Distribution of Iron in Reticulocytes After Inhibition of Heme Synthesis With Succinylacetone: Examination of the Intermediates Involved in Iron Metabolism

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Succinylacetone (SA) is an inhibitor of heme synthesis that acts on the enzyme δ-aminolevulinic acid dehydratase. When reticulocytes are incubated with 59Fe-transferrin (59Fe-Tf) in the presence of SA, there is an accumulation of 59Fe in the mitochondrion and in a cytosolic non-heme intermediate that has been described as a putative Fe transporter (Adams et al, Biochim Biophys Acta 1012:243, 1989). Considering these observations, the present study was designed to examine the intermediates of Fe metabolism in control and SA-treated reticulocytes. This investigation showed that in the cytosol of control cells, most 59Fe was incorporated into hemoglobin (Hb) with a minor amount entering ferritin. In addition, a previously unrecognized cytosolic intermediate was identified (band X) that was absent when heme synthesis was inhibited with SA. Upon reincubation of SA-treated reticulocytes with protoporphyrin IX, band X initially increased in intensity and then decreased later in the incubation. In contrast, when 59Fe-labeled control cells were reincubated in the presence of SA and unlabeled diferric Tf, there was no decrease in this cytosolic pool of Fe, suggesting that it was not an intermediate supplying Fe for either ferritin or heme synthesis. Finally, there is little low molecular weight (M,) Fe in reticulocytes, and our studies suggest that the low-M, Fe present does not behave as an intermediate. Moreover, after inhibition of heme synthesis with SA, 59Fe in the low-M, compartment was markedly decreased, suggesting that this component may be heme or a low-M, heme-containing molecule. Considering the apparent lack of a cytosolic Fe transporter in rabbit reticulocytes, an alternative model of intracellular Fe transport is proposed that does not implicate a potentially toxic intermediate pool of low-M, Fe complexes.

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MMATURE ERYTHROID CELLS are avid consumers of iron (Fe), which is required for the synthesis of hemoglobin (Hb). Since the pioneering experiments of Jandl et al., it has been well established that Hb-synthesizing cells such as reticulocytes acquire Fe from plasma transferrin (Tf) by receptor-mediated endocytosis. However, despite immense progress in understanding the regulation of cellular Fe uptake and storage, virtually nothing is known about the pathway followed by Fe after its release from Tf in the endosome. After Fe is released from Tf, it is transported through the endosomal membrane into the cytosol, where it has been proposed to enter a common intracellular transit pool (referred to here as low-M, Fe) may then supply Fe for the synthesis of heme and other Fe-containing molecules. Since this pool of Fe is believed to have major physiologic roles, its identification and characterization is of considerable interest.

Although the intermediates in the intracellular Fe uptake pathway have not been characterized, it is well established that in erythroid cells most Fe is targeted to mitochondria, where the enzyme ferrochelatase inserts Fe into protoporphyrin IX to form heme. Several studies have demonstrated that inhibition of heme synthesis in reticulocytes using succinylacetone ([SA] an inhibitor of the enzyme, δ-aminolevulinic acid dehydratase) or isonicotinic acid hydrazide ([INH] an inhibitor of δ-aminolevulinic acid synthase) results in the accumulation of non-heme 59Fe in the mitochondrion and in a cytosolic intermediate. This accumulation of nonheme 59Fe in the mitochondrion can be subsequently used for heme synthesis when protoporphyrin is available. Moreover, 59Fe bound to the putative cytosolic carrier can be chased into heme in the absence of SA or into the mitochondrion in the presence of SA. These latter results have been interpreted as indicating that Fe bound to a cytosolic intermediate is selectively amplified due to inhibited heme synthesis in the mitochondrion. Hence, examination of the distribution of Fe in SA-treated reticulocytes compared with control cells may facilitate detection of the route taken by Fe before it enters the mitochondrion.

The present study was designed to investigate the nature of Fe accumulated in control and SA-treated rabbit reticulocytes and their role as intermediates in the synthesis of heme. The results demonstrate firstly that the accumulation of Fe that occurs in the cytosol of SA-treated rabbit reticulocytes does not act as an intermediate for heme synthesis or donate...
Fe to ferritin. Secondly, we have identified a previously unrecognized cytosolic intermediate that may be involved in the transfer of heme from the mitochondrion to the cytosolic site where heme is inserted into globin. Alternatively, this component may be a short-lived hemoprotein of as-yet-unknown function. Thirdly, we have found little low-M, Fe in reticulocytes, and the results suggest that this low-M, Fe does not act as an intermediate, but behaves as an end product.

Finally, considering the efficiency of Fe uptake into heme and the apparent lack of a cytosolic Fe transporter in rabbit reticulocytes, a model of intracellular Fe transport is proposed that, in contrast to an earlier proposal, does not imply a potentially toxic pool of low-M, Fe complexes.

