Acquisition of Iron from Transferrin Regulates Reticulocyte Heme Synthesis

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Fe-salicylaldoxime isonicotinoylhydrazone (SIH), which can donate iron to reticulocytes without transferrin as a mediator, has been utilized to test the hypothesis that the rate of iron uptake from transferrin limits the rate of heme synthesis in erythroid cells. Reticulocytes take up $^{59}$Fe from $[^{59}$Fe]SIH and incorporate it into heme to a much greater extent than from saturating concentrations of $[^{59}$Fe]transferrin. Also, Fe-SIH stimulates [2-$^{14}$C]glycine into heme when compared to the incorporation observed with saturating levels of Fe-transferrin. In addition, $\delta$-aminolevulinic acid does not stimulate $^{59}$Fe incorporation into heme from either $[^{59}$Fe]transferrin or $[^{59}$Fe]SIH but does reverse the inhibition of $^{59}$Fe incorporation into heme caused by isoniazid, an inhibitor of $\delta$-aminolevulinic acid synthase. Taken together, these results suggest the hypothesis that some step(s) in the pathway of iron from extracellular transferrin to intracellular protoporphyrin limits the overall rate of heme synthesis in reticulocytes.

It is generally thought that the formation of ALA is the rate-limiting step in heme biosynthesis (1). Although this is well-documented in hepatocytes (2), there is some evidence that ALA synthase activity does not control heme synthesis in erythroid cells. It has been suggested (3-5) that some step(s) in the pathway of iron from extracellular transferrin, the physiological donor of iron to erythroid cells (6), to protoporphyrin limits the overall rate of heme synthesis in reticulocytes. It has been shown that lack of Fe-transferrin reduces the incorporation of glycine into heme (3), that elevation of non-heme iron pools in reticulocytes increases the incorporation of glycine into heme (3), and that neither ALA nor protoporphyrin increases $^{59}$Fe incorporation into heme in reticulocytes (4, 5).

Recently, we reported (7) that significantly more $^{59}$Fe is incorporated into heme from $[^{59}$Fe]pyridoxal benzoylhydrazone, which can be used as a source of iron for heme synthesis independent of transferrin and transferrin receptors, than from saturating concentrations of $[^{59}$Fe]transferrin. These results, together with the finding that exogenous ALA is not incorporated into heme in Friend erythroleukemia cells in which ALA synthase, ALA dehydratase, and protoporphobilinogen deaminase have been induced maximally with dimethyl sulfoxide (8), show that ALA synthase activity is probably not rate-limiting for heme synthesis in erythroid cells.

In the present study, we have exploited our recent finding that reticulocytes can utilize iron from various acyl hydrazones (7, 9) without transferrin as a mediator, to further examine the hypothesis that the rate of uptake of iron from transferrin limits the rate of heme synthesis in erythroid cells.

MATERIALS AND METHODS

Reticulocytes—Reticulocytes were obtained from chronically bled rabbits, prepared, washed, and incubated as previously (4, 7). In all the experiments reported here, reticulocytes were depleted of transferrin by incubating them five times for 15 min each at 37 °C with 20 volumes of saline containing glucose and bovine serum albumin (9). Each time the cells were collected by centrifugation (3900 rpm for 5 min). Twenty-five $\mu$l of packed cells were incubated in a final volume of 250 $\mu$l of medium (10) containing the indicated substances and with [2-$^{14}$C]glycine or with $^{59}$Fe bound to either transferrin or salicylaldoxime isonicotinoylhydrazone (SIH).

Studies with $^{59}$Fe—$[^{59}$Fe]FeCl$_3$ in 0.5 M HCl was converted to ferric citrate by the addition of a 20-fold molar excess of sodium citrate. $[^{59}$Fe]Transferrin was prepared by mixing the $[^{59}$Fe]ferric citrate with transferrin in a molar ratio of 1 mol of Fe to 0.75 mol of transferrin. Solid NaHCO$_3$ was added to a final concentration of 0.1 M, the pH was adjusted to 7.4, and the solution was kept at room temperature for 3 h and then dialyzed against a large excess of phosphate-buffered saline, pH 7.4. Since some of the $^{59}$Fe originally added was lost during dialysis, the amount of the iron-transferrin complex was estimated using a molar absorption coefficient of 4620 at 470 nm (6).

