Hemin Control of Globin Synthesis

ISOLATION OF A HEMIN-REVERSIBLE TRANSLATIONAL REPRESSOR FROM HUMAN MATURE ERYTHROCYTES*

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MICHAEL L. FREEDMAN,† MICHAEL GERAGHTY,§ AND JUDITH ROSMAN
From the Department of Medicine, New York University School of Medicine, New York, New York 10016

SUMMARY

Hemin mature erythrocyte postribosomal supernatant contains a hemin-reversible translational repressor of globin synthesis as measured in the rabbit reticulocyte cell-free system. Incubation of human supernatant minus hemin at 37° for 2 hours results in conversion of the repressor to a hemin-irreversible form. The presence of 35 μM hemin retards this conversion.

Both hemin-reversible and -irreversible forms of the repressor may be partially purified by precipitation of supernatant at pH 5. Incubation of the partially purified hemin-reversible repressor minus hemin at 37° for 1 hour or longer results in conversion to the irreversible form. Hemin (35 μM) also retards this conversion. The activity of the repressor is destroyed by incubation with trypsin or by boiling and is therefore presumed to be a protein. Both forms of the repressor are eluted at the same point on a Sephadex G-200 column corresponding to a molecular weight of 3.0 x 10̇.

The isolation of a hemin-reversible translational repressor from an erythroid cell which has lost its protein synthetic capability is evidence that this inhibitor is a physiological regulator of globin synthesis.

Hemin has been shown to be required by both intact rabbit reticulocytes (1-6) and their cell-free preparations (7-13) for maximal globin synthesis. When intact cells are incubated in the absence of either hemin or iron for hemin synthesis (3-5) the polyribosomes are converted to single ribosomes (6) not attached to mRNA (14), indicating that the control is at the site of initiation of protein synthesis. Both hemin and iron-trasferrin prevent and reverse this inhibition of initiation under appropriate conditions in the intact cell (3-5). When cell-free preparations are incubated in the absence of hemin, a hemin-reversible protein translational repressor of globin chain initiation has been reported to form in the postribosomal supernatant at the same time globin synthesis stops (15, 16). Prolonged incubations of the lysates minus hemin result in conversion of this reversible repressor to a hemin-irreversible form (9-12, 15, 16). Hemin markedly retards the formation of both the reversible and irreversible forms of the repressor. It has been postulated that the reversible repressor may be the regulator through which globin synthesis is controlled by hemin in the intact cell (15).

In the present study, we confirm these findings in the rabbit reticulocyte system and furthermore isolate and characterize similar hemin-reversible translational repressor activity from mature human erythrocyte postribosomal supernatants. These findings support the view that hemin is a physiological regulator of globin synthesis via prevention of the activity of a translational repressor.

EXPERIMENTAL PROCEDURES

Reticulocytosis was induced in rabbits by the injection of phenylhydrazine. The blood was collected in heparin by cardiac puncture, washed three times with an isotonic salt solution and the buffy coat removed. The cells were lysed by addition of an equal volume of ice-cold deionized water and the stroma removed by centrifugation at 25,000 X g for 15 min (10). The resultant supernatant is referred to as "lysate" hereafter.

Cell-free Incubations—The incubation conditions were similar to those described previously (10-13). All sulfhydryl compounds were omitted from the incubation, as these were inhibitory. A standard incubation contained the following ingredients added in this order: (a) 20 μl of 4.2 X 10^4 μM hemin in 50 mM Tris-Cl (pH 7.9) or 20 μl of Tris-Cl without hemin. Hemin was obtained from the Sigma Chemical Co. It was dissolved in 0.2 ml of 1 N KOH and then 1 ml of 0.2 M Tris-Cl (pH 7.8), and 2.6 ml of water were added. The pH was adjusted to 7.8 with 1 N HCl. The concentration was determined spectrophotometrically as previously described (6); (b) 10 μl of lysate; (c) 40 μl of a test solution of postribosomal supernatant or partially purified repressor (see below); (d) 80 μl of master mix. Each milliliter of master mix contained the following ingredients: 7 mg of creatine phosphate disodium salt and 0.5 mg (50 enzyme units) of creatine phosphokinase, both obtained from the Sigma Chemical Co., 10 μCi of L-[U-14C]leucine (33 nmole) in 0.1 ml of 0.01 N HCl; 0.1 ml of a mixture of nonradioactive amino acids as previously described (15); 0.1 ml of a mixture of ATP and GTP to deliver 3 and 0.6 μmole, respectively (these were made up together in water and neutralized to pH 6.9); 0.5 ml of salts so that the final concentration of the master mix was 0.225 μM KCl; 6 mM MgCl₂ and 30 mM Tris-Cl (pH 7.8); and 0.2 ml of water.