MATERIALS AND METHODS

Materials. Iron 59 (as ferric chloride in 0.1 mol/L HCl) was purchased from Dupont NEN (Boston, MA). TF was purchased from Boehringer (Mannheim, Germany). Eagle’s modified minimal essential medium (MEM) was obtained from Gibco Laboratories Ltd (Grand Island, NY). Bovine serum albumin ([BSA] 98% to 99% pure, fatty acid-free), leupeptin, HEPES, protoporphyrin IX, phenylmethylsulfonyl fluoride (PMSF), and Triton X-100 were obtained from Sigma Chemical Co (St. Louis, MO). SA (4.6-diolethanoic acid) was obtained from US Biochemical Corp (Cleveland, OH). Bio-Gel P-10 and P-150 were obtained from Bio-Rad Laboratories Ltd (Mississauga, Ontario, Canada). Sephadex G-25, Sephaeryl S-300, and Percoll were obtained from Pharmacia (Uppsala, Sweden). Desferrioxamine (DFO) was obtained from Ciba-Geigy Pharmaceutical Co (Summit, NJ). All other chemicals were of analytic reagent quality.

Reticulocytes. Reticulocytosis was induced in young New Zealand white rabbits by repeated phlebotomy via cardiac puncture using a protocol approved by the McGill University Animal Care Committee. Reticulocyte-rich blood was collected in heparin, and reticulocytes were purified on 63% Percoll gradients at 4°C using a protocol approved by the McGill University Animal Care Committee. Reticulocyte-rich blood was collected in heparin, and reticulocytes were purified on 63% Percoll gradients at 4°C using the method of Watson and Doherty. The cells were then washed three times in a large excess of ice-cold phosphate-buffered saline (PBS) by centrifugation at 1,000 rpm for 5 minutes at 4°C. Reticulocytes were identified based on staining with new methylene blue, and cell counts were determined using an improved Neubauer counting chamber. The final preparation of cells was composed of 60% to 95% reticulocytes. For all incubations, cells were suspended in MEM containing 25 mmol/L HEPES (pH 7.4), 10 mmol/L NaHCO3, and 1% BSA.

Control studies demonstrated that the uptake and distribution of Fe into intracellular compartments was similar in reticulocytes induced by phlebotomy versus phenylhydrazine. However, in the present study, phlebotomy was used, since the blood cell suspension obtained using phenylhydrazine appeared to be more susceptible to hemolysis.

Protein purification and labeling. Apo TF was prepared and labeled with 59Fe to produce 59Fe2-TF, as described previously. BSA was dialyzed extensively before use against 0.15 mol/L NaCl adjusted to pH 7.4 using 1.4% NaHCO3.

Cellular uptake of iron from 59Fe-TF. General incubations were performed at 37°C in a shaking water bath. However, zero-time samples in binding studies were obtained following addition of ice-cold 59Fe-51Tf (3.75 mmol/L) to reticulocytes incubated in an ice bath.

Iron uptake by reticulocytes was measured in cells (packed cell vol: 100 to 500 µL) preincubated for 30 minutes at 37°C in the absence or presence of SA (2 mmol/L) in a total volume of 45 mL. After this incubation, 59Fe-Tf (3.75 µmol/L) was added to the cell suspension, and the incubation was continued for up to 4 hours at 37°C. The cells were subsequently washed four times in a large excess of ice-cold PBS (pH 7.4) by centrifugation at 1,000 rpm for 5 minutes at 4°C. In some experiments, labeled and washed reticulocytes were reincubated for up to 2 hours at 37°C in the presence of protoporphyrin IX (4 µmol/L) or SA (2 mmol/L) and unlabeled diferric Tf (3.75 µmol/L). For studies using protoporphyrin IX, all experimental manipulations were performed in tubes wrapped in aluminum foil to prevent the photodynamic generation of free radicals by this compound. Stock solutions of SA and protoporphyrin IX were prepared in MEM immediately before experiments.

For studies examining the distribution of intracellular 59Fe by polycrylamide gel electrophoresis (PAGE), 59Fe-labeled reticulocytes (100 to 500 µL) were then lysed by one round of freezing and thawing in the presence of 100 µL ice-cold 0.14 mol/L NaCl/10 mmol/L HEPES (pH 7.4) containing PMSF (1 mmol/L) and leupeptin (100 µmol/L; this buffer containing protease inhibitors will subsequently be referred to as the NaCl/HEPES/PI buffer). It should be noted that throughout this study we have used HEPES as the buffer of choice due to its negligible affinity for Fe.

The lysates were centrifuged at 16,000 g for 40 minutes at 4°C to separate the stromal mitochondrial membrane (SMM) fraction from the cytosol. The separated SMM was then resuspended and washed by centrifugation at 16,000 x g for 40 minutes at 4°C in 1 mL ice-cold NaCl/HEPES/PI buffer. To solubilize the washed SMM fraction, 100 µL ice-cold NaCl/HEPES/PI buffer was added containing 1.5% of the non-ionic detergent, Triton X-100, and the tube was then subjected to vortex mixing. The soluble supernatant was separated from undissolved material by centrifugation at 16,000 g for 40 minutes at 4°C. 59Fe-heme extraction from reticulocytes. In experiments in which 59Fe incorporation into reticulocytes and heme were compared, 59Fe-heme content was measured following its extraction with acid methyllyethylketone using the modified method of Teale. Washed and packed 59Fe-labeled reticulocytes (50 µL) were lysed by adding 0.5 mL doubly distilled water and performing one round of freezing and thawing. Reticulocyte lysate was then mixed with the 400 µL 10% Tween 20, followed by the addition of 1 mL 0.2N HCl and then 2 mL ice-cold methyllyethylketone. Following vigorous shaking and centrifugation (5,000 rpm for 5 minutes), the 59Fe-heme-containing top layer was transferred to a separate tube. The remaining aqueous phase was extracted once again with 1 mL methyllyethylketone, which, following sample centrifugation, was combined with the first heme-containing fraction. Non-heme Fe was calculated as the difference between total cell Fe and heme Fe.

