Regulation of Heme Synthesis in Erythroid Cells: Hemin Inhibits Transferrin Iron Utilization but Not Protoporphyrin Synthesis

By Prem Ponka and Herbert M. Schulman

The inhibition of δ-aminolevulinic acid (ALA) synthase activity by heme is commonly thought to regulate the overall rate of heme synthesis in erythroid cells. However, since heme inhibits erythroid cell uptake of iron from transferrin, we have tested the hypothesis that in reticulocytes heme regulates its own synthesis by controlling the cellular acquisition of iron from transferrin rather than by controlling the synthesis of ALA. We found that heme added to reticulocytes in vitro inhibits not only the total cell incorporation of $^{59}$Fe from transferrin but also the incorporation of $^{[2^{-14}]C}$-glycine and transferrin-bound $^{59}$Fe into heme. However, hemin did not inhibit $^{[2^{-14}]C}$-glycine incorporation into protoporphyrin. Furthermore, cycloheximide, which increases the level of non-hemoglobin heme in reticulocytes, also inhibited $^{[2^{-14}]C}$-glycine into heme but not into protoporphyrin. With high concentrations of ferric pyridoxal benzoylhydrazone (Fe-PBH), which, independent of transferrin and transferrin receptors, can be used as a source of iron for heme synthesis in reticulocytes, significantly more iron is incorporated into heme than from saturating concentrations of Fe-transferrin. This suggests that some step (or steps) in the pathway of iron from extracellular transferrin to protoporphyrin limits the overall rate of heme synthesis in reticulocytes. In addition, hemin in concentrations that inhibit the utilization of transferrin-bound iron for heme synthesis has no effect on the incorporation of iron from Fe-PBH into heme. Our results indicate that in reticulocytes heme inhibits and controls the utilization of iron from transferrin but has no effect on the enzymes of porphyrin biosynthesis and ferrochelatase. This mode of regulation of heme synthesis may be a specific characteristic of the hemoglobin biosynthetic pathway.

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MATERIALS AND METHODS

Chemicals

Hemin chloride and protoporphyrin were obtained from Porphyрин Products (Logan, Utah), 4,5-dioxoheptanoic acid (succinylacetone) (SA) from US Biochemical Corp (Cleveland) and purified human apo-transferrin from Behringwerke (Marburg, W Germany). All other chemicals used were of the highest purity available. $^{59}$FeCl$_3$ (10 to 25 mCi/mg) and $^{[2^{-14}]C}$-glycine (40 to 60 mCi/mmol) were purchased from New England Nuclear Corp (Boston).

Rabbit Reticulocytes

Reticulocytosis was induced in male rabbits by repeated bleeding (10 mL/kg/d) by cardiac puncture with the animals under ketam-

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Supported by grants from the Medical Research Council of Canada.

Submitted April 30, 1984; accepted Oct 11, 1984.

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0006-4971/85/6504-0014$03.00/0

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0006-4971/85/6504-0014$03.00/0
ine-HCl (Ketalar, Parke-Davis Canada, Scarborough, Ontario) anesthesia. Blood was defibrinated by shaking it with glass beads for 15 minutes and filtering it through cheesecloth. Cells were collected by centrifugation at 1,650 g for five minutes at 4 °C. The reticulocyte content was enriched by suspending the top quarter of the cell pellet in a washing buffer (5% bovine serum albumin, 0.137 mol/L NaCl/0.005 mol/L KCl/0.001 mol/L Na₂HPO₄/0.010 mol/L KH₂PO₄/0.001 mol/L MgSO₄ 0.1% dextrose, pH 7.0) and centrifuging. This process was repeated three times. Enriched reticulocytes, free of leukocytes, were suspended in incubation mixture containing essential amino acids as defined by Schulman.²

In experiments examining the effect of hemin, bovine serum albumin was excluded from the incubation mixture. Twenty-five microliters of packed cells were incubated in a final volume of 250 µL of medium containing the indicated substances and [2-¹⁴C]-glycine or [59Fe] transferrin or pyridoxal benzoylhydrazone (PBH). Unless stated otherwise, the cells were preincubated with test substances for 30 minutes before radioactive precursors were added.

