic factors, and intracellular iron pathways in a cell free system. Incubation of ^{59}Fe-ghosts with unlabeled reticulocyte lysate results in mobilization of the membrane bound iron, and its utilization for hemoglobin and ferritin synthesis. An iron binding component migrating near the low molecular weight range on a Sephadex G-100 column was rechromatographed on G-25 and emerged with the void volume. Chromatographic behavior suggested a molecular weight near 5,000. The component was subjected to gel electrophoresis and migrated as a single TCA precipitable band that stained with Coomassie blue. A Lowry test for protein was positive. The component was found to reversibly bind ferrous ion, and to be metabolically active.

The assimilation of iron by the developing erythrocyte occurs via a direct donation of iron by serum transferrin to a membrane bound iron receptor site (1). While there is evidence for membrane iron binding complexes (2), little is known about the mechanisms or control of transmembrane iron transport, or the factors regulating the release of iron from the membrane to the cytoplasm. The strong Lewis acid nature of iron suggests a role for a cytoplasmic iron transport agent; however, there is little agreement on this subject (3,4,5,6). The lack of a well documented specific cytoplasmic iron carrier is consistent with the hypothesis of Morgan and Appleton which describes the entrance of transferrin into the cell (7). As a part of our studies of cellular iron metabolism, we have taken a new experimental approach to the questions of intracellular iron transport. Rabbit reticulocytes have been incubated with ^{59}Fe-transferrin, then cooled rapidly to 4°

[+]We dedicate these studies to the memory of our friend and colleague Dr. Robert H. Hayashikawa.

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and ghosts, containing specifically bound ^{59}Fe, have been prepared. The mobilization and subsequent fate of the ^{59}Fe have been examined. The results presented below indicate that a low molecular weight iron binding component is involved. The iron of this complex is readily utilized for hemoglobin and ferritin biosynthesis.

EXPERIMENTAL AND RESULTS

Transferrin- was prepared from fresh Pel-Freeze rabbit serum by $(NH_4)_2SO_4$ fractionation and ion exchange chromatography (8,9,10). Spectrophotometry and gel electrophoresis indicated a purity in the 98% range. Published methods were used for the saturation with ^{59}Fe (11).

^{59}Fe Labeled Reticulocyte Ghosts- Forty ml of blood was collected in heparinized syringes from each of three rabbits, which had been made reticulocytic (20%) by phlebotomy, or in one case by phenylhydrazine. The cells were washed twice by centrifugation with buffered glucose-physiological saline solution (0.15 M NaCl, 0.01 M glucose, 5 mM Tris, pH 7.45). To two volumes of packed cells was added one volume of ^{59}Fe-transferrin in the glucose-NaCl-Tris buffer so that the final concentration and radioactivity were 5×10^{-5} N and 3×10^5 cpm/ml, respectively. The mixture was incubated with shaking at 37° for 15 minutes, then cooled rapidly to 4°, and the cells washed five times by centrifugation in ice cold buffer. Ghosts were prepared from these cells by lysis in 20 mOsM Tris at 4° (12). After five washes the slightly pink ghosts were counted in a gamma well scintillation counter. Control experiments with iodinated (13) transferrin indicated that the ghosts retained fewer than 0.05 transferrin molecules per iron bound.

Mobilization of Iron From Reticulocyte Ghosts- was studied by incubation of ^{59}Fe-ghosts in standard buffer or unlabeled reticulocyte lysate for 45 minutes at 37° with shaking. The mixture was then cooled to 4° and the ghosts tightly pelleted by centrifugation at 43,000 x g for 20 minutes. An aliquot of the supernatant and the pellet were then assayed for ^{59}Fe and the percent iron mobilization from the ghosts was calculated. In Table 1 is shown the fractional iron mobilization from the ghosts for various incubation media. From these data, and other experiments, we have arrived at the following tentative conclusions. The binding of iron to the ghost is quite stable since only 6% of the iron is released to buffer after 45 minutes of incubation. Factors contained in the lysate, however, have a marked ability to cause the release of the iron to the soluble fraction. Of the metabolites