SIH was synthesized by Schiff base condensation of salicylaldoxime and isonicotinic acid hydrazide (11). SIH was dissolved with a few drops of 1 N NaOH and diluted with incubation medium. Following pH adjustment to 7.4, further medium was added to obtain the required SIH concentration. SIH was labeled with $^{59}$Fe using the same procedure as described previously for the preparation of $[^{59}$Fe]pyridoxal isonicotinoylhydrazone (9) but with an Fe-SIH ratio of 1:1. Following incubation of reticulocytes with $^{59}$Fe-labeled ligands, $^{59}$Fe was determined in cells which had been washed with phosphate-buffered saline and in heme extracted from them with acid methyl ethyl ketone (12).

[2-$^{14}$C]Glycine Incorporation into Heme and Globin—Reticulocytes were incubated as above except that the $^{59}$Fe-labeled ligand was replaced by either nonradioactive Fe-transferrin (20 $\mu$m with respect to iron) or nonradioactive Fe-SIH (50 or 100 $\mu$m). Controls consisted of cells incubated without Fe-ligand. Following 30 min of preincubation of reticulocytes in the presence or absence of Fe-ligands, 1.5-2.0 $\mu$l of [2-$^{14}$C]glycine (New England Nuclear) were added to each 250-$\mu$l sample. At indicated time intervals, cells were washed in ice-cold phosphate-buffered saline, lysed in the cold with 50 $\mu$l of distilled H$_2$O kept on ice for 20 min, and frozen overnight.

After thawing, protein was precipitated by adding 1 ml of ice-cold acidified acetone (3% concentrated HCl in acetone, v/v) and kept on ice for 15 min. Following centrifugation, heme-containing supernatants were transferred into 25-ml tubes. The precipitate was washed twice with acidified acetone, and the supernatants were pooled with the original extract.

The protein was dissolved in 1 ml of 1 N NaOH, re-precipitated by
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adding 3 ml of 10% trichloroacetic acid, and collected on 0.45-μm pore size mixed cellulose acetate and nitrate filters (Millipore Corp.) which were thoroughly washed with 5% trichloroacetic acid. Filters were transferred to scintillation vials, dissolved in 1 ml of Celsolve (ethylene glycol monooethyl ether), mixed with 10 ml of Biofluor (New England Nuclear), and counted. Since almost all of the protein made in reticulocytes is globin, the radioactivities of 14C-protein are referred to as 14C-globin.

Heme was precipitated from the pooled acidified acetone extracts by the addition of 3 ml of distilled water. After a short while, 17 ml of distilled water were added and heme was collected on 0.22-μm pore size mixed cellulose acetate and nitrate filters (Millipore Corp.) which were then treated as described for 14C-globin. In control experiments, 2-14C-glycine was mixed with unlabeled reticulocyte lysate prepared from the same number of cells used in our experiments. The radioactivity of the heme extracted from this mixture by the procedure described above never exceeded 0.4% of the radioactivity added, indicating that the nonspecific binding of 2-14C-glycine to the Millipore filters is negligible.

2-14C-Glycine Incorporation into Heme and Protoporphyrin—Reticulocytes were incubated as above with 2-14C-glycine and at indicated time intervals washed in phosphate-buffered saline, and their content of 14C-heme and 14C-protoporphyrin was determined using a procedure modified (7) from that of Schwartz and Wikoff (13).

2-14C-Glycine uptake by reticulocytes was measured as described by Blostein and co-workers (14). After incubating 100 μl of reticulocytes in 700 μl of buffer (100 mM NaCl, 20 mM Tris-HCl, 12 mM glucose, 1 mM MgCl2, pH 7.4) for 30 min at 37 °C, 200 μl of 2-14C glycine (about 2.5 μCi/sample) were added to a final concentration of 1 μM. At 5, 15, 30, and 60 min, 50-μl samples were withdrawn, mixed with 1 ml of ice-cold buffer, layered on 150 μl of n-butylphthalate, and centrifuged at 18,000 × g for 15 s. The 14C content of aliquots of the supernatant buffer and of the 5% trichloroacetic acid-soluble fraction of cell lysate was determined. The results were calculated for the same volume of extracellular and intracellular water which was assumed to be 70% of the volume of reticulocytes as determined by hematocrit (14).

