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# HEMIN FEEDBACK INHIBITION AT RETICULOCYTE δ-AMINOLEVULINIC ACID SYNTHETASE AND δ-AMINOLEVULINIC ACID DEHYDRATASE

Nader G. Ibrahim, Naomi R. Gruenspecht and Michael L. Freedman
Dept. of Medicine, New York University School of Medicine, New York, NY 10016

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SUMMARY: Addition of hemin (5-200 μM) to a rabbit reticulocyte iron-free incubation medium, resulted in a progressive inhibition of heme synthesis as measured by incorporation of ($^{14}$C)-glycine. In contrast when ($^{14}$C) δ-aminolevulinic acid incorporation into heme was studied, significant inhibition below that of the ($^{14}$C)-glycine control only occurred with hemin concentrations greater than 100 μM. Hemin progressively inhibited cellular and mitochondrial δ-aminolevulinic acid synthetase activity, as well as cellular δ-aminolevulinic acid dehydratase activity. The results indicated that elevated levels of hemin initially control heme synthesis by feedback inhibition at the rate-limiting enzyme of heme synthesis, δ-aminolevulinic acid synthetase. Hemin inhibition of δ-aminolevulinic acid dehydratase is only significant for the entrire heme synthetic pathway when greater than one-third of this enzyme's activity is inhibited.

Previous studies with intact reticulocytes (1, 2), and the purified enzyme (3) from these cells have suggested that hemin directly inhibits δ-aminolevulinic acid synthetase activity. However, recently this view was challenged and it was proposed that hemin does not directly inhibit δ-aminolevulinic acid synthetase but rather decreased the incorporation of transferrin-iron into reticulocytes, and that the enzyme requires iron for maximal activity (4). The present study, therefore, was undertaken in an attempt to resolve this conflict. Our results in iron-transferrin free media show that hemin inhibits δ-aminolevulinic acid synthetase activity both in intact reticulocytes and their partially purified mitochondrial preparations. In addition hemin also inhibits δ-aminolevulinic acid dehydratase, but this is only significant for the entire heme synthetic pathway when greater than 33% of the enzyme activity is inhibited.

METHODS: Measurement of Heme Synthesis in Reticulocytes: Reticulocyte-rich blood was collected in heparin from phenylhydrazine-treated rabbits by needle puncture of an ear artery. The preparation and washing of the reticulocytes were as previously described (2). Incubations (4 ml) were performed at 37° for 60 min. The incubation medium, preparation of hemin and measurement of heme synthesis using L-2 ($^{14}$C)-glycine (50 nmoles, 2.5 μCi) and L-4-($^{14}$C) ALA (100 nmoles 5 μCi) was as previously described (2)

δ-aminolevulinic Acid Synthetase Assay: The preparation of reticulocytes and incubation medium was identical to the above with following modifications; 1 mM EDTA was added to inhibit δ-aminolevulinic acid dehydratase and the total volume of incubation was 2 ml.

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At the end of the 60 min incubation at 37°, 0.6 ml of the incubation mixture was added to 0.2 ml of 20% TCA. The tubes were left on ice covered with aluminum foil for 10 min and then centrifuged at 2000 rpm for 10 min. The amount of ALA formed was estimated in 0.4 ml of the TCA supernatant from incubated intact cells by a modification of the original method of Mauzerall and Granick (5). δ-aminolevulinic acid was converted to pyrrol compound by condensation with acetyl acetone in sodium acetate buffer, pH 4.7. The aminoacetone was removed by methylene chloride, in this step there is approximately a 5% loss of δ-aminolevulinic acid. The color was developed by the addition of an equal volume of Ehrlich-Hg reagent; the peak of pyrrol was scanned on an Aminco-DW 2 spectrophotometer from a wavelength of 500-650 nm. The peak at 553 nm represents δ-aminolevulinic acid (6). The millimolar extinction coefficient was assumed to be 58 $cm^{-1}$. The amount of protein was determined by the method of Lowry, et al (7).