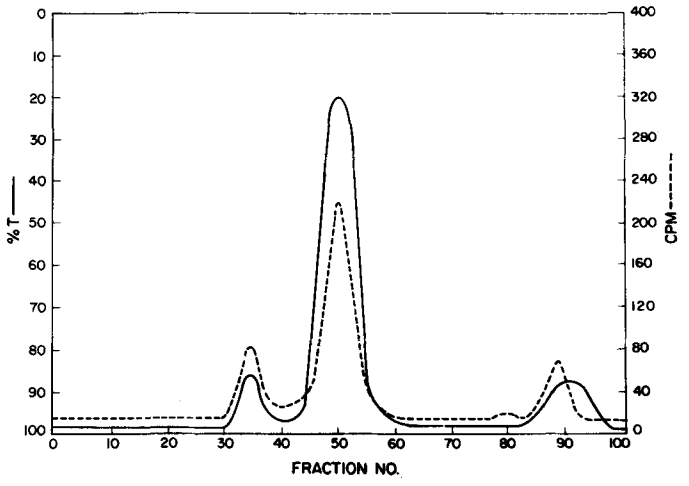

Fig 1. Elution profile obtained from Sephadex G-100 chromatography of the supernatant obtained from an incubation of ^{59}Fe-ghosts with unlabeled reticulocyte lysate. The 2 x 100 cm column was equilibrated and eluted with 5 mM Tris-HCl in 0.15 M NaCl at pH 7.45.

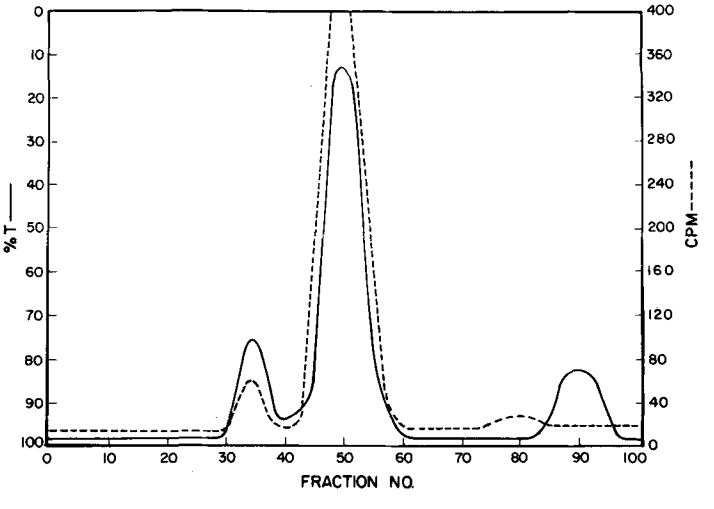

Fig 2. Elution profile obtained from lysate of whole reticulocytes treated with ^{59}Fe-transferrin and then washed prior to lysis. Conditions are as described for Fig 1.

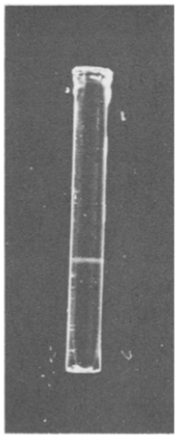

Fig 3. Electrophoretic pattern of the iron binding component on 7% acrylamide gel. The run was made at 5 mamp for 2 hours in a Tris-glycine buffer at pH 9.4. The gel was subsequently fixed in TCA and stained with Coomassie brilliant blue. The component migrated toward the anode.

and cofactors tested, ATP had the most pronounced iron release ability. ATP in buffer alone was able to mobilize the iron via chelation, however, in the presence of lysate ATP enhanced iron mobilization, but the iron was bound to other cytoplasmic constituents. Reducing agents such as NADH, NADPH, and ascorbate had little or no effect. Protoporphyrin in the presence of lysate and ATP had an inhibitory effect.

