CONCISE REPORT

Cytosol Intermediates in the Transport of Iron

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Three 59Fe-labeled nonheme components of the cytosol were identified when rabbit reticulocytes were incubated with 59Fe-labeled plasma under conditions in which the iron supply was not limiting. Two of these components were identified as ferritin and transferrin. The latter was characterized by gel filtration as having apparent molecular weight higher than transferrin, indicating that the transferrin may be complexed to another moiety. The third component, referred to as iron-binding protein-I (IBP-I), is as yet uncharacterized. When the reticulocytes were incubated with unlabeled plasma after pulse-labeling with 59Fe-labeled plasma, 59Fe radioactivity in these cytosol components decreased; after 15 min of chase, the 59Fe in ferritin, transferrin, and IBP-I fell to 64.6%, 28.5%, and 65.8% of the initial values, respectively. A good correlation existed between the decrease of 59Fe in these three nonheme compartments and the associated increase in 59Fe-heme. The data presented suggest that cytosol ferritin, transferrin, and IBP-I are intermediates in the transport of 59Fe from the plasma membrane to the mitochondria.

TWO HYPOTHESES are currently considered to explain iron transport from the reticulocyte plasma membrane to the mitochondrion. The first holds that iron is transported into the cell still attached to its plasma protein carrier, transferrin.1,2 The second postulates that transferrin delivers iron to the plasma membrane and that cytoplasmic factors mobilize the membrane-bound iron for transport to the mitochondria.3,4

In this article we have analyzed the passage of iron through the reticulocyte cytosol by examining the kinetics of 59Fe-labeling of cytosol components in reticulocytes. Rabbit reticulocytes were labeled in a series of short incubations with 59Fe-labeled plasma, followed by chase incubations with unlabeled plasma. In these experiments, cytosolic intermediates in the iron uptake process should be rapidly labeled during incubations with 59Fe-labeled plasma, with loss of 59Fe radioactivity in the subsequent incubations with unlabeled plasma. Using this approach, 59Fe was identified not only in ferritin but also in transferrin and a previously undescribed cytoplasmic component with an apparent molecular weight of about 60,000.

MATERIALS AND METHODS

Incubation of Reticulocytes With 59Fe-Plasma

The iron saturation of transferrin in rabbit plasma was reduced by extensive dialysis against 0.15 M sodium phosphate buffer (pH 5.0). The plasma was adjusted to pH 7.4 with dilute NaOH and NaHCO, added to a final concentration of 20 mM. 59Fe-nitrilotriacetate at a 1:2 molar ratio of 59Fe to nitrilotriacetate was then added to obtain 80%-100% iron saturation of the transferrin. To assure an adequate supply of 59Fe, reticulocytes, induced in rabbits as previously described,4 were incubated with a sufficient volume of 59Fe-labeled plasma (usually 50 μl of reticulocyte-rich cells in 500 μl of 20% plasma) so that less than 10% of the total 59Fe was incorporated by the cells during the period of incubation.

Chase experiments were performed by incubating reticulocytes with 59Fe-labeled plasma for up to 15 min; these cells were then rapidly washed and within 60 sec resuspended in normal nonlabeled plasma for varying lengths of time.