Preparation of Reticulocyte Mitochondria: Reticulocyte hemolysates were prepared by adding 2 ml of cold deionized water to 1 ml of washed packed cells for 30 seconds. The mixture was then homogenized by 3 strokes with a teflon homogenizer and sucrose, tris - HCl, EDTA buffer, pH 7.4 was added to the lysate to a final concentration of 0.25 M sucrose, .01 M Tris HCl and .001 M EDTA. Rabbit reticulocyte mitochondria were isolated from hemolysates by centrifugation at 650 x g for 20 minutes and the supernatant was carefully removed. This supernatant was then layered over 2 ml of 0.5 sucrose in Tris - HCl (.01 M) EDTA (0.001 M) pH 7.4, and centrifuged at 27,000 x g for 15 minutes. The supernatant was discarded and the resultant pellet was resuspended and homogenized gently with a teflon glass homogenizer. The suspension was centrifuged once more at 22,000 x g. The final pellet was resuspended in the same buffer to give a final concentration of 10 mg mitochondrial protein per ml.

The mitochondria were incubated in a medium containing 50 mM glycine, 50 mM sodium phosphate dibasic, 10 mM $MgCl_2$, 1 mM EDTA pH 7.4 and pyridoxal phosphate 0.2 mM (6). The reaction was carried out in glass test tubes in final volume of 0.6 ml for 60 minutes at 37°C in shaking waterbath. The reaction was terminated by addition of 0.3 ml of 15% TCA. The tubes were allowed to sit on ice for 10 minutes and then centrifuged for 10 minutes at 1500 x g. δ-aminolevulinic acid formation was measured in the resultant supernatant as described previously for the whole cells. Hemin concentration was added as in Table I.

δ-aminolevulinic Acid Dehydratase Assay: δ-aminolevulinic acid dehydratase activity was determined by using 50 µl of whole reticulocytes in a final incubation volume of 0.5 ml according to the method of Sassa, et al (8). The incubation medium contained 0.05 M sodium phosphate pH 6.6 and 0.005 M δ-aminolevulinic acid. Dithiothreitol (0.01 M) and hemin were added as shown in Table 2. The mixture was agitated and incubated in a shaking water bath at 37°C for 1 hour. The reaction was terminated by the addition of 0.8 ml, 5% TCA, 0.1 M $HgCl_2$. After the tubes stood on ice for 10 min. they were centrifuged at 1000 x g for 10 min. 0.4 ml of the supernatant was removed to another set of tubes and an equal volume of modified Ehrlich reagent was added (5). The absorbance was determined within 15 min. The spectrum was recorded from 450-650 nm. The molar absorption coefficient of porphobilinogen at 550nm is $6.1 \times 10^4$.

RESULTS: Incorporation of ($^{14}C$)-glycine measures the entire heme synthetic pathway while incorporation of ($^{14}C$) δ-aminolevulinic acid measures the pathway beyond δ-aminolevulinic acid synthetase. As previously reported, and confirmed in this study, (Table I) δ-aminolevulinic acid synthetase is the rate-limiting enzyme in heme synthesis (8). Thus,

TABLE 1

HEMIN INHIBITION OF L-2-($^{14}$C) GLYCINE AND L-4-($^{14}$C) ALA INCORPORATION INTO RABBIT RETICULOCYTE HEME (n = 6)[a]

| 14C Precursor | No Hemin in Incubation | | Hemin (μM) in Incubation | | | | | |
|---|---|---|---|---|---|---|---|---|
| | pmoles $^{14}$C/mg heme ± SEM | % Control[b] | 50 | | 100 | | 200 | |
| | | | pmoles $^{14}$C/mg heme ± SEM | % Control | pmoles $^{14}$C/mg heme ± SEM | % Control | pmoles $^{14}$C/mg | %Control |
| Glycine | 592±122 | 100 | 314±44.9 | 53 | 249±16.2 | 42 | 184±13.8 | 31 |
| ALA | 807±113 | 136 | 823±32.1 | 139 | 597±28.5 | 101 | 442±25.6 | 78 |