Incubations were performed at 34° in plastic tubes in a rotating water bath. Additions were performed at room temperature and...
the incubation started at 34°C immediately after the addition of master mix.

**Formation and Isolation of Repressor Activity in Postribosomal Supernatants**—Either rabbit reticulocyte or human mature erythrocyte lysates were centrifuged at 225,000 × g for 90 min at 4°C to remove the ribosomes. The resultant postribosomal supernatants were then incubated at 37°C in the presence of either 35S-hemin or an equal volume of the 50 mM Tris buffer for the times shown in the individual experiments. The incubation minus hemin results in formation of the translational repressor. Additions to the cell-free incubation systems were made at room temperature.

In experiments where the repressor was isolated, the postribosomal supernatant containing the repressor was diluted with an equal volume of deionized water and slowly titrated at 0°C to pH 5.0 with 0.1 M acetic acid. The precipitate was centrifuged in the cold at 1,600 × g for 15 min and the pellet was dissolved in a volume equal to the original lysate of 36.7 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes) buffer, pH 7.2. A small amount of undissolved material was removed by centrifugation at 34,000 × g for 15 min. The clarified solution had a pH of 7.1. This procedure removed over 95% of the hemoglobin in the supernatant. This partially purified repressor was diluted with the Hepes buffer, as described with individual experiments, and added to the cell-free incubations.

**Determination of Molecular Weight of Repressor on Sephadex G-200 Column**—A Sephadex G-200 column (100 × 2.6 cm), equilibrated with 3.67 mM Hepes (pH 7.2) and 0.0276 M KCl, was developed with this buffer at a rate of 16 ml per hour at 4°C. The indicated markers were applied separately to the column in 4-ml fractions containing 4 ml. Fractions, containing 4 ml, were collected and the elution volume determined by the peak of absorbance at 280 nm (γ-globulin, bovine serum albumin), 260 nm (blue dextran), or 415 nm (rabbit hemoglobin). The fraction of irreversible repressor was determined as the peak of inhibitory activity after the application of 1 ml of isolated repressor (mixed with 3 ml of water) from a human postribosomal supernatant preincubated for 2 hours minus hemin. To locate hemin-reversible repressor, an aliquot of 1 ml of isolated repressor from nonpreincubated postribosomal supernatant was mixed with 3 ml of water and applied to the column. Each column fraction was incubated at 37°C for 3 hours and the peak of inhibitory activity was determined.

**Measurement of Incorporation of Radioactivity into Hemoglobin and Globin**—In most experiments, hemoglobin synthesis was measured by removing 25-μl samples from the incubation mixture into 6 ml of ice-cold 0.02 M NaCl containing 20 μmoles of L-[14C]leucine. Two milliliters of 20% trichloroacetic acid were added to this mixture as it was agitated vigorously. After 15 min at 0°C, 20 min at 85°C, and another 15 min at 0°C, the solution was filtered through a Millipore filter (0.45 μm pore size). The precipitate was washed with 5% cold trichloroacetic acid, dried, and counted in a gas-flow counter with Micromil window (efficiency 10%). In one experiment, as noted in the figure legend, heme was released and globin precipitated with acidified acetone (3 μl 0.15 M HCl). The globin was washed with 2 ml of 5% trichloroacetic acid at 85°C for 15 min and then with 3 ml of acetone. The dried precipitate was dissolved in 0.20 ml of formic acid and counted with 10 ml of Aquasol (New England Nuclear Corp.) in a Beckman liquid scintillation spectrometer (efficiency 90%).

