EVIDENCE FOR AN INHIBITOR IN THE CONTROL OF GLOBIN SYNTHESIS BY HEMIN IN A RETICULOCYTE LYSATE

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SUMMARY. The synthesis of hemoglobin by a reticulocyte lysate is stimulated several fold by the addition of hemin. The ability to respond to hemin is completely lost if the lysate is held for a short time at 34°C, but only slightly reduced if held at 0°C. Hemoglobin synthesis by reconstituted lysates prepared by combinations of ribosomal and supernatant fractions from hemin-responsive and -unresponsive lysates indicated that the factor mediating hemin stimulation is in the supernatant fluid. Addition of supernatant fraction from an unresponsive lysate to an incorporating system containing a responsive lysate markedly decreased the stimulation of globin synthesis by hemin. These results suggest that hemin promotes globin synthesis by preventing the formation of an inhibitor of chain initiation in the cell sap.

Hemin is known to be a factor which controls globin synthesis in erythroid cells. It also appears to be involved in the mechanism for initiation of hemoglobin synthesis in the developing embryo (1, 2). In reticulocytes, iron deficiency brings about a disaggregation of polyribosomes, a change which may be prevented by the addition of hemin as well as iron (3, 4, 5). This has been reported (3, 5) to be inconsistent with proposed mechanisms in which hemin had a role in releasing completed chains from polyribosomes. Furthermore, studies with radioactive iron (6) or hemin (7, 8) showed that hemin is not associated with nascent peptide chains attached to polyribosomes, and suggested that it may function in chain initiation (7).

Hemin has been shown to increase the extent of globin synthesis in cell-free preparations from pigeon erythrocytes (9) and rabbit reticulocytes (10, 11). A common feature of these stimulations is the marked decrease in response to hemin when it is added a short time after the start of incubation. On storage, the ability of a

reticulocyte lysate to be stimulated by hemin is lost much more rapidly than is the ability to synthesize protein in the absence of added hemin (10, 11). By comparing the properties of lysates which could be stimulated by hemin with those of heated lysates which could not, we have partially characterized the mode of action of hemin in promoting globin synthesis.

METHODS

The general procedure, with minor modifications, was that described by Adamson, et al. (10). Washed reticulocytes from a phenylhydrazine treated rabbit (5) were lysed with an equal volume of water and the lysate freed of debris by centrifugation for 15 min. at 25,000 × g. The lysate was used immediately or rapidly frozen in small portions and stored at -80°C. These portions could be stored for 2-3 weeks with but small losses in response to hemin. They were used immediately after thawing. The protein synthesizing system consisted of 10 μl of a hemin solution,[a] 60 or 70 μl of lysate and 40 or 30 μl of master mix,[b] added in this order. The ribosome concentrations in representative lysates were determined spectrophotometrically after pelleting and resuspending the ribosomes, with the use of an extinction coefficient at 260 mμ of 12.0 O.D./mg/ml, (ref. 15).

Incubations were carried out for 90 minutes at 34°C. At this time incorporation of labeled leucine was essentially complete. Twenty-five μl samples were used

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[a] Three to six mg of recrystallized hemin were dissolved in 3 ml of 0.1 N NaOH, 0.6 ml of 1 M Tris buffer, pH 7.4, was added, and the solution was neutralized with 3 ml of 0.1 N HCl. This was centrifuged for 10 min. at 2000 × g and assayed spectrophotometrically (12). The solution was diluted to the desired concentrations with an equivalent NaCl-Tris buffer.

[b] The master mix contained the following ingredients in amounts to yield the indicated final conc.: KCl (75 mM); $MgCl_2$ (2 mM); ATP (1 mM); GTP (0.2 mM); mercaptoethanol (6 mM); creatine phosphate (15 mM); creatine kinase (45 E.U./ml); L-leucine-1-C^{14} (0.3 mM, 5 μC/μM) and 18 other C^{12}-L-amino acids. The composition of the amino acid mixture was the same as that reported for rabbit hemoglobin (13, 14).

for assay of the radioactivity incorporated into hot CCl_3COOH insoluble material retained on Millipore filters. These were counted in a Nuclear-Chicago gas flow counter with Micromil window (eff. $\approx$ 30%).

RESULTS

Preliminary investigations confirmed the earlier reports (10, 11) that reticulocyte lysates lose the ability to be stimulated by hemin much more rapidly than they lose the ability to synthesize globin in the absence of added hemin. These rates of loss increase with temperature and are characteristic of individual preparations. Therefore an initial test was often necessary to determine the time required for complete loss of hemin stimulation. In order to ascertain whether the heat labile factor responsible for hemin stimulation is associated with the ribosomes, the soluble fraction or both, ribosomal and supernatant fractions from responsive and unresponsive lysates were interchanged and the synthesizing capacities of the mixtures were determined (Table I). The labile component was found to be in the soluble fraction.