(a) Incubation at 37° for 60 minutes in duplicate

(b) Control = glycine control = 100%

there is a greater (1.36 fold) incorporation of precursor when ($^{14}$C) δ-aminolevulinic acid is used to bypass the rate limiting step. If an agent selectively inhibits δ-aminolevulinic acid synthetase, incorporation of ($^{14}$C)-glycine would be decreased but incorporation of ($^{14}$C)-δ-aminolevulinic acid would be unaffected. If an agent inhibits heme synthesis at several steps including δ-aminolevulinic acid synthetase, both ($^{14}$C) glycine and ($^{14}$C) δ-aminolevulinic acid incorporation would be decreased. However, in terms of cellular activity, the decrease in incorporation of δ-aminolevulinic acid is only significant when it is below that of the glycine control. It is necessary, therefore, to compare incorporation of ($^{14}$C) δ-aminolevulinic acid to that of ($^{14}$C) glycine control which measures the entire pathway. Thus, as shown in Table I, the heme synthetic pathway capability is represented by the glycine control of 592± 122 p moles $^{14}$C incorporated/mg heme (100%). Hemin (50, 100, 200 μM) inhibited glycine in these experiments by 47%, 58% and 69% respectively. When ($^{14}$C) δ-aminolevulinic acid was used as precursor and compared to the glycine control of 100%, there was no inhibition until 200 μM (22%). If ($^{14}$C) δ-aminolevulinic acid incorporation in the presence of 50, 100, 200 μM hemin was compared to the control ($^{14}$C) δ-aminolevulinic acid incorporation (without hemin), 100 μM hemin give 26% inhibition while 200 μM hemin gave 45% inhibition. However, this comparison does not take into account the greater activity of the pathway beyond δ-aminolevulinic acid synthetase. It appears, therefore, that the major inhibitory action of hemin is at δ-aminolevulinic acid synthetase, but that the pathway is also inhibited beyond this enzyme.

TABLE 2

HEMIN INHIBITION OF RETICULOCYTE ALA-S AND ALA-D ACTIVITY

| Hemin in Incubation (μM) | ALA-S Activity [a] (%) | ALA-D Activity [b] (% Control) | |
|---|---|---|---|
| | | -DTT | +DTT |
| 0 | 100 | 100 | 100 |
| 5 | 96 | 91 | 94 |
| 25 | 86 | 87 | 87 |
| 50 | 61 | 86 | 84 |
| 100 | 56 | 64 | 66 |
| 200 | 50 | 30 | 40 |

(a) ALA-S
Control = 259 pmoles/ALA formed/mg protein/hour = 100%

(b) ALA-D
Control +DTT = 27.2 nmoles PB6 formed/mg protein/hour =100%

Control - DTT = 24.4 nmoles PB6 formed/mg protein/hour = 100%

In order to confirm that δ-aminolevulinic acid synthetase was being inhibited by hemin, this enzyme activity was measured both in intact reticulocytes and their isolated mitochondria. The results in Table 2 show that 5-200 μM hemin progressively inhibited the enzyme in intact cells. As shown in Table 3, hemin (25-100 μM) al so progressively inhibited the enzyme in isolated mitochondria.

δ-aminolevulinic acid dehydratase was also found to be inhibited by hemin. Table 2 shows the comparative progressive percent inhibition of δ-aminolevulinic acid synthetase and dehydratase in the presence of hemin.

DISCUSSION: The reticulocyte has an unusually large demand for heme, as its major product is hemoglobin. It, therefore, must have a mechanism to synchronize heme and protein synthesis to avoid an excess of either. It has been shown in this and other laboratories that if heme synthesis is primarily decreased, protein synthesis is secondarily decreased (9-12). This control is exerted via an inhibitor of chain initiation, the "hemin-controlled repressor" (11-17).