PAGE coupled with 59Fe autoradiography. 59Fe distribution in the cytosol and SMM fraction of control and SA-treated reticulocytes was examined by PAGE coupled with autoradiography using the modified procedure of Bottomley et al and Menguad and Horwitz. A 5- to 15-µL sample of the cytosol or SMM containing approximately 1,000 to 10,000 cpm 59Fe and 80 µg protein was applied to a 5% native polycrylamide gel containing 1.5% of the nonionic detergent, Triton X-100, but no sodium dodecyl sulfate or 8-mercaptoethanol. In addition to Triton X-100, this separating gel contained 0.375 mol/L Tris hydrochloride (pH 8.8) and 0.1% ammonium persulfate. The separating gel was used without a stacking gel and was prepared from a stock solution containing 29% (wt/vol) acrylamide and 1% (wt/vol) bisacrylamide. It is important to note that a non-denaturing gel was used, because Fe-binding proteins retain the 59Fe label under such conditions. Electrophoresis was performed at 10 to 15 mA per gel at constant current. The temperature of the gel was maintained at 4°C by circulating ice-cold water through the Mighty Small II electrophoresis unit (Hoefer Scientific Instruments,
San Francisco, CA). After the electrophoresis was complete, the gel was fixed in 25% methanol, 5% formaldehyde, and Tris (50 mmol/L, pH 7.4) for 60 minutes at 4°C. The gel was then dried and autoradiography was performed over 24 hours at ~80°C using Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) and an intensifying screen. Densitometric data were collected with an LKB 2222-020 UltraScan XL Laser Densitometer, and the data were analyzed by GelScan XL software. Control studies demonstrated that 98% to 100% of the 59Fe applied to the gel was recovered. In addition, no 59Fe was detected in the running buffer or in the fixation buffer.

Gel-filtration chromatography. In some experiments, 59Fe distribution in the cytosol of control and SA-treated reticulocytes was examined using gel-filtration chromatography. Gel chromatography was performed at 4°C using 0.75 x 28-cm packed with either Bio-Gel P-10, Bio-Gel P-150, or Sephacryl S-300, which was prewashed with 1% BSA and equilibrated with 0.14 mol/L NaCl/10 mmol/L HEPES (pH 7.4). Samples were eluted using the equilibration buffer, and fractions of 0.5 mL were collected under gravity.

Low-M, intracellular iron assay. We used the spinning-column technique, which enables rapid separation of low- and high-M, molecules in many samples simultaneously. Briefly, reticulocytes were labeled with 59Fe-Tf (3.75 μmol/L) for 1 to 20 minutes at 37°C in the absence and presence of SA (2 mmol/L) as described earlier. After this procedure, the cells were washed at 4°C and resuspended in 100 μL ice-cold 0.14-mol/L NaCl/10 mmol/L HEPES (pH 7.4). The reticulocytes were then permeabilized with mixing of glass powder using modifications to the technique described by Augustin et al. Control experiments demonstrated that permeabilization of reticulocytes with glass powder did not result in any change in endosomal integrity, since there was no leakage of 125I-Tf from the endosomal compartment into the soluble cytosol. Since this technique appeared to maintain the integrity of organelle membranes, it was used in preference to more aggressive techniques such as hypotonic lysis or lysis using nonionic detergent. These latter methods probably damage organelle membranes, which could result in artifactual release of 59Fe from organelles.

One volume of the glass powder (consisting of glass fragments 30 to 80 μm in length) was mixed with 7 vol ice-cold 0.14-mol/L NaCl/10 mmol/L HEPES (pH 7.4), and 70 μL of this suspension was then added to 100 μL 59Fe-labeled reticulocytes. Vortex mixing (70 seconds per sample at 4°C) of this suspension with the glass powder permeabilized the cells. After centrifugation at 16,000 g for 40 minutes at 4°C to separate the cytosol from the SMM, 100 μL cytosol was applied to a spinning column (1 mL packed Sephadex G-25 Super Fine—prewashed with 1% BSA in 0.14-mol/L NaCl/10 mmol/L HEPES, pH 7.4), which was then centrifuged at 1,600g for 5 minutes at 4°C. The columns were then washed twice with 100 μL ice-cold 0.14-mol/L NaCl/10 mmol/L HEPES (pH 7.4). The eluate from the spinning column contained high-M, molecules (>5,000 Daltons, the exclusion limit of Sephadex G-25), whereas 59Fe-containing molecules retained on the gel were low-M, (<5,000 Daltons). The low-M, 59Fe-containing molecules that were present in the gel were analyzed by removing the gel from the column and placing it into counting tubes. Radioactivity was measured on an LKB 1282 Compugamma counter. Preliminary studies using this technique demonstrated that the recovery in the eluate of high-M, molecules such as Blue Dextran 2000, BSA, and 125I-Tf was 98% to 99%. In contrast, retention in the gel of low-M, complexes such as 59Fe-NTA (1:5), 59Fe-citrate (1:100), 59Fe-ATP (1:1,500), and 59Fe-DFO (1:1) was 98% to 99%.