RESULTS AND DISCUSSION

Effect of Fe-Transferrin or Fe-SIH on 2-14C-Glycine Incorporation into Heme and Globin—We reported recently (7) that both the uptake of 59Fe and its incorporation into heme by reticulocytes are much greater from 200 μM 59Fe-pyridoxal benzoylhydrazone than from saturating concentrations (20 μM) of 59Fe-transferrin. This suggested that the amount of heme synthesized by the cells can be increased by supplying iron to the cells in a form which does not depend on the transferrin pathway of iron acquisition. However, it can be argued that the increased uptake of 59Fe from 59Fe-pyridoxal benzoylhydrazone as compared to 59Fe-transferrin, leads to an increase in the specific activity of an intracellular 59Fe precursor pool and, therefore, that the increased 59Fe in heme does not reflect a higher rate of heme synthesis. To overcome this problem, the rate of heme synthesis was measured using 2-14C-glycine as a precursor. At the same time, it was also used to estimate the rate of globin synthesis. Unlabeled iron was supplied either bound to transferrin or complexed to SIH because preliminary experiments showed that relatively low concentrations (100 μM) of 59Fe-SIH gave the highest labeling of reticulocyte heme when compared to 59Fe bound to various other acyl hydrazones.2

Fig. 1 shows the results of a typical experiment, and Fig. 2 summarizes the results of several experiments in which reticulocytes were incubated with 2-14C-glycine and without any added iron or with iron bound to either transferrin or SIH. Fe-transferrin was added at 20 μM (with respect to iron) since higher concentrations did not increase the incorporation of 2-14C-glycine into heme or globin. The results in Figs. 1 and 2 clearly indicate that with 50 or 100 μM Fe-SIH as a source of iron the incorporation of 2-14C-glycine into heme is, on average, about 40% higher than with the saturating concentration of Fe-transferrin as the iron source. An increase was found in all our experiments, and in some it was as high as 70%. To exclude the possibility that this increase in the

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Fig. 2. Effect of Fe-SIH, added in two different concentrations, on [2-14C]glycine incorporation into heme and globin. Also shown is [2-14C]glycine incorporation into heme and globin in reticulocytes incubated without iron-ligand. In each experiment (the number of experiments is given at the bottom of each column), triplicate samples for each group were run, and results are expressed as percentages (±S.E.) of values obtained with reticulocytes incubated with 20 μM Fe-transferrin (Fe-Tf). The time of incubation with [2-

\[ ^{14} \text{C} \]\text{glycine was 60 min.}

The rates of globin synthesis appear to be the same with either transferrin or SIH used as iron-ligands, suggesting that Fe-SIH causes the accumulation of non-hemoglobin heme in reticulocytes. Even with transferrin as the source of iron, low levels of non-hemoglobin heme can be found in the cytosol and mitochondria of reticulocytes (15, 16). This newly formed non-hemoglobin heme would have a much higher specific activity than hemoglobin heme and may explain the discrepancy we find in the incorporation of [2-14C]glycine into heme compared to globin (Fig. 1). Theoretically, the heme to globin ratio of [2-14C]glycine incorporation should be 0.8; however, in 15 experiments, we obtained an average ratio of 2.1 with a range from 1.7 to 2.5.

Effect of ALA on 59Fe Incorporation into Heme—Table 1 shows that ALA does not stimulate the incorporation of transferrin-bound 59Fe into heme, demonstrating that ALA synthesis is not the limiting step in heme formation in reticulocytes. However, when ALA synthase activity is inhibited with isoniazid (3, 4), added ALA causes normal utilization of 59Fe for heme synthesis independent of the ligand to which the 59Fe is bound. This experiment also shows that exogenous ALA can be utilized by reticulocytes for heme synthesis and as such should be useful in examining the contribution of ALA synthase to the overall rate of heme production.

Effect of N-Methyl Protoporphyrin on [2-14C]Glycine Incorporation into Heme and Protoporphyrin—To determine whether the iron provided to reticulocytes as Fe-SIH is inserted into protoporphyrin enzymatically, we utilized N-methyl protoporphyrin, an inhibitor of ferrochelatase (17, 18).

Fig. 3 shows that N-methyl protoporphyrin inhibited [2-14C]glycine incorporation into heme equally effectively with either transferrin or Fe-SIH as iron sources, indicating that ferrochelatase can function with the iron from both ligands. As expected, the inhibition of ferrochelatase stimulated [2-14C]glycine incorporation into protoporphyrin when iron was de-
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FIG. 4. A, effect of 100 μM Fe-SIH (○) on the incorporation of transferrin-bound 
"Fe (final concentration, 20 μM) into reticulocytes (Rtc) and heme. B, effect of 20 μM Fe-transferrin (○) on the incorporation of SIH-bound "Fe (final concentration, 100 μM) into reticulocytes and heme. Also shown is the effect of succinylacetone (△). Control (●) consisted of cells incubated with a given ligand but without other additions. Unlabeled Fe-ligands were added to the cells at the same time as "Fe-ligands. Results are averages of duplicates.

livered to the cells by either ligand. It is of interest that, in the absence of the inhibitor, prolonged incubation with Fe-SIH results in lower [2-14C]glycine incorporation into protoporphyrin than that found with Fe-transferrin, suggesting that iron provided by Fe-SIH is more effectively utilized for heme synthesis (Fig. 3).