Preparation and Gel Filtration of 59Fe-Labeled Cytosol

Since 59Fe in the putative cytosol intermediates is chased rapidly (see Results), an experimental procedure was established to minimize the time elapsed between the end of the incubation with 59Fe-plasma and isolation of cytosol components. After incubation with plasma, cells were washed with phosphate-buffered saline (pH 7.4) by 3 10-sec centrifugations at 3500 g using a Fisher model 59 bench top centrifuge. No cell lysis was incurred during this procedure. The cell pellet was then lysed with 10 volumes of 20 ideal milliosmolar Tris-HCl (pH 7.6) and the cytosol separated by centrifugation at 10,000 g for 5 min. The stroma was again washed with 10 volumes of lysin buffer. This was centrifuged and the supernatants pooled. An aliquot of cytosol was either directly loaded onto a Biogel A-1.5 column (0.9 x 40 cm) or frozen on an acetone-dry-ice mixture and stored at -80°C. The Biogel columns were equilibrated with 20 imosM Tris-HCl (pH 7.6), 0.005% NaN3. Fractions of 0.56 ml were collected and radioactivity determined in a Searle 1185 double-channel automatic gamma counter. Molecular weights were estimated by calibration of the columns with standard proteins (Pharmacia Company). Control experiments using purified 59Fe-labeled transferrin as a source of 59Fe gave cytosol gel filtration patterns similar to those obtained with 59Fe-labeled plasma. 59Fe radioactivity present as heme in the Biogel A-1.5 column fractions, the stroma, and an aliquot of whole cytosol was assayed by cyclohexanone extraction;7 the efficiency of heme extraction, determined with 59Fe-labeled hemoglobin purified by CM-Sephadex chromatography,7 was at least 90%. Nonehme 59Fe radioactivity in the gel filtration fractions, stroma or whole cytosol was determined by subtraction of the heme 59Fe radioactivity from the total 59Fe radioactivity in each of these fractions. 5Fe-ferritin and 5Fe-transferrin in the gel filtration fractions were assayed specifically by immunoprecipitation with goat anti-rabbit ferritin and anti-rabbit...
transferrin under conditions assuring at least 80% precipitation of the labeled antigens.

RESULTS

Kinetics of $^{59}$Fe-Labeling in Pulse-Chase Experiments

Figure 1 shows the distribution of $^{59}$Fe in heme and nonheme components of the cytosol and in the stroma of rabbit reticulocytes during 15 min of pulse-labeling with $^{59}$Fe-plasma, followed by a 15-min chase with unlabeled plasma. At the end of the pulse period (15 min), approximately 55% of the total iron uptake by the cell had been incorporated into heme. The $^{59}$Fe in the nonheme cytoplasmic and stromal compartments represented 21% and 23% of the total iron uptake, respectively. After 15 min of chase incubation, the heme fraction increased to 74% and the nonheme cytoplasmic and stromal fraction decreased to 14% and 12% of the total $^{59}$Fe uptake, respectively. With longer chase periods, the $^{59}$Fe in the stromal compartment was reduced further with little decrease in the cytoplasmic compartment. These data suggest a flux of iron through the stromal and cytosol nonheme compartments into hemoglobin.

Gel Filtration of Reticulocyte Cytosol

In order to gain further insight into the nature of the nonheme cytosol compartment, $^{59}$Fe-labeled cytosol components were separated according to apparent molecular size by gel filtration. Figure 2A shows the $^{59}$Fe radioactivity pattern of cytosol from reticulocytes incubated for 15 min with $^{59}$Fe-labeled plasma. The most prominent peak of radioactivity is in hemoglobin, potentially obscuring other minor peaks in this area. As seen in Fig. 2B, nonhemoglobin $^{59}$Fe cytosol components are more prominent with shorter incubation periods; after 5 min of incubation with $^{59}$Fe-labeled plasma, two areas of radioactivity other than...
hemoglobin are evident: (1) a component eluting at the elution volume of rabbit ferritin (fractions 24–27) and (2) a second component between fractions 33–39. In addition, a small amount of radioactivity was variably found in fractions 52–55.