The inability of the soluble fraction from an unresponsive lysate to support hemin stimulation of globin synthesis by ribosomes from a responsive lysate may be due either to the loss of a labile factor or to the production of an inhibitor. Addition of supernatant fraction from an unresponsive lysate to an incorporating system containing a responsive lysate should distinguish between these two possibilities. If such a supernatant fraction had lost a labile factor it should act merely as a diluent and its effect on hemin stimulation would be no worse than an equivalent dilution with water. However, as is shown in Fig. 1, dilution of a responsive system with supernatant fraction from an unresponsive lysate markedly decreased the stimulation of globin synthesis by hemin. In contrast, no inhibition was observed on equivalent dilution with either water or a supernatant fraction from a responsive lysate. The results indicate that the supernatant fraction of an unresponsive lysate contains an inhibitor of protein synthesis which is formed or activated on incubation.

TABLE I

Hemin Stimulation of Globin Synthesis by Original and Reconstituted Lysates

	Hemin Concentration 0	30 μM	
	Leucine Incorporation		Stimulation
	cpm	cpm	per cent
Lysate (0^{o})	908 [a]	5828	540
Lysate (34^{o})	552	546	0
R (0^{o}) + S (0^{o})	832	4710	470
R (34^{o}) + S (0^{o})	657	2605	300
R (0^{o}) + S (34^{o})	512	517	0
R (34^{o}) + S (34^{o})	461	502	10

The ribosomes from two 1 ml portions of a lysate held at 0^{o}C for 45 min. and two 1 ml portions of a lysate held at 34^{o}C for 45 min. were pelleted by centrifugation for one hour at 56,000 RPM in a Spinco SW56 rotor. Centrifugation was performed at 4^{o}C in tubes cut down to 3 mm above the surface of the liquid to prevent collapse of the tubes. The small volumes permitted a short settling time essential for studies where inactivation is occurring. The tubes were removed from the swinging buckets by a special extractor (description in preparation). The supernatant fluids were decanted and held in ice and the tubes containing the ribosomes were swabbed with a cotton applicator. The supernatant fluids were poured back onto the unwashed ribosomal pellets in the combinations indicated above. The ribosomes were resuspended with a glass rod and portions from these reconstituted lysates and the original lysates were tested for protein synthesizing capacity in the absence and presence of hemin. Supernatant fluids had no capacity to synthesize protein in the absence of added ribosomes.

(0^{o}), (34^{o}): temperatures at which lysates were held.
R, ribosomal fraction; S, supernatant fraction, from corresponding lysates.

[a] 1000 cpm represents the incorporation of 23 leucine molecules per ribosome. This was calculated with the use of an average value, 2.5 ± 0.5 mg/ml, for the concentration of ribosomes and assuming that dilution of added leucine by the endogenous amino acid was negligible.

Recently, the presence of a ribonuclease which is inhibited by hemin was demonstrated in reticulocyte lysates (16, 17). The purified enzyme, at a concentration

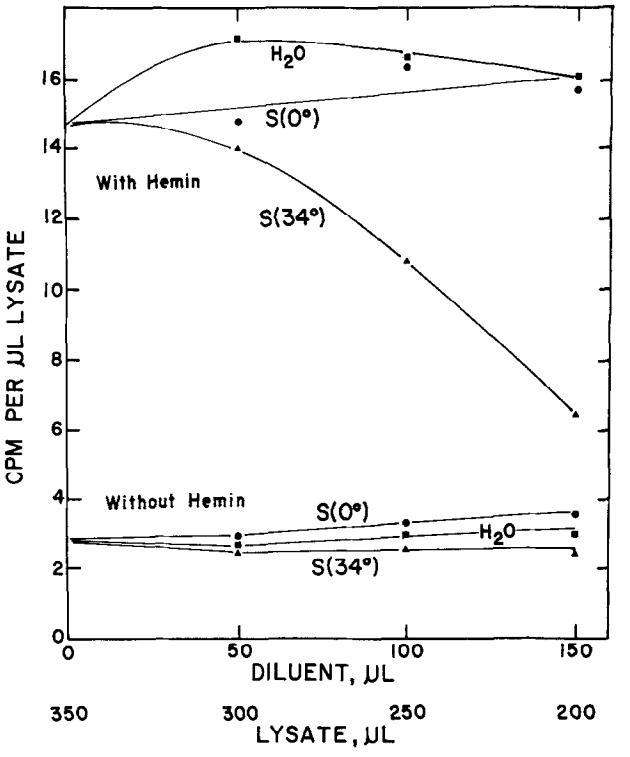

Figure 1. Effect of Diluents on Hemoglobin Synthesis by a Reticulocyte Lysate.