**RESULTS**

**Formation of Translational Repressor Activity in Rabbit Reticulocyte Postribosomal Supernatants**—In confirmation of previous reports (7–13), hemin was found necessary in rabbit reticulocyte lysate cell-free preparations for maximum protein synthesis (Fig. 1). In the absence of hemin (Fig. 1B), the rate and extent of hemoglobin synthesis were markedly diminished after 5 min of incubation at 34°C. In order to confirm that this was due to the formation of a translational repressor, postribosomal supernatants were incubated at 37°C for varying times, and aliquots were added to the incorporating lysate system.

In Fig. 1, the effects of 5-, 30-, and 60-min preincubation of the rabbit reticulocyte postribosomal supernatant are shown. Both the 5- and 30-min preincubated supernatants did not inhibit the extent of incorporation in the 1-hour incubation plus hemin; only the 60-min preincubated postribosomal supernatant inhibited the extent of incorporation (Fig. 1A). However, in the minus hemin incubation (Fig. 1B), all three preincubated supernatants inhibited the extent of incorporation. Addition of nonpreincubated reticulocyte supernatants did not alter the kinetics of incorporation, which indicates that there is no detectable preformed repressor in these cells. The early rate of incorporation (first 6 min) are shown in Fig. 2. Both in the presence (Fig. 2A) and absence (Fig. 2B) of hemin, inhibition of the rate of hemoglobin synthesis was apparent after approximately 2 min in the presence of all three preincubated

1 The abbreviation used is: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
supernatant. Either addition of nonpreincubated reticulocyte supernatant, or water, gave identical control kinetics. It was confirmed, therefore, that preincubation for 5 to 30 min without hemin results in formation of a hemin-reversible translational repressor while preincubation of 1 hour results in hemin-irreversible repressor (15, 16).

**Translational Repressor Activity in Human Mature Erythrocyte Post-ribosomal Supernatants**—In Fig. 3, the effect of adding postribosomal supernatant from normal human mature erythrocytes is shown in the plus (Fig. 3A) and minus (Fig. 3B) hemin-incorporating systems. Human supernatant depressed the rate of incorporation from 2 to 10 min in the presence of hemin but after that time, the rate quickly increased. The extent of incorporation was often slightly greater in the presence of all the seven human lysates tested plus hemin. In the minus hemin incubation, both rate and extent were inhibited. When the supernatant was incubated at 37° for 2 hours minus hemin, irreversible repressor formed which depressed both rate and extent in the plus and minus hemin systems (Fig. 3). Incubation of the supernatant minus hemin for 15 and 30 min at 37°, however, did not significantly alter the kinetics from the non-preincubated pattern (data not shown).

In Fig. 4, the early kinetics of incorporation in the presence of fresh human supernatant is shown. After 2 min there was significant inhibition of the rate of incorporation in both the plus (Fig. 4A) and minus (Fig. 4B) hemin systems. Similar kinetic data were found with the three rabbit mature erythrocyte postribosomal supernatants tested (data not shown). However, since we were able to obtain similar activity in human cells, further isolation and characterization studies were all performed with human material.

In order to show that hemin prevents the formation of this irreversible repressor, human post-ribosomal supernatants were preincubated either in the presence of 35 µM hemin in Tris-Cl buffer, or with Tris-Cl buffer alone (minus hemin). The lysates were then diluted with deionized water, added to the cell-free lysate system, and assayed by measuring incorporation into hemoglobin at 1 hour at 34°. As shown in Fig. 5, no inhibition was found in human postribosomal supernatants diluted 10-fold which had been preincubated for 1 hour at 37° in the presence of hemin. Inhibitory activity was still identifiable at a 1:10 dilution in the sample incubated without hemin. Thus, it is clear that hemin retards the formation of the irreversible repressor. Further experiments of this type were performed with repressor isolated by pH 5 precipitation.