Fate of the Mobilized Iron- was determined by Sephadex G-100 gel filtration chromatography of the supernatant of each incubation mixture. Typical results obtained from the incubation of ^{59}Fe ghosts with reticulocyte lysate are shown in Fig 1. The components centered at fractions 35, 50, and 90 have been identified as ferritin, hemoglobin, and low molecular weight components, respectively. It will be seen that the bulk of the iron mobilized from the membrane has been utilized for ferritin and hemoglobin biosynthesis. Another peak of radioactivity is seen centered at fraction 80. We have not yet identified this component, however, long term incubations suggest that the iron is on a direct pathway to either hemoglobin or ferritin biosynthesis. In all incubations of ^{59}Fe ghosts with lysate we have found a fourth peak of radioactivity which slightly preceeds the low molecular weight fraction, and it is this fraction which we have isolated and partially characterized. When the ^{59}Fe ghosts are incubated with buffer, the elution profile shows only trace amounts of hemoglobin and ferritin.

As a control experiment we incubated whole cells with ^{59}Fe-transferrin,

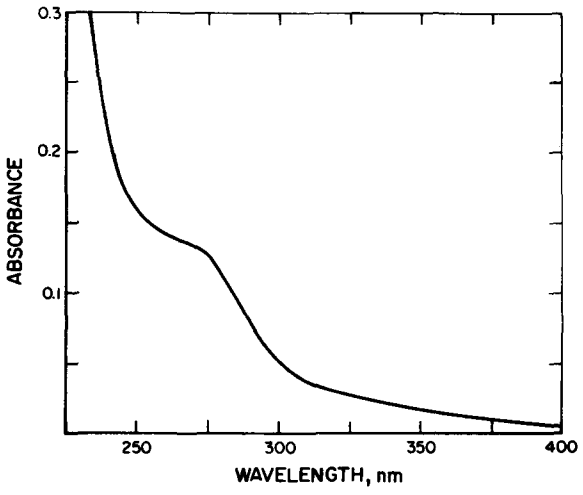

Fig 4. Spectrum of the isolated iron binding component determined with a Cary 118C spectrophotometer.

lysed the cells in 20 mOsM Tris-HCl, and passed the lysate down the same Sephadex G-100 column. The results are shown in Fig 2. It will be seen that hemoglobin is of higher specific activity and ferritin of lower specific activity in the whole cell experiment compared to the ^{59}Fe-ghost incubation. The amount of radioactive iron in fraction 80 is increased, while the radioactivity of the fourth peak centered at fraction 88 is greatly diminished. Our tentative interpretation of these results is that the radioactive component centered at fraction 88 is an iron binding intermediate with a rapid iron turnover in the whole cell. Its concentration is enhanced in the incubation experiments since there is a high availability of membrane bound ^{59}Fe and a decreased hemoglobin biosynthetic rate.

Isolation of the Fraction 88 Iron Binding Component- Fractions 82 through 93 from four experiments, similar to that shown in Fig. 1, were pooled and lyophilized. This material was taken up in a minimum amount of distilled water and applied to a Sephadex G-25 column equilibrated and eluted with 5 mM Tris-HCl in 0.15 M NaCl at pH 7.45. The elution profile from this column indicated two UV absorbing peaks. The first migrated with the void volume and proved to be the iron binding component. A second peak composed of low molecular weight components was found to have the residual radioactivity. Sephadex G-25 chromatography of fresh lysate from a ^{59}Fe-ghost incubation with no prior lyophilization, yielded all of the radioactivity in the void volume, even when potential chelating agents such as ATP had been added. This indicates that cytoplasmic iron is not bound to low molecular weight

Table 1. Percent of specifically bound ^{59}Fe mobilized from reticulocyte ghosts by various incubation media. The enhancement factor is relative to buffer alone. (‡ at 1 mM)

INCUBATION MEDIUM	^{59}Fe MOBILIZED	ENHANCEMENT FACTOR
Buffer	5.9%	1.00
Lysate	23.5%	4.0
Buffer + ATP‡	29.5%	5.0
Buffer + Protoporphyrin	5.1%	0.9
Lysate + ATP‡	43.6%	7.4
Lysate + NADH‡	24.9%	4.2
Lysate + Protoporphyrin	21.5%	3.6
Lysate + ATP + Protoporphyrin	29.3%	5.0

chelates, but is bound to complexing agents that are totally excluded by the G-25 gel.