TABLE 3

HEMIN INHIBITION OF ALA-S FROM ISOLATED RETICULOCYTE MITOCHONDRIA (n=4)[a]

| | Control | Hemin 5 μM | Hemin 25 μM | Hemin 50 μM | Hemin 100 μM |
|---|---|---|---|---|---|
| Mean | 8.77[b] | 9.19 | 7.54 | 6.13 | 5.44 |
| SEM | 0.85 | 0.68 | 0.25 | 0.58 | .79 |
| % Control | 100% | 105% | 86% | 70% | 62% |

a) n = number of experiments on different reticulocytes performed in duplicate.

b) Results expressed as nmoles ALA formed/mg protein/ 1 hour incubation at 37°.

When protein synthesis is primarily inhibited (e.g. cycloheximide (18) or supranormal temperatures (19) ) heme synthesis is secondarily inhibited. This secondary effect on heme synthesis has been proposed to be the result of increased levels of intracellular heme inhibiting the first and rate-limiting enzyme of heme synthesis, δ-aminolevulinic acid synthetase (20). There are three proposed mechanisms by which the amount of δ-aminolevulinic acid formed in a cell can be regulated by hemin at the level of the enzyme δ-aminolevulinic acid synthetase. The first involves a decrease in activity of the enzyme by end produce feedback inhibition (1-3). The second is that there is a change in the quantity of the eznyme present, through variation of the rate of synthesis of mRNA (end-product feedback repression (21)). Since reticulocytes are anculeate cells, there is no mRNA synthesis and therefore, end product feedback repression is not possible in these cells. The third mechanism has been proposed by Neuwirt et al and recently summarized (4). These investigators have proposed that hemin interferes with transferrin-iron uptake into reticulocytes and that δ-aminolevulinic acid synthetase requires iron for its activity.

In the studies by Neuwirt et al (4) they attempted to exclude the possibility of hemin feedback inhibition by showing that when glycine incorporation into heme was decreased, δ -amino-levulinic acid incorporation was also decreased. This is in contrast to other studies (1,2). However, it should be noted that very high non-physiological concentrations of hemin (100-400 μM) were used by Neuwirt et al (4). To achieve maximal protein synthesis in the presence of an iron

chelating agent ( αα-dipyridyl) only 50 μM hemin is necessary (11,12). Furthermore, Neuwirt et al (4) compared δ-aminolevulinic acid incorporation in the presence of hemin to that of δ-aminolevulinic acid incorporation in the absence of hemin, rather than to a glycine control.

In our present study we used a range of hemin concentration to include the "physiological" 50 μM. Furthermore, we have used an iron-transferrin-free medium. On the basis of our data both with intact reticulocytes and their isolated mitochondria it is clear that hemin can control δ-aminolevulinic acid synthetase activity by end-produce feedback inhibition.

It is also clear from our data, that hemin similarly inhibits the activity of δ-aminolevulinic acid dehydratase. This is in agreement with previously reported findings (22-24). However, since δ-aminolevulinic acid synthetase is the rate limiting enzyme (Table I),approximately one-third of δ-aminolevulinic acid dehydratase may be inhibited (Table 2) before there will be any decrease in the total heme synthetic capability of the cell starting from glycine. Thus, any study using comparative incorporation of glycine and δ-aminolevulinic acid must take into account that it is only glycine incorporation which measure the total cellular rate of heme synthesis. This inhibition of δ-aminolevulinic acid dehydratase, as well as the high concentrations of hemin used by Neuwirt et al (4) appears to explain the disparity between their results and those of Karibian and London (1) and Freedman, et al (2).

Our results indicate that in the reticulocyte as hemin levels begin to rise for any reason, the first effect would be feedback inhibition at δ-aminolevulinic acid synthetase and a decrease in heme synthesis. If hemin levels continue to rise the feedback inhibition at δ-aminolevulinic acid dehydratase will become important and serve as an additional protective mechanism to balance heme and protein synthesis.

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