RESULTS

Kinetics of iron uptake by rabbit reticulocytes. Incubation of reticulocytes at 37°C with 59Fe-Tf (3.75 μmol/L) showed that Fe was taken up at a rate ranging from 102,000 to 187,000 atoms Fe/cell/min (five separate experiments), and the data from one representative experiment are shown in Fig 1A. The uptake of 59Fe by reticulocytes at time zero (Fig 1A) can probably be accounted for by the binding of 59Fe-Tf to the Tf receptor. It should be noted that 59Fe taken up by reticulocytes appeared in heme with remarkable efficiency. Indeed, 59Fe-heme can be detected as early as 30 seconds after the onset of the incubation, and at this time point it already represented 20% of the total 59Fe in the reticulocyte (Fig 1A and B). Moreover, after an incubation period of 210 seconds, 75% of total reticulocyte 59Fe was present in heme, and this value increased to 88% after 10 minutes. In contrast, non-heme 59Fe (which includes Tf-bound Fe) only increased slightly as a function of incubation time, and after 10 minutes represented 12% of total reticulocyte 59Fe (Fig 1A and B). By examining the molar ratio of heme 59Fe to total 59Fe in reticulocytes, it is apparent that after 300 seconds of incubation a steady state is established between the flux of 59Fe coming into the cell and 59Fe incorporation into heme (Fig 1B). In separate experiments, we established that 59Fe incorporation into reticulocytes and heme, as well as [2-14C]glycine incorporation into both heme and globin, were linear for at least 4 hours (data not shown).

In agreement with earlier studies, 22,24 incubation of reticulocytes with 59Fe-Tf in the presence of the heme synthesis inhibitor, SA, inhibited 59Fe incorporation into heme by about 90% and slightly stimulated 59Fe uptake by 10% to 30%. Furthermore, after a 2-hour incubation of reticulocytes with SA and 59Fe-Tf, 59Fe uptake into the SMM fraction was approximately seven times greater than that seen for the control (data not shown).

Distribution of 59Fe in reticulocytes in the presence and absence of the heme synthesis inhibitor, SA. Initial experiments examined 59Fe distribution in the cell cytosol (Fig 2A) and SMM fraction (Fig 2B) of rabbit reticulocytes after incubation with 59Fe-Tf for up to 120 minutes in the absence (control) and presence of SA. It is obvious from Fig 2 that there are great differences in 59Fe distribution in reticulocytes in the presence and absence of the heme synthesis inhibitor. In the cytosol of control cells, two main bands are evident, a band labeled “X” and a band corresponding to visible Hb (Fig 2A). Most 59Fe in control cells was incorporated into Hb, whereas there was virtually no 59Fe entering femtin. Band X increased slowly as a function of incubation time, but did not appear to increase in intensity after 60 minutes of incubation with 59Fe-Tf. This apparent plateau of radioactivity in band X may suggest that this component is an intermediate. Alternatively, these data could also be interpreted as indicating that the rate of degradation of band X is equal to its rate of synthesis.

In contrast to control reticulocytes, little 59Fe was incorporated into Hb or band X in the presence of SA (Fig 2A). This observation indicated that band X may be a heme-containing molecule.
However, further studies are required to directly determine the presence of heme in this band. It is also important to note the presence of two additional bands in the cytosol of cells treated with SA. One of these bands comigrated with either human or rabbit ferritin standards, and another diffuse band (labeled "Y") increased as a function of incubation time and was the most prominent band after 120 minutes of incubation with $^{59}$Fe-Tf (Fig 2A). This latter band may correspond to a low-$M_\text{r}$ component identified by Ponka et al.\textsuperscript{22} and Adams et al.\textsuperscript{24} in SA-treated reticulocytes. A band also appeared between ferritin and the origin in SA-treated reticulocytes (Fig 2A). However, this latter band was not consistently present, and its nature remains unknown.

The distribution of $^{59}$Fe in the SMM fraction of control versus SA-treated reticulocytes was also markedly different (Fig 2B). In cells treated with SA, a diffuse band (labeled "Z") increased in intensity as a function of incubation time, whereas no such band was present in the SMM fraction of control cells (Fig 2B). It is clear from Fig 2A and B that band Y in the cytosol and band Z in the SMM migrate in the gel to approximately the same position and both appear as diffuse bands. Although this suggests some similarity, we have no definitive evidence that these two species are identical. In the SMM fraction of both control and SA-treated cells, two bands appeared at the top of the gel and increased markedly in intensity as a function of incubation time in SA-treated reticulocytes (Fig 2B). It should be noted that the top "band" in Fig 2B represents the origin.