**Studies With [⁵⁹Fe]**

[⁵⁹Fe]Cl₂ in 0.5 mol/L HCl was converted to ferric citrate by the addition of a 20-fold molar excess of sodium citrate. [⁵⁹Fe]-transferrin was prepared by mixing the [⁵⁹Fe]-ferric citrate with transferrin in a molar ratio of 1.00 mol Fe:0.75 mol transferrin. After addition of solid NaHCO₃ to a final concentration of 0.1 mol/L, the pH was adjusted to 7.4 and the solution was kept at room temperature for three hours. The final concentration of [⁵⁹Fe]-transferrin was 20 µmol/L with respect to Fe.

PBH was synthesized by Schiff base condensation between pyridoxal and benzoic acid hydrazide.²² PBH was dissolved with a few drops of 1N NaOH and diluted with incubation medium. Following pH adjustment to 7.4, further medium was added to obtain the required PBH concentration. PBH was labeled with [⁵⁹Fe] by the same procedure described previously for the preparation of [⁵⁹Fe]-PBH except that the Fe:PBH ratio was 1:1.

After incubation of reticulocytes with [⁵⁹Fe]-labeled ligands, [⁵⁹Fe]-transferrin was 20 µmol/L with respect to Fe. After incubation of reticulocytes with [⁵⁹Fe]-labeled ligands, [⁵⁹Fe] was determined in cells that had been washed with PBS and in heme extracted with acid methylethylketone by the method of Teale.²³

\[ [²⁴C]⁻Glycine Incorporation Into Heme and Protoporphyrin \]

Reticulocytes were incubated as described above except that [⁵⁹Fe]-transferrin was replaced by nonradioactive iron-transferrin (20 µmol/L with respect to Fe); 1.5 to 2.0 µCi of [²⁴C] glycine was added per 250-µL sample. At indicated time intervals, cells were washed in PBS, and their content of [⁵⁹Fe]-heme and [⁵⁹Fe]-protoporphyrin was determined by means of a procedure modified from Schwartz and Wikoff.²⁴

Samples of packed reticulocytes (25 to 30 µL) were lysed with 500 µL of distilled water and kept frozen overnight. After the samples were thawed, they were mixed with 10 µL of 1 N NaOH containing 10 µg of carrier protoporphyrin and 350 µL of 10% Tween 20. The samples were then mixed with 1 mL of cold ethylacetate-glacial acetic acid (3:1), vigorously shaken, and after settling for 30 minutes at 4 °C, they were centrifuged at 2,800 rpm for five minutes. After the transfer of the upper porphyrin-containing layer into a clean tube, the extraction was repeated and the top layers were combined. After two extractions, the bottom layer was essentially colorless and was discarded. The pooled ethylacetate extracts were washed three times each with 1 mL of 3% sodium acetate.

Protoporphyrin and heme were separated by the addition of 3 mL of 1.5 N HCl to the washed ethylacetate extracts. After being vigorously shaken and centrifuged (2,800 rpm for five minutes), the top ethylacetate layer was transferred to a clean tube and mixed again with 3 mL of 1.5 N HCl. After being shaken and centrifuged, the upper and lower layers were separated. Pooled lower layers contained protoporphyrin and coproporphyrin, whereas upper ethylacetate layers contained heme. When [⁵⁹Fe]-heme or [⁵⁹Fe]-protoporphyrin were added separately to reticulocyte lysates, we found the recoveries of both heme and protoporphyrin by this extraction procedure to be ~50%. Although the recovery is low, it is highly reproducible. Similar recoveries of heme by use of this extraction procedure were previously reported.²⁵

The solutions of heme were decolorized in scintillation vials with 300 µL 30% H₂O₂ and then mixed with 10 mL scintillation cocktail (Biofluor, New England Nuclear). This amount of H₂O₂ does not decrease the efficiency of counting ¹⁴C by more than 5%. The HCl solutions of protoporphyrin were pooled and mixed with 7 mL of saturated sodium acetate. The pH was always above 5.0. Protoporphyrin was extracted with 2 mL of ethylacetate-glacial acetic acid. 3:1. The extraction was repeated once, and the ethylacetate extracts were pooled and washed twice with 1 mL of 0.1 N HCl to remove coproporphyrin. The protoporphyrin solutions were decolorized and mixed with scintillation cocktail as above.