**Isolation of Repressor by pH 5 Precipitation**—When a non-preincubated supernatant was brought to pH 5 with acetic acid, hemin-reversible repressor was isolated in the precipitated proteins and separated from the bulk of hemoglobin (Fig. 6). Inhibition of the early rate of hemoglobin synthesis was apparent after 2 min in the plus hemin system, and after 1 minute in the minus hemin system. When this isolated hemin-reversible repressor was incubated in the absence of hemin for 1 hour, hemin-irreversible repressor formed (Fig. 6A). Hemin markedly retarded the conversion from the reversible to the irreversible form of the repressor (Fig. 7).

Irreversible repressor could also be isolated by pH 5 precipitation of supernatants preincubated minus hemin (Fig. 8).

**Fig. 3.** The effect of human mature erythrocyte postribosomal supernatants on hemoglobin synthesis in the rabbit reticulocyte cell-free system at 34°. A, incubation with 35 µM hemin; B, without hemin. ●—●, addition of 40 µl of water; △—△, 40 µl of fresh supernatant from nonpreincubated human mature erythrocytes; ■—■, 40 µl of human supernatant preincubated for 2 hours at 37°.

**Fig. 4.** The effect of fresh human erythrocyte postribosomal supernatant on the early kinetics of hemoglobin synthesis in the rabbit reticulocyte cell-free system at 34°. A, with 35 µM hemin; B, without hemin. ●—●, addition of 40 µl of water; △—△, 40 µl of fresh human erythrocyte supernatant.

**Fig. 5.** Hemin retardation of the formation of irreversible translational repressor in human mature erythrocyte postribosomal supernatants. Aliquots of a human supernatant were preincubated at 37° for 1 hour either in the presence of 35 µM hemin in Tris-Cl buffer or Tris-Cl buffer alone (without hemin). Aliquots of these preincubated supernatants were diluted with water as shown and 40 µl of these dilutions were added to the rabbit reticulocyte cell-free system plus hemin. Incubations were for 1 hour at 34°. The results are expressed as per cent stimulation over that of a control minus hemin incubation. A value of 100% is equal to the extent of incorporation in a control plus hemin incubation without added human supernatant. A value of 0% represents no stimulation above a control minus hemin incubation. ●—●, preincubation of supernatant minus hemin; △—△, preincubation with 35 µM hemin.
Fig. 6. Isolation of hemin-reversible repressor from human erythrocyte supernatants. Hemoglobin synthesis was measured in the rabbit reticulocyte cell-free system. A, incubation with 35 μM hemin; B, without hemin. The isolation of the repressor by pH 5 precipitation is given under “Experimental Procedures.” O—O, addition of 40 μl of water; △—△, 40 μl of repressor from non-preincubated human erythrocyte supernatant; ■—■, 40 μl of the same repressor preincubated at 37° for 1 hour minus hemin.

Fig. 7. Hemin retardation of the conversion of isolated hemin-reversible human erythrocyte repressor to the hemin-irreversible form. The reversible repressor was isolated by pH 5 precipitation from non-preincubated human erythrocyte post-ribosomal supernatant. O—O, isolated repressor preincubated for 1 hour at 37° minus hemin; △—△, the same repressor preincubated for 1 hour at 37° with 35 μM hemin. The results are expressed as in Fig. 5. Incorporation in this experiment was measured by isolation of globin (see “Experimental Procedures”). The 100% (plus hemin) value was 198,181 cpm. The 0% (minus hemin) value was 73,085 cpm.

Much less repressor was isolated if the supernatant was preincubated with hemin.

Inactivation of Repressor by Trypsin—Irreversible repressor isolated from a supernatant incubated at 37° minus hemin for 1 hour was diluted to a point where it inhibited the stimulation of hemin by 50%. This has been previously defined as 1 unit of inhibitory activity (10) and in this experiment represented a dilution of 10^5. Serial dilutions were made of this inhibitory unit and treated with trypsin to investigate the possibility that the human repressor is a protein. As is shown in Fig. 9, incubation of the repressor with trypsin (with subsequent addition of soybean antitrypsin) removed its inhibitory activity. When the trypsin-antitrypsin complex was added to either the control or to the repressor, there was no change. This provides evidence that the translational repressor from human cells is a protein.
Similar removal of inhibitory activity was achieved by bringing the repressor to the boiling point.