Effect of Fe-SIH and Fe-Transferrin on "Fe Uptake and Utilization—Since reticulocyte iron uptake from Fe-transferrin and Fe-SIH (as well as other Fe-acyl hydrazones) (7, 9) seems to occur by two independent pathways, it was of interest to examine the effect of each of the iron ligands on the uptake of iron from the other. As shown in Fig. 4A, after 15 min of incubation, the reticulocyte uptake of "Fe from transferrin is markedly inhibited by 100 μM Fe-SIH when compared to controls incubated with [59Fe]transferrin. The inhibition of "Fe incorporation into heme commences earlier, shortly after the beginning of the incubation with Fe-SIH, and the inhibition is more pronounced. It seems reasonable to conclude that large amounts of unlabeled iron from Fe-SIH significantly decrease the specific activity of "Fe taken up from transferrin, resulting in decreased "Fe incorporation into heme. However, this "dilution" of intracellular "Fe cannot explain the effect of Fe-SIH on the total uptake of "Fe from transferrin. First, increasing the intracellular non-heme iron pool in reticulocytes by incubating them with inhibitors of heme synthesis does not cause a decrease in iron uptake from transferrin (3, 5). Second, iron uptake from transferrin is not affected by Fe-SIH when heme synthesis is inhibited by succinylacetone (4,6-dioxoheptanoic acid), a specific inhibitor of ALA dehydratase (5, 24) (Fig. 4A). As discussed previously, the addition of 100 μM Fe-SIH probably leads to the intracellular accumulation of non-hemoglobin heme which is known to inhibit the uptake of iron from transferrin by reticulocytes (19-23). Lack of inhibition of iron uptake from transferrin due to Fe-SIH when succinylacetone is present supports the idea that the accumulated heme in Fe-SIH-treated cells blocks the incorporation of "Fe from transferrin (Fig. 4A). On the other hand, 20 μM Fe-transferrin has no effect on the uptake or incorporation of "Fe into heme by reticulocytes incubated with 100 μM [59Fe]SIH. The uptake of "Fe from this ligand does not seem to be regulated, which may explain the higher incorporation of "Fe into heme when compared to that from [59Fe]transferrin (Fig. 4B).

It should be mentioned that when compared to cells incubated only with Fe-transferrin (20 μM), 50 μM Fe-SIH and 50 μM Fe-SIH plus 20 μM transferrin increased [2-14C]glycine incorporation into heme, on average, by about 40% (Fig. 2) and 32% (not shown), respectively, without significantly stimulating globin synthesis (not shown).

We think our data clearly exclude ALA synthase activity as a limiting step in reticulocyte heme synthesis. On the contrary, they indicate that some step(s) in the pathway of iron from extracellular transferrin to protoporphyrin limits the overall rate of heme synthesis in reticulocytes. This controlling step, which is probably a specific part of the heme biosynthetic pathway in erythroid cells (23), may involve the activity or processing of transferrin receptors, the rate of release of iron from transferrin or the reduction, and/or transport of iron from transferrin to ferrochelatase.

The inhibition of globin synthesis by iron deprivation (Fig. 2) is due to a reduction of the synthesis of heme which is required for the initiation of globin mRNA translation (25, 26). Excess heme formed in the presence of 100 μM Fe-SIH does not seem to stimulate globin synthesis above the rate seen with saturating concentrations of Fe-transferrin (Fig. 2), possibly because globin mRNA has become limiting. This may occur since in erythroblasts the transcription of globin mRNA, which is one of the factors determining the amount of reticulocyte globin mRNA, also requires heme (27, 28). In conclusion, during erythroid cell development, the rate of iron uptake determines the rate of heme synthesis, to which globin mRNA transcription and translation have been coordinated. Heme, in turn, limits its own synthesis by inhibiting iron uptake from transferrin (19-23) and probably also by reducing the numbers of transferrin receptors in erythroblasts (29).

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