The identities of extracted protoporphyrin and heme were confirmed by paper chromatography²⁶ using commercially obtained heme and protoporphyrin IX as standards. In one experiment, [⁵⁹Fe]-heme and [⁵⁹Fe]-protoporphyrin were added separately to reticulocyte lysates, which were then extracted as above. After paper chromatography, [⁵⁹Fe]-heme and [⁵⁹Fe]-protoporphyrin were found only in spots corresponding to their respective standards.

\[ [²⁴C]⁻Glycine Uptake by Reticulocytes \]

Glycine uptake by reticulocytes was measured essentially as described by Benderoff et al.²⁷ One hundred microliters of reticulocytes was suspended in 700 µL buffer (150 mmol/L NaCl, 20 mmol/L Tris-HCl, 12 mmol/L glucose, 1 mmol/L MgCl₂, pH 7.4) containing the indicated substances for 30 minutes and then 200 µL [³⁴C] glycine (about 2.5 µCi per sample) was added to final concentrations of 0.1 mmol/L or 1 mmol/L. At indicated time intervals, 50-µL samples were withdrawn, mixed with 1 mL ice cold buffer, layered over 150 µL n-butyl-phthalate in Eppendorf tubes and centrifuged at 18,000 g for 15 seconds. ¹⁴C was determined both in aliquots of the supernatant buffer and the 5% trichloroacetic acid (TCA) soluble fraction of reticulocyte lysate. The results were calculated for the same volume of intracellular ("In") and extracellular ("Out") water. Intracellular water was assumed to be 70% of the packed volume of reticulocytes as determined by hematocrit.²⁸

**RESULTS**

Effects of Succinylacetone (SA) and 2,2'-Bipyridine on [²⁴C]⁻Glycine Incorporation Into Heme and Protoporphyrin

Because the major goal of this study was to elucidate the step (or steps) at which hemin controls heme synthesis, the effects of two well-defined inhibitors of heme synthesis on the incorporation of [²⁴C] glycine into heme were measured first.

Figure 1 shows that 1 mmol/L of SA inhibits [²⁴C] glycine incorporation into both heme and protoporphyrin. This is not unexpected because SA inhibits ALA dehydratase²⁹ and therefore inhibits the synthesis of protoporphobilinogen. On the other hand, 2,2'-bipyridine inhibits [²⁴C] glycine incorporation into
HEME. CONTROL 2,2'-BIPYRIDINE -SA

15 30 60 [2.14C]-glycine incorporation into heme and protoporphyrin (PROTO).

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Fig 3. The effect of hemin, added in different concentrations on the uptake and utilization of transferrin-bound 59Fe and on the incorporation of [2.14C]-glycine into heme and protoporphyrin (PROTO).

Effect of Heme on Heme Synthesis

Figure 3 shows the results of a typical experiment and Table 1 summarizes the results of several experiments in which reticulocytes were incubated with and without hemin in the presence of either 59Fe-transferrin or [2.14C]-glycine. Hemin, in a concentration-dependent manner, inhibited 59Fe incorporation into whole cells and heme and [2.14C]-glycine incorporation into heme. However, at 60 minutes 10 µmol/L and 25 µmol/L hemin stimulated [2.14C]-glycine incorporation into protoporphyrin by 13% and 17%, respectively, and 50 µmol/L hemin, which inhibited 59Fe incorporation into heme by 80%, inhibited [2.14C]-glycine incorporation into protoporphyrin by only 8% (Table 1).