The nonheme \(^{59}\)Fe compartments in the cytosol are better visualized and quantified after the gel filtration fractions are extracted for heme and the heme radioactivity subtracted from the total \(^{59}\)Fe radioactivity. Figure 3A shows the nonheme \(^{59}\)Fe radioactivity for a 15-min pulse-labeling experiment. To further identify the three nonheme \(^{59}\)Fe components, ferritin and transferrin were immunoprecipitated in the gel filtration fractions with goat anti-rabbit ferritin and anti-rabbit transferrin antibodies, respectively. Figure 3B and C show the results of these assays. Fifty-five percent of the \(^{59}\)Fe in the first peak is precipitated by antiferritin antibody; in control experiments, the efficiency of this immunoprecipitation reaction was 80%. Therefore other components beside ferritin may contribute to the total iron content in this peak, to be referred to as the "ferritin peak," although ferritin is the major component. Further reference to the size of this ferritin peak is related to that determined specifically by precipitation with antiferritin antibody. Using goat anti-rabbit transferrin, one major \(^{59}\)Fe-containing peak was detected in the area between the ferritin and hemoglobin peaks (fractions 34–39). This peak of \(^{59}\)Fe radioactivity will be referred to as the "transferrin peak"; the apparent molecular weight of the transferrin peak is about 180,000. The third nonheme \(^{59}\)Fe peak, which was found by subtraction of \(^{59}\)Fe heme from the labeled hemoglobin region, is an as yet unidentified component that is precipitated neither by antiferritin nor antitransferrin antibodies. We will refer to this component as iron-binding protein I (IBP-I). IBP-I could also be demonstrated by first removing hemoglobin by ion-exchange chromatography on CM-Sephadex. \(^{59}\)Fe-labeled cytosol was passed through CM-Sephadex and eluted with 0.1 \(M\) Na phosphate (pH 7.0). Of the eluted \(^{59}\)Fe radioactivity, that which was heme-extractable accounted for 74.6% of the radioactivity applied to the CM-Sephadex. As 11.6% of the nonheme material was found to be retained by the CM-Sephadex quantification of the nonheme \(^{59}\)Fe was not possible and hence the more cumbersome procedure described above of heme extraction of gel filtration fractions was used.

**Kinetics of \(^{59}\)Fe Labeling in the Nonheme Cytosol Components**

The changes in radioactivity in the ferritin, transferrin, and IBP-I peaks as a function of time of incubation are shown in Fig. 4A. Radioactivity in the three peaks increases rapidly during the first 5 min of incubation and then rises at a slower rate over the subsequent 10 min. It is apparent from the rapid rate and the magnitude of labeling of \(^{59}\)Fe-hemoglobin (Fig. 1) that demonstration of these cytosol components is possible only with very short periods of labeling (requiring appropriately rapid technical manipulations) or with subtraction of \(^{59}\)Fe heme radioactivity from the gel filtration pattern.

The kinetics of flow of \(^{59}\)Fe out of these cytosol intermediates are shown in the chase experiments in Fig. 4B. The transferrin peak is most rapidly depleted of \(^{59}\)Fe; about 50% of the radioactivity is chased out of this peak within 5 min. In contrast, IBP-I is about 30% depleted of \(^{59}\)Fe at 10 min. Ferritin is depleted to a similar extent; by 10 min after the chase, ferritin-\(^{59}\)Fe has decreased by about 33% with little loss thereafter. Hence, all three of these components have a pool of rapidly mobilizable \(^{59}\)Fe, a finding compatible with the conclusion that these cytosol components are intermediates in the \(^{59}\)Fe transport process. However, part of the cytosol nonheme \(^{59}\)Fe is not rapidly chased; after 15 min of chase, \(^{59}\)Fe is mainly contained in the IBP-I and ferritin iron peaks. The failure to completely
deplete these compartments of $^{59}$Fe in part reflects the continued flux of $^{59}$Fe into the cytosol compartment from the stroma (see Fig. 1). The decrease of $^{59}$Fe in IBP-I, transferrin, and ferritin accounts for about one-third of the radioactivity appearing in heme during the chase period; the remainder of the increase in $^{59}$Fe in heme can be accounted for by the decrease in stromal $^{59}$Fe.