Characterization of the Iron Binding Component- was carried out with the material obtained from the combination of G-100 and G-25 chromatography as described above. When this fraction was lyophilized, resolubilized, and subjected to gel electrophoresis a single band was obtained, which was TCA precipitable and stained with Coomassie brilliant blue. Further indication of the protein nature of the iron binding component was a positive Lowry (14) test obtained on a Tris-HCl free sample. An amount of iron binding component equivalent to that obtained from a single incubation mixture contained 100 μgm of protein based on a serum albumin standard curve. The UV spectrum of the iron binding component which is shown in Fig. 4 is also consistent with the suggested protein nature.

The form of the iron associated with the iron binding component was investigated using bathophenanthroline sulphonate (BPS), a ferrous specific chromogenic agent. Addition of BPS to the amount of iron binding component isolated from one incubation mixture gave an absorbancy increase at 533 nm corresponding to 35 ngm of ferrous ion released. Acidification of the solution caused a five fold increase in this value and the appearance of the pink ferrous-BPS color. Addition of ascorbate, an agent capable of reducing

Fe^{3+}, gave an additional 5% increase in absorbancy. Organic extractions indicated the absence of heme (15). It is apparent that the iron binding component is able to compete successfully with BPS for iron, and that upon acidification, but without prior reduction, the iron binding component releases ferrous ion. Approximately 95% of the iron associated with this component is in the ferrous form, despite lyophilization, resolubilization, and chromatography in an aerobic atmosphere. It is not possible to calculate a meaningful minimum molecular weight since we have no assurance that the component is saturated with iron.

In order to test for the ability of this component to reversibly bind iron we attempted to purify the apo-protein on Sephadex G-25 at pH 3. We were surprised to find that the component adhered quantitatively to the gel at this pH. Switching to a Tris-HCl buffer at pH 8.0 resulted in the elution of the component from the column, with no iron attached. After lyophilization and resolubilization an aliquot of iron, carefully maintained in the ferrous state, was added to this component. Rechromatography on a G-25 column resulted in the emergence of the component with the void volume and co-chromatography of radioactive iron. The chromatographic behavior of the iron binding component on Sephadex G-100, G-50, and G-25 indicate a molecular weight in the range of 5,000 daltons.

Biological Activity- The incubation of ^{59}Fe ghosts with the isolated iron binding component resulted in the mobilization of 21% of the specifically bound ^{59}Fe. G-100 chromatography of this incubation mixture revealed radioactivity appearing in fraction 88, as anticipated. In the intact cell low molecular weight chelating agents such as amino acids and nucleotides may have some role in iron mobilization.

The incubation of ^{59}Fe labeled iron binding component with fresh, unlabeled reticulocyte lysate, followed by chromatography of the mixture on G-100, indicated that all of the counts initially associated with this component were utilized for ferritin and hemoglobin biosynthesis. This is an interesting observation in view of the fact that the incubation system was mitochondria free, and the enzyme ferrochelatase is generally thought to be a mitochondrial enzyme (16).

DISCUSSION

While this study represents the first isolation and partial characterization of a cytoplasmic iron transport agent in developing erythrocytes, there are several reports in the literature which appear to support our

findings. Serafini, et. al. (3) demonstrated a high specific activity iron fraction by paper electrophoresis, that was not identifiable as any known iron protein. Barova et. al. (4) and Primosigh et. al. (5) demonstrated that substances in the molecular weight range that would include the component described above, became labeled and donated iron for hemoglobin synthesis. Allen and Jandl (6) suggested that a protein other than hemoglobin bound iron, however, one third of that iron was in the form of heme.

The propensity of iron to oxidize, polymerize, and bond non-specifically to a variety of biomolecules dictates the need for specific transport agents, such as serum transferrin. The discovery of a cytoplasmic iron transport agent, therefore, is consistent with our expectations based on iron chemistry.

The ^{59}Fe-ghost incubation system and isolation of a low molecular weight iron binding component provide a few answers, but also a host of new questions. Studies underway in this laboratory are aimed at a further study of the physical and chemical properties of this component and its role in intracellular iron metabolism.

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