Hemin can be taken up by erythroid cells, incorporated into hemoglobin, and can undoubtedly also

The incorporation of 14C into heme is inhibited by 2,2'-bipyridine by about 55% and causes an accumulation of 14C-protoporphyrin, whereas SA inhibits the incorporation of 14C into heme by about 40%; almost all of the 14C that does appear in heme can be accounted for by the decrease in 14C-protoporphyrin, however. In 15 experiments, the ratios of 14C-heme to 14C-protoporphyrin varied between 0.15 and 2.28 after 60 minutes of incubation with [2.14C]-glycine. Although difficult to explain with certainty, this might reflect variations in ferrochelatase levels or activities in reticulocytes of different maturity.
increase the size of the intracellular pool of non-hemoglobin heme. The level of intracellular non-hemoglobin heme can also be increased in reticulocytes by inhibiting globin synthesis. Figure 4 shows that cycloheximide inhibited [2-14C]-glycine incorporation into heme by 33% but had no effect on [2-14C]-glycine incorporation into protoporphyrin.

**Effect of Heme on Uptake of Glycine by Reticulocytes**

Because a change in the uptake of a labeled substrate would alter the rate of its appearance in products of its metabolism, we examined the effect of heme on glycine uptake by reticulocytes. Figure 5 shows that hemin inhibited the transport of [2-14C]-glycine added to final concentrations of 0.1 mmol/L and 1.0 mmol/L into both control and SA-treated reticulocytes. At a concentration of 0.1 mmol/L of [2-14C]-glycine, the inhibition of the glycine uptake was found with a concentration of hemin as low as 10 μmol/L.

**Effect of Heme on Uptake and Utilization of Iron From Fe-PBH**

Although transferrin is considered an obligatory donor of iron for erythroid cells, we demonstrated previously that Fe-PIH, independent of transferrin and transferrin receptors, can provide iron for heme synthesis in reticulocytes. More recently, we found that Fe-PBH, an analogue of Fe-PIH, is more effective than Fe-PIH as an iron donor, (P.P., H.M.S., unpublished observations, 1984). The uptake of iron from transferrin by reticulocytes in vitro is saturable with respect to Fe-transferrin concentrations, maximum rates being obtained within the physiologic range of plasma iron concentration (20 μmol/L). At physiologic concentrations of iron, the uptake of iron from Fe-PIH and Fe-PBH is less than from transferrin; however, the rate of iron uptake from the synthetic ligands is not saturable in concentrations from 20 to 200 μmol/L (P.P., H.M.S., unpublished observations, 1984).

With 200 μmol/L of Fe-PBH, the uptake of iron and its incorporation into heme is much greater than with the saturating concentration (20 μmol/L) of Fe-transferrin (Table 2). It seems that the amount of heme synthesized by the cells can be increased by supplying

### Table 1. Effect of Hemin on 55Fe Incorporation Into Reticulocytes and Heme and on [2-14C]-Glycine Incorporation Into Heme and Protoporphyrin

<table>
<thead>
<tr>
<th>Hemin Concentration (μmol/L)</th>
<th>Reticulocytes 55Fe</th>
<th>Heme 55Fe</th>
<th>2-14C-Glycine Heme</th>
<th>2-14C-Glycine Protoporphyrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>81.0 ± 2.5</td>
<td>76.9 ± 0.3</td>
<td>54.7 ± 8.0</td>
<td>113.1 ± 1.2</td>
</tr>
<tr>
<td>25</td>
<td>57.4 ± 7.1</td>
<td>53.4 ± 7.3</td>
<td>27.3 ± 3.0</td>
<td>117.0 ± 7.9</td>
</tr>
<tr>
<td>50</td>
<td>22.9 ± 6.7</td>
<td>19.2 ± 7.1</td>
<td>29.0 ± 4.3</td>
<td>91.8 ± 8.3</td>
</tr>
</tbody>
</table>

Cells were incubated for 60 minutes. The results of three experiments are presented as the mean percentage of control values ± SEM.

Fig 4. The effect of 1 mmol/L cycloheximide on the incorporation of [2-14C]-glycine into heme (A) and protoporphyrin (B).