**Molecular Size of Human Translational Repressor**—The molecular weight of the human translational repressor was estimated with a Sephadex G-200 column. Irreversible repressor was isolated by pH 5 precipitation of a human supernatant incubated minus hemin for 2 hours at 37°. Reversible repressor was isolated by pH 5 precipitation of a nonincubated supernatant. The reversible repressor was assayed by conversion of the column fraction to the irreversible repressor and measuring incorporation for 1 hour at 34°; prior to this conversion, these fractions were not inhibitory. Both of the repressors were eluted similarly, at an elution volume just after the void volume of this Sephadex G-200 column. The molecular weight, therefore, is approximately 3 x 10^5. There was no inhibitory activity found in the fractions corresponding to low molecular weight components. Indeed, most fractions from this column showed stimulatory activity. The stimulatory activity, however, was not further investigated in the present study.

These results indicate that no low molecular weight component is required for repressor formation. Furthermore, it appears that there is no detectable size change in the transformation from the hemin-reversible to the irreversible form of the repressor. Previous estimates of the molecular size of the rabbit reticulocyte repressor have been 3 x 10^4 (17) using Sephadex G-200 and 5 x 10^4 (17, 18) using Sepharose 6B.

**DISCUSSION**

Evidence has been presented (9-12, 15-19) and confirmed in the present study that hemin exerts its control in the rabbit reticulocyte cell-free system by preventing the formation of a translational repressor. Reticulocyte lysates do not have pre-formed repressor but when the lysates are incubated in the absence of hemin, a hemin-reversible repressor first forms from a proinhibitor state (15, 16). Prolonged incubation then results in conversion to a hemin-irreversible repressor. It is clear, therefore, that hemin does not merely inhibit an inhibitor but it prevents the formation of an inhibitor. This implies that the reticulocyte depends upon an adequate supply of hemin in order to maintain globin synthesis. If hemin availability is diminished due to lack of heme synthesis, the translational repressor would form and stop protein synthesis.

One test of this hypothesis then would be the identification of this translational repressor in cells which have lost both heme synthesis and protein synthesis. Such a cell is the erythrocyte which in maturing from the reticulocyte, loses both mitochondria and protein synthetic capability. The identification of a hemin-reversible translational repressor in this cell is strong evidence that indeed this is a physiological control mechanism of globin synthesis.

Evidence has been presented in the present study that the human mature erythrocyte contains a protein translational repressor of approximately 3 x 10^4 molecular weight which is similar to that previously described in rabbit reticulocyte lysates (17, 18). This repressor is reversed by hemin and with prolonged incubations minus hemin is converted to a form that is hemin-irreversible. Hemin also markedly retards the formation of the irreversible form of the repressor. Examination of the early kinetics of globin synthesis shows that the human translational repressor does not inhibit significantly the rate of incorporation for the first 1 to 2 min at 34°. It is this portion of the synthesis curve which predominantly represents chain elongation (20). After this time period, the synthesis curve also reflects chain initiation and it is this portion of the curve that is most altered by the human repressor until the reversibility with hemin occurs. This provides evidence that the site of action of the human erythrocyte translational repressor is upon initiation. The stimulatory effect on extent of hemoglobin synthesis seen with the human supernatant and the fractions derived from them might result from factors that support initiation. However, the present study was not concerned with this stimulatory activity and its identity is still unknown.

It is apparent that hemin control would be a very sensitive way for cells to regulate their amount of protein synthesized. As the concentration of intracellular hemin would fall, either due to lack of iron or a defect in the heme synthetic pathway, the translational repressor would stop protein synthesis. Recently, evidence has been presented that hemin, or iron for hemin synthesis, is also required by reticulocytes (21, 22), Krebs II ascites tumor cells (21), and platelets (22) for non-globin protein synthesis. It is possible, therefore, that hemin via prevention of the activity of a translational repressor is a universal requirement in mammalian protein synthesis. Further experiments are necessary in other cell types to confirm this hypothesis.

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