Previous investigations examining the distribution of $^{59}$Fe in control and SA-treated reticulocytes have used the gel-filtration chromatography technique.\textsuperscript{23,25} In this study, we also examined the distribution of $^{59}$Fe in reticulocytes using gel-filtration columns prepared with Bio-Gel P-10, Bio-Gel P-150, and Sephacryl S-300. For each type of gel examined, 40% to 50% of the applied $^{59}$Fe became bound when the cytosol of SA-treated cells was added to the column. In contrast, when cytosol from control reticulocytes was passed through the gel, only 5% of $^{59}$Fe was adsorbed. For example, using Bio-Gel P-150, 45% of $^{59}$Fe from the cytosol of SA-treated cells became bound to the gel. In this context, it is relevant that Garrick et al.\textsuperscript{25} using agarose-based columns, also found lower recoveries of $^{59}$Fe from the cytosol of SA-treated rat reticulocytes versus control cells: 98% recovery from control cells versus 72% recovery from SA-treated reticulocytes. When DFO (2 mmol/L) was incubated for 30 minutes at 4°C with the cytosol of SA-treated cells and this solution was then passed through the gel, only 5% of added $^{59}$Fe remained on the column (Fig 3). This experiment suggested that in the absence of DFO, much of the cytosolic Fe that was amplified in SA-treated cells became bound to the chromatography media. Therefore, the Fe-containing fraction that was bound to the gel may be equivalent to band Y identified after native PAGE (Fig 2A). Considering the high adsorption of $^{59}$Fe to gel-filtration media, this technique was not appropriate to examine intracellular distribution of $^{59}$Fe in SA-treated cells, and the PAGE-autoradiography method was used in preference.

After gel-filtration chromatography of the cytosol of SA-treated cells, it is obvious that residual radioactivity remained in a peak that comigrated with Hb (Fig 3). Further studies are required to demonstrate directly that the $^{59}$Fe in this peak was actually bound to Hb as $^{59}$Fe-heme.
Distribution of $^{59}$Fe in control and SA-treated reticulocytes after reincubation in the presence of protoporphyrin IX. To test whether any $^{59}$Fe-containing components in control or SA-treated reticulocytes acted like intermediates for heme synthesis, the cells were labeled with $^{59}$Fe-Tf in the absence (control, C) and presence of the heme synthesis inhibitor, SA (S).

Examining first the distribution of $^{59}$Fe in the cytosol of control cells, the most obvious change upon reincubation with protoporphyrin is that band X slowly disappeared and after 2 hours was almost not visible (Fig 4A). Similar data were obtained when $^{59}$Fe-labeled control reticulocytes were reincubated in MEM without protoporphyrin, suggesting that band X is not protoporphyrin-deficient. The chase of $^{59}$Fe in band X during reincubation could indicate that this component is a cytosolic intermediate involved in the transport of heme. Alternatively, band X may be a hemoprotein with a short half-life. It is also of interest that in the SMM fraction of both control and SA-treated cells there was a marked decrease in the intensity of a band at the top of the gel after reincubation with protoporphyrin (Fig 4B). These data suggest that this component may be a mitochondrial or membrane intermediate involved in heme synthesis.

More substantial changes in the distribution of $^{59}$Fe are evident upon examining cells that had been treated with SA and then reincubated with protoporphyrin (Fig 4A). One of the most notable changes in the cytosolic distribution of $^{59}$Fe is the marked increase in the intensity of the Hb band upon reincubation with protoporphyrin (Fig 4A). This increase in Hb-$^{59}$Fe is accompanied by a pronounced decrease in the intensity of band Z in the SMM (Fig 4A), suggesting that this latter component is supplying $^{59}$Fe for heme synthesis. It is also relevant to note that band X in the cytosol of SA-treated reticulocytes is not present before reincubation with protoporphyrin. However, upon reincubation with protoporphyrin there was an increase in the intensity of band X up to 30 minutes, followed by a decrease up to 120 minutes (Fig 4A). Once again, these data suggest that component X may be a cytosolic heme-containing intermediate that may be involved in heme transport. Interestingly, band Y in SA-treated reticulocytes also decreased slowly upon reincubation with protoporphyrin, disappearing from the cytosol after 60 minutes of reincubation (Fig 4A). In addition, previous studies have suggested that ferritin acts as an Fe source for heme synthesis. However, our present results do not support this hypothesis, since there was no decrease in the intensity of the ferritin band in SA-treated reticulocytes upon reincubation with protoporphyrin IX.
Reincubation Time (min): during reincubation with protoporphyrin occurred at a faster rate than when the cells were reincubated with MEM only, changes in the distribution of $^{59}$Fe were identical to those found when the cells were reincubated with protoporphyrin IX. However, changes in the distribution of $^{59}$Fe during reincubation with protoporphyrin occurred at a faster rate than when the cells were reincubated with MEM only (data not shown). The fact that the same alterations in $^{59}$Fe distribution were found upon reincubation in MEM suggests that this component may be a cytosolic heme-containing intermediate involved in the transport of heme to the site of Hb synthesis. In contrast to the marked changes in cytosolic distribution of $^{59}$Fe upon reincubation of SA-treated reticulocytes with protoporphyrin (Fig 4A), there was no change in cytosolic distribution of $^{59}$Fe in SA-treated reticulocytes reincubated with SA and unlabeled diferric Tf (Fig 5). In addition, there was no change in the distribution of $^{59}$Fe in the SMM fraction of control or SA-treated reticulocytes upon reincubation with SA and unlabeled Tf (data not shown).

Effect of the iron chelator, DFO, on the distribution of $^{59}$Fe in reticulocytes. It was relevant to examine whether DFO (1 mmol/L) incubated with the cell lysate for 30 minutes at 4°C could chelate $^{59}$Fe from any of the components identified using native PAGE. Interestingly, DFO markedly depleted $^{59}$Fe from both band Y in the cytosol and band Z in the SMM fraction of SA-treated cells, while having little effect on other $^{59}$Fe-containing components, including band X (Fig 6). The fact that DFO failed to remove $^{59}$Fe from band X further supports our suggestion that Fe in this component may be inserted into heme. It is also pertinent to note that bands Y and Z comigrated with $^{59}$Fe-citrate, and after treatment of the lysate with Pronase (1 mg/mL/24 h at 4°C), neither of these bands were affected, in contrast to other $^{59}$Fe-containing components on the gel (data not shown).