Fig 5. The effect of hemin, added in different concentrations on [2-14C]-glycine transport into reticulocytes. Upper portion of the figure shows uptake of 1 mmol/L glycine, and lower portion of the figure shows uptake of 0.1 mmol/L glycine, respectively (C: control; △: 10 μmol/L hemin; Δ: 25 μmol/L hemin; ○: 50 μmol/L hemin).

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iron to the cells in a form that does not depend on the transferrin pathway of iron utilization. This could mean that the rate of iron utilization for heme synthesis in reticulocytes is not limited by either the rate of protoporphyrin synthesis or the activity of ferrochelatase. It suggests that the number of transferrin receptors, the processing of transferrin-receptor complexes, the rate of release of iron from transferrin, or the movement of iron from transferrin to ferrochelatase may be the limiting factor for heme synthesis. Therefore, since hemin inhibits the utilization of iron from transferrin, it was of interest to see if hemin affected the utilization of iron from Fe-PBH. Figure 6 shows that 50 μmol/L hemin had virtually no effect on the incorporation of iron from Fe-PBH into heme, whereas a similar concentration of hemin inhibited the incorporation of iron from transferrin into heme by more than 75%.

These results suggest that under normal conditions, the iron for heme synthesis in reticulocytes is derived directly from transferrin and that intracellular iron stored in ferritin and other compounds is not available for this purpose. This idea is in accord with results published recently by us. 29

**DISCUSSION**

It is generally believed that end product feedback inhibition regulates heme biosynthesis. 2,3,32,34 Burnham and Lascelles first demonstrated that hemin inhibits both the activity and synthesis of ALA synthase in *Rhodopseudomonas spheroides*. 35 Karibian and London 1 later showed that heme synthesis in erythroid cells is subject to negative feedback control, and they suggested that heme regulates ALA synthase activity in these cells also. Other studies, however, have shown that heme inhibits and controls iron uptake from transferrin in erythroid cells. 5,13 In reticulocytes, heme does not inhibit the binding of transferrin to receptors 6 but seems to interfere with the release of iron from transferrin, 6,7 suggesting that the overall rate of heme synthesis in erythroid cells may be regulated by feedback inhibition of the uptake of iron from transferrin.

There is considerable experimental evidence for this hypothesis. First, the rate of iron uptake from transferrin limits the rate of heme synthesis in reticulocytes. This is supported by the following observations: (1) lack of Fe-transferrin reduces the utilization of glycine for heme synthesis; 15 (2) elevation of the non-heme iron pools in reticulocytes increases the incorporation of glycine into heme; 5 (3) addition of ALA or protoporphyrin to reticulocytes does not increase 55Fe incorporation into heme; 16,29; and (4) the increased uptake of iron from Fe-PBH as compared to transferrin is accompanied by an increase in 55Fe (Table 2) incorporation into heme. Second, heme has no effect on the utilization of intracellular non-heme 55Fe for heme synthesis, 14,15 but does inhibit the incorporation of transferrin-bound 55Fe into heme (Figs 3 and 6) and this inhibition is not reversed by ALA. 16 Third, hemin not only inhibits glycine but also ALA incorporation into heme. 16,18 Fourth, hemin, in concentrations in which it inhibits heme synthesis in intact cells 21 has no effect on the synthesis of ALA by mitochondria isolated from reticulocytes 14 or erythroid fetal liver 16 or by homogenates of mouse spleen erythroid cells. 37 There are reports that heme inhibits ALA synthase purified from erythroid cells; 38,39 however, the concentration of hemin used was so high that the activities of other enzymes, such as succinate dehydrogenase, isocitrate dehydrogenase, and NADH-cytochrome c reductase would also be inhibited. 30

Even though, when taken together, these results strongly suggest that ALA synthase is not the rate-limiting enzyme and site of regulation of heme synthesis in reticulocytes, this view has been questioned. 18,41 Ibrahim et al 18 concluded that hemin inhibits ALA