DISCUSSION

A pool of nonheme iron in the cytosol representing iron in transit from the plasma membrane to the mitochondria has been postulated for some time.$^{9,10}$ The evidence has been reviewed by Jacobs.$^{11}$ Most studies give support for $^{59}$Fe bound to a low molecular weight substance.$^{12,13}$ Data suggesting higher molecular weight carriers have been scanty. Najean et al.$^{14}$ and Mazur and Carleton$^{15}$ postulated a pool of ferritin that could deliver iron for heme synthesis, and others have subsequently presented evidence suggesting that iron does pass through ferritin on the way to heme.$^{3,5,16}$ The studies reported here demonstrate a labile pool of $^{59}$Fe bound to three moieties in the cytosol of rabbit reticulocytes. These moieties are rapidly labeled with $^{59}$Fe and, to a greater or lesser extent, the iron can be rapidly chased into heme. By this kinetic analysis, these components appear to be intermediates in the transport of iron through the cytosol of erythroid cells.

The Ferritin Peak

Recently, we have provided evidence that places ferritin directly on the route of iron delivery from membrane to mitochondrion.$^5$ Ferritin can mobilize $^{59}$Fe from $^{59}$Fe-labeled reticulocyte plasma membranes and can donate $^{59}$Fe for heme synthesis. The present studies further confirm an active role for ferritin, as $^{59}$Fe accumulates rapidly in immunoprecipitable ferritin and at least one-third of the $^{59}$Fe accumulated is rapidly exchanged during the chase period. The apparently nonchaseable ferritin iron may reflect either dilution of the $^{59}$Fe with a large core of preexisting nonradioactive iron in ferritin or the existence of two distinct pools of ferritin iron, one used in the intracellular transport of iron for heme synthesis and the other for iron storage.

The Transferrin Peak

Although precipitable with antitransferrin antibody, this peak eluted in gel filtration columns as if it had a molecular weight larger than that of transferrin. Perhaps this material represents transferrin bound to another cellular component, as has been previously suggested.$^{17}$

Two features of the transferrin peak suggest that it acts as a carrier of iron in rabbit reticulocyte cytosol: (1) an apparent steady-state of labeling is reached and (2) iron is readily chased out of this component. It is possible, however, that this peak does not represent a true component of the cytosol but is a part of a transferrin-receptor complex in the plasma membrane and is released as the result of cell lysis. We have recently isolated a transferrin-membrane protein complex with similar gel filtration characteristics from rabbit reticulocyte membranes.$^4$ Alternatively, transferrin within the cytosol may represent transferrin that has been pinocytosed into the reticulocyte.$^{1,2}$

The Iron-Binding Protein I

This iron-binding moiety, to the best of our knowledge, is a newly described entity. Its detection depends on short pulse-labeling periods, rapid preparation of the cytosol, and subtraction of heme-$^{59}$Fe radioactivity from the total $^{59}$Fe radioactivity, although a similar radioactive peak is obtained when cytosol hemoglobin is eliminated by chromatography on CM-Sephadex prior to gel filtration on Biogel A-1.5.

On the basis of the present data it is not possible to determine if the pathway of iron transport proceeds sequentially through the three nonheme iron cytosol components or involves one or more of these independently and in parallel. Elucidation of this point will require an experimental approach allowing identifica-
tion of cytosol components during very short labeling periods. Clearly, though, the net decrease of \(^{59}\)Fe radioactivity in stroma and the cytosol nonheme compartments is equal to the net gain in radioactivity in heme. It is not clear why a low molecular iron carrier, such as described by others\(^9\) and by us,\(^5\) was not detected in these studies. In most prior studies\(^5\) heme synthesis was inhibited by isonicotinic acid. Perhaps the low molecular weight material is of such rapid turnover that it cannot usually be identified in experiments in which heme synthesis is not inhibited. Alternatively, this material may not be directly involved in the normal process of iron transport but may represent a compartment of nonheme iron that increases with inhibition of heme synthesis.

**NOTE ADDED IN PROOF**

Ulvik and Romslo (Biochim Biophys Acta 588:256r, 1979) have recently provided evidence that iron in ferritin may be mobilized by mitochondria and serve as a substrate for mitochondrial ferrochelatase.

**REFERENCES**

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