A hemin-responsive lysate was diluted (as shown in the abscissa) with water; a ribosome-free supernatant fraction, S (0^{o}), from a responsive lysate or a ribosome-free supernatant fraction, S (34^{o}), from an unresponsive lysate. A 150 µl aliquot of a master mix was added to each of these diluted lysates and 100 µl aliquots of these mixtures then added (in duplicate) to 10 µl of a buffer (with or without hemin—final hemin concs. 0 or 30 µM). Protein synthesis was measured as described in Methods. The supernatant fractions were prepared as described in the legend of Table I. Control incubations with supernatant fractions and no lysate showed no incorporation. The ordinate reflects the specific activity of the undiluted lysate.

comparable to that found in lysates, completely degraded washed polyribosomes in one hour at 37^{o}C, (ref. 17), and it was suggested that hemin may promote globin synthesis by inhibiting the destruction of polyribosomes (16, 17). Therefore, a comparison was made of polyribosome profiles of lysates before and after a comparable incubation (Fig. 2). No evidence for disaggregation of polyribosomes was observed when lysates were incubated for two or three times as long as necessary for destruc-

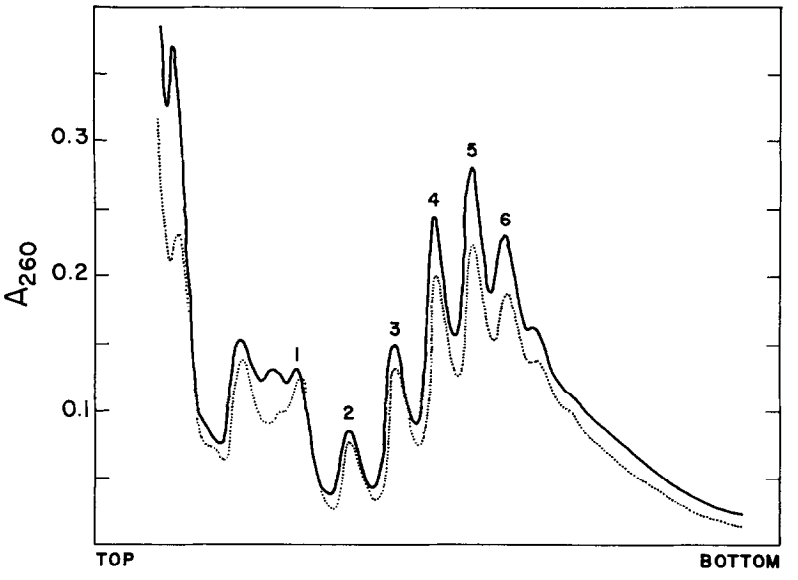

Figure 2. Effect of Preincubation on the Polyribosome Profile of a Lysate.

Lysates, 250 µl, diluted with 750 µl of buffer, were layered on 15→30% linear sucrose gradients and centrifuged at 4°C and 25,000 RPM for 3 hours in a Spinco SW25.1 rotor. The tubes were punctured and the gradient displaced upward with a 50% sucrose solution delivered by a motor-driven syringe. They were monitored continuously at 260 mµ with a 5 mm Oak Ridge flow cell and a Gilford spectrophotometer. Broken line, (••••••), lysates held for 60 min. in ice after thawing, 360% hemin stimulation. Solid line, (———), lysates incubated for 60 min. at 34°C after thawing, no hemin stimulation. Concurrent experiments showed that the hemin response of this lysate was completely destroyed by a 30 min. incubation.

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tion of the hemin-mediated stimulation. This observation, together with the fact that 30 µM hemin, which we observe to be maximal for hemin stimulation, inhibits ribonuclease activity by only 25 to 30% (16, 17), makes it highly unlikely that the role of hemin in this stimulation is principally that of a ribonuclease inhibitor.

DISCUSSION

These results indicate that hemin stimulates globin synthesis by preventing the formation of a soluble inhibitor. Since hemin is considered to promote chain initiation (7, 11), the inhibitor formed in its absence is most likely an inhibitor of this step in protein synthesis. It is well known that globin chains are unstable in the absence of