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**Table 2. Reticulocyte Uptake and Utilization of 55Fe Bound to Transferrin or PBH**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Fe Concentration (μmol/L)</th>
<th>Reticulocytes</th>
<th>Fe Incorporation (pmol/10^6 rtc/h ± SEM)</th>
<th>% in Heme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>60 minutes</td>
<td></td>
</tr>
<tr>
<td>Transferrin</td>
<td>20</td>
<td>4.81 ± 0.50 (n = 5)</td>
<td>3.71 ± 0.19</td>
<td>77</td>
</tr>
<tr>
<td>PBH</td>
<td>200</td>
<td>20.22 ± 4.20 (n = 7)</td>
<td>9.68 ± 2.93</td>
<td>48</td>
</tr>
</tbody>
</table>

Reticulocytes were depleted of transferrin and incubated for 60 minutes with 55Fe, added at indicated concentrations, bound to either transferrin (15 μmol/L) or PBH (200 μmol/L); rtc, Reticulocytes; n, No. of experiments.
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synthase activity in reticulocytes; however, more recently he and his co-workers demonstrated that hemin induces ALA synthase activity in erythroid precursor cells during differentiation. It is difficult to imagine opposite responses of ALA synthase to heme in erythroid precursor cells and reticulocytes. In their experiments with reticulocytes, using transferrin-free medium, they found that 50 to 100 μmol/L hemin inhibited [2-14C]-glycine incorporation more than [4-14C]-ALA incorporation into heme. However, their conclusion that heme controls ALA synthase activity in reticulocytes may not be justified. The results of studies of heme synthesis in reticulocytes incubated without transferrin as a source of iron are questionable. In addition, because hemin inhibits glycine transport (Fig 5), their measurements of the effect of hemin on the rate of heme synthesis using [2-14C]-glycine incorporation were probably incorrect. Our experiments with 1.0 and 0.1 mmol/L glycine show that hemin inhibits glycine uptake more at the lower concentration of glycine (Fig 5). The inhibition of transport could have been quite considerable in their experiments, for they used only 12.5 μmol/L glycine. Furthermore, such a low concentration of glycine may not reflect ALA synthase activity accurately because the K_m for glycine of the enzyme is 4 to 19 mmol/L. Because of these difficulties of interpretation, we decided to test further the hypothesis that heme regulates heme synthesis in erythroid cells by controlling the entry of iron into the cells and/or the delivery of iron to ferrochelatase.

When hemin was added to reticulocytes in relatively low concentrations (10 to 25 μmol/L), it inhibited 59Fe and [2-14C]-glycine incorporation into heme but stimulated [2-14C]-glycine incorporation into protoporphyrin (Fig 3). By correcting for the decreased uptake of [2-14C]-glycine caused by hemin (Fig 5), we were able to demonstrate that even 50 μmol/L hemin leads to accumulation of 14C-protoporphyrin. Although 50 μmol/L hemin inhibits glycine uptake by the cells, it does not significantly affect its incorporation into protoporphyrin. It is apparent that the level of 14C-protoporphyrin in the cells is determined not only by the flux of [2-14C]-glycine into the cells but also by the rate of 14C-protoporphyrin incorporation into heme. We believe that these results clearly show that heme does not inhibit any enzyme in the heme biosynthetic pathway between ALA synthase and protoporphyrinogen oxidase, but does inhibit heme synthesis by blocking the uptake of iron from transferrin. Even though heme has been shown to inhibit the activity of ferrochelatase from liver mitochondria, our results indicate that it does not inhibit ferrochelatase in reticulocytes since hemin has no effect on the incorporation of iron from Fe-PBH into heme.

The mechanism by which heme regulates heme synthesis may be different in erythroid and nonerythroid cells, a conclusion that is in agreement with a review by Kappas et al. The inhibition of iron uptake that is caused by heme seems to be a property only of erythroid cells since it is observed with reticulocytes (references 4 through 9 and this study), fetal mouse liver erythroblasts, human erythroblasts, and hemo-

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ACKNOWLEDGMENT

We are grateful to Ania Wilczynska for advice, Cheryl Partridge for technical assistance, Sandy Fraiberg for typing the manuscript, and Christine Lalonde for the illustrations. We are indebted to Dr R. Blostein for helpful discussion regarding glycine uptake measurements.
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