Hence, these data suggest that after native PAGE, a non-protein-bound $^{59}$Fe component was found in the cytosol (band Y) and SMM (band Z) of SA-treated reticulocytes.

Low-M, $^{59}$Fe in control and SA-treated reticulocytes. It is assumed that Fe passes through a low-M, intracellular Fe pool before it is incorporated into heme and other Fe-containing molecules. Due to the pronounced effects of SA on heme synthesis, one may expect a change in this pool, which would thereby facilitate detection of the route taken by Fe to reach the mitochondrial heme biosynthetic pathway.

To examine the cytosolic distribution of $^{59}$Fe in low- and high-M, molecules in control and SA-treated reticulocytes, we used the spinning-column technique. This method is very rapid, which minimizes the possibility of the adsorption and oxidative damage that can occur during conventional gel-filtration chromatography. Our present study demonstrates that a small amount of $^{59}$Fe was present in a low-M, form in control or SA-treated reticulocytes (Fig 7), with most $^{59}$Fe being incorporated into the membranes and high-M, molecules. Surprisingly, when heme synthesis was inhibited by SA, the amount of $^{59}$Fe in the low-M, compartment was markedly less than that found in control reticulocytes (see inset, Fig 7). These data are consistent with the suggestion that the low-M, component may be either heme or a low-M, component containing heme. It is known that Sephadex G-
used in the spinning column has a high avidity for uncommitted cytosolic heme. In addition, if the Fe in the low-M, compartment acted like an intermediate for heme synthesis, 

Fe uptake into this compartment should plateau as a function of time early during the incubation with Fe-Tf. Indeed, after 5 minutes of incubation with Fe-Tf, a steady state is established between the flux of Fe coming into the cell and Fe incorporation into heme (Fig 1B). Hence, presumably, if a cytosolic intermediate is involved in transporting Fe through the cytosol, Fe in this compartment would also have reached a steady state by this time. In contrast, Fe uptake into the low-M, compartment was linear as a function of incubation time up to 20 minutes (see inset, Fig 7), and this linear increase suggested that the Fe is incorporated into an end product.

The existence of minor amounts of low-M, Fe in SA-treated reticulocytes, as assessed by the spinning-column technique, was in marked contrast to results obtained using native PAGE. However, when Tris (0.375 mol/L, pH 8.8) was used instead of HEPES (10 mmol/L, pH 7.4) as the buffer in the spinning column, the amount of low-M, Fe in SA-treated reticulocytes increased from 7% to 39%. Because Tris was used at this concentration in the gel for PAGE, this would explain the presence of low-M, Fe using this technique. Hence, during PAGE, the Fe-chelating properties of Tris probably resulted in the removal of Fe from a high-M, component in the cytosol of SA-treated cells, which may have a relatively low affinity for Fe. We cannot rule out that this high-M, component, which would be in the eluate of the spinning column, may be the 8- to 13,000-Dalton protein previously described by others in SA-treated reticulocytes. It is also important to note that when PAGE was performed using HEPES as buffer, the bands obtained were not well defined, and hence this technique could only be used with Tris-containing buffer.

DISCUSSION

In this investigation we have attempted to gain insight into the nature of the intracellular Fe pools responsible for Hb synthesis in reticulocytes. Indeed, a low-M, intermediate Fe pool17 is thought to play important physiologic and pathophysiologic roles,18-21 and its biochemical characterization is therefore of great importance. The present study was stimulated by reports of a cytosolic Fe-containing molecule that was only present in reticulocytes when heme synthesis was inhibited using INH or SA. Moreover, Fe bound to this cytosolic component could be chased into heme in the absence of SA or into the mitochondrion in the presence of SA, suggesting that it may be a cytosolic transporter of Fe. In addition, this putative transporter had a M, of 8 to 13,000 Daltons and exhibited an absorbance at 280 nm.

Our present study has confirmed the presence of an Fe-
containing molecule in the cytosol of SA-treated reticulocytes (band Y, Fig 2A) which is not found in control cells.\textsuperscript{13,22,24} However, when reticulocytes were incubated with \textsuperscript{59}Fe-Tf plus SA and then chased in the presence of SA and unlabeled diferric Tf, there was neither a decrease in this cytosolic pool of \textsuperscript{59}Fe nor any change in the cytosolic distribution of Fe (Fig 5). If this molecule was a cytosolic transporter supplying Fe either for incorporation into ferritin or for heme synthesis, one would expect that its Fe could be chased into either of these compartments. Hence, our present results do not support a role for this Fe-containing molecule as a cytosolic transporter. It is also prudent to note that this Fe-containing compartment in the cytosol (band Y) had properties similar to those of the \textsuperscript{59}Fe that accumulated in the SMM (band Z). Considering these data, it is possible that the Fe in the cytosol may have "leaked" from the mitochondrion due to damage during isolation. This would explain why this cytosolic component is chased during reincubation with protoporphyrin, namely the marked decrease of \textsuperscript{59}Fe in the SMM (Fig 4B) would lead to an apparent decrease in the cytosolic Fe-containing compound.

It is of interest that after SA treatment some \textsuperscript{59}Fe was still comigrating with Hb (Figs 2A, 3, and 5). Since SA is a competitive inhibitor of heme synthesis, it cannot be expected to completely inhibit heme synthesis, and this may account for the small amount of \textsuperscript{59}Fe incorporated into the Hb fraction. While further studies are required to directly determine that this \textsuperscript{59}Fe in Hb was actually \textsuperscript{59}Fe-heme, it is clear that this Fe-containing pool was not acting as an intermediate in heme synthesis or for Fe incorporation into ferritin, since it could not be chased during reincubation of SA-treated reticulocytes with SA and unlabeled diferric Tf (Fig 5).
Previous reports\textsuperscript{24,25} have demonstrated the presence of an 8- to 13-kD protein that had the properties of a cytosolic Fe transport intermediate in the rat reticulocyte. Because we have examined the rabbit reticulocyte, we cannot exclude that such intermediates exist in the rat system, and further work implementing the techniques used in the present study should clarify the role of cytosolic Fe intermediates in the rat.

Apart from the cytosolic Fe-containing molecule that was found in SA-treated reticulocytes (band Y), another component that displayed the properties of an intermediate was found in the cytosol of control cells (band X). This component has not been reported in previous investigations and was not found when heme synthesis was inhibited with SA (Fig 2A), suggesting that it may contain \(^{59}\)Fe-heme. Upon reincubation of SA-treated reticulocytes with protoporphyrin, band X increased in intensity (Fig 4A). In contrast, when \(^{59}\)Fe-labeled control reticulocytes were reincubated in the presence of SA and unlabeled diferric Tf, there was a marked decrease in the intensity of band X (Fig 5). These reports suggest that component X may be an intermediate involved in the transfer of heme in the cytosol. However, further experiments are required to determine directly that band X contains heme. Once heme is synthesized, it is transported out of the mitochondrion to combine with globin chains in the cytosol, which then assemble into the functional \(\alpha_2\beta_2\)-tetramer.\textsuperscript{42} Further, it is known that release of heme from the mitochondrion depends on the presence of cytosolic protein,\textsuperscript{43} but the mechanism of heme transport is not well understood. It is relevant that glutathione-S-transferase has been postulated to be involved in the cytosolic transport of heme in rat liver\textsuperscript{44} and human erythrocytes.\textsuperscript{45} Alternatively, band X may be a short-lived hemoprotein of as-yet-unknown function, and further studies are clearly required to determine the identity of component X.

A previous study in guinea pig reticulocytes identified a 17,000-M, component that had a specific activity \((^{59}\text{Fe/heme})\) 20-fold greater than that of Hb.\textsuperscript{46} This component contained heme and was considered to be a Hb \(\alpha\)-chain pool for which the heme may be a precursor to the heme in Hb.\textsuperscript{46} Such a heme-containing pool could correspond to the putative cytosolic heme intermediate identified in this report (band X). However, in our present study and also in previous investigations,\textsuperscript{22} no such low-M, pool of \(\alpha\)-chains was demonstrated in rabbit reticulocyte cytosol by gel-filtration chromatography. Hence, band X does not appear to correspond to a heme-containing \(\alpha\)-chain pool.

It should be noted that after inhibition of heme synthesis using SA, there was a marked accumulation of \(^{59}\)Fe in the SMM (Fig 2B). Previous studies\textsuperscript{13,21,22} using INH- or SA-treated reticulocytes also demonstrated an accumulation of \(^{59}\)Fe in the SMM, and analysis of marker enzymes suggested that the \(^{59}\)Fe was present in the mitochondrion. Definitive evidence that \(^{59}\)Fe accumulates in the mitochondrion of SA-treated reticulocytes was provided by Adams et al,\textsuperscript{24} using both electron microscopy and marker-enzyme analysis. Considering the large accumulation of Fe in the mitochondrion in the presence of SA, it could be suggested that there is no coupling between mitochondrial Fe uptake and protoporphyrin synthesis. However, previous investigations have shown that Fe uptake from Tf increases after inhibition of heme synthesis,\textsuperscript{20,22,24} and it is known that heme decreases Fe uptake from Tf into the whole cell\textsuperscript{20,24,25} and the mitochondrion.\textsuperscript{52} Hence, the intracellular level of heme controls Fe uptake from Tf, and it has been commonly assumed that the heme deficit is a likely cause of non-heme Fe accumulation in erythroblast mitochondria in some forms of sideroblastic anemia.\textsuperscript{53,54} Indeed, the Fe metabolism of erythroid mitochondria shows unique characteristics—an accumulation of nonheme Fe is never found in the mitochondria of nonerythroid cells.\textsuperscript{5}

It is apparent from our present investigation that there is only a small amount of low-M, Fe in reticulocytes. In fact, our experiments using the spinning-column technique suggest that the low-M, Fe present does not behave as an intermediate and may correspond to an end product (Fig 7). After inhibition of heme synthesis with SA, \(^{59}\)Fe in the low-M, compartment is markedly decreased (see inset, Fig 7), suggesting that this component may be heme or a low-M, hemeccontaining molecule.

The absence of a low-M, Fe pool that acts as an intermediate in the synthesis of heme (Fig 7) does not conform to the hypothesis proposed by Jacobs.\textsuperscript{17} However, it is relevant to note that earlier reports by other investigators and one of the present authors also failed to identify low-M, Fe intermediates in the cytosol of rabbit or rat reticulocytes\textsuperscript{35,35,37} and human K562 cells.\textsuperscript{52} Moreover, Blackburn and Morgan\textsuperscript{16} demonstrated a low-M, Fe-binding molecule in reticulocytes, but demonstrated that it had no role as an intermediate in the Fe uptake process. Previous studies that have reported the presence of low-M, Fe complexes in cells have been performed in the presence of buffers that contain high concentrations of chelating agents (such as Tris, citrate, acetate, or phosphate)\textsuperscript{13,16,22,38,59} or under nonphysiologic conditions. For example, Weaver and Pollack\textsuperscript{14} reported the existence of AMP-Fe and ATP-Fe complexes in guinea pig reticulocytes. However, they incubated \(^{59}\)Fe-Tf directly with reticulocyte hemolysate to create AMP/ATP complexes, which is clearly not physiologic (ie, Tf does not enter the cytoplasm, but remains enclosed within an endocytotic vesicle).\textsuperscript{3} Bartlett\textsuperscript{14,15} and Konopka et al\textsuperscript{11} also demonstrated large amounts of Fe nucleotides in erythroid cells, but only after acid extraction, which is again unphysiologic. Another problem with these previous studies reporting low-M, Fe complexes is that almost all used conventional gel-filtration chromatography. This technique suffers from several problems: first, it is inherently slow, which can result in oxidation of sensitive components\textsuperscript{32,66} and artificial release of Fe from proteins; and second, a large proportion of the applied sample remains bound to the column, resulting in an artificial distribution of \(^{59}\)Fe in the eluate (eg, Weaver and Pollack\textsuperscript{18} found that \(^{59}\)Fe recovery from their gel-filtration column was only 41%). Furthermore, as stressed by Romslo,\textsuperscript{6} despite the many reports of low-M, Fe-binding components in the cytosol of cells, none of these studies have convincingly demonstrated a role for these complexes as intermediates in the Fe uptake process from Tf.
A quantitatively significant cytosolic pool of low-M, Fe complexes would pose considerable problems to the cell in terms of oxidative stress due to the formation of oxygen radicals via the Fenton and Fe-catalyzed Haber-Weiss reactions. These reactions would be particularly pronounced in reticulocytes with their high content of oxy-Hb (=5 mmol/L). It is known that extremely low levels of nonheme Fe in sickle erythrocytes may be responsible for lipid peroxidation. Further, if one considers the amount of Fe (102,000 to 187,000 atoms Fe/cell/min) that must transverse the reticulocyte to finally be incorporated into Hb and the potential of this Fe to stimulate free radical reactions, it is clear that mechanisms must be present to minimize the concentration of potentially toxic low-M, Fe complexes.

Although our present results demonstrate that the Fe-containing components identified do not appear to act as intermediates in the Fe uptake process from Tf, it cannot be excluded that our techniques may not be sufficiently sensitive to detect a quantitatively small but kinetically rapid pool of Fe that is being quickly transported to the mitochondrion for heme synthesis. However, based on the apparent lack of a cytoplasmic Fe transport intermediate in rabbit reticulocytes and the extremely efficient use of Tf-bound Fe for heme synthesis, we suggest an alternative model of intracellular Fe transport in rabbit reticulocytes that does not imply a quantitatively significant low-M, Fe pool as suggested by Jacobs and others. We propose that after Fe is released from Tf in Hb-synthesizing cells, it is passed directly from protein to protein (probably in hydrophobic environments) until it reaches ferrochelatase in the mitochondrion. According to this model, Fe bypasses the cytosol, since it is transferred from one membrane protein to another. Iron transfer between proteins could be facilitated by the direct interaction of the endosome containing Fe-Tf with the mitochondrion. Such docking between these organelles may then facilitate Fe transfer via membrane-bound channels or carriers, for instance. In this regard, it is of interest that Li et al. have recently demonstrated that H+-ATPase from reticulocyte endosomes can act as an Fe transporter in reconstituted liposomes. Direct interaction between the endosome and the mitochondrion would mediate a highly efficient transfer of Fe and preclude the need for a potentially toxic low-M, cytoplasmic Fe pool. Moreover, an intracellular trafficking network involving the interaction of organelles for transport of molecules is not an unusual concept. Indeed, the specific interaction of secretory vesicles originating from the endoplasmic reticulum with the cis-Golgi stack is well known. Recent work has demonstrated that vesicles and their acceptor membranes contain targeting molecules that interact specifically to initiate docking between these compartments. Experiments by Voelker and others have suggested that the transport of phospholipids occurs by direct contact between membranes of the endoplasmic reticulum and the mitochondrion. In addition, Vance and Rusinol et al. have recently characterized an endoplasmic reticulum-like membrane fraction termed the mitochondrion-associated membrane, which acts as a bridge for phospholipid transport between these organelles. Association of 125I-Tf with mitochondria has been observed in previous investigations, and it is intriguing that the direct interaction of Tf-containing endocytotic vesicles with mitochondria has been demonstrated using electron microscopy. In conclusion, further work examining the interaction of Tf-containing endocytotic vesicles with mitochondria appears to be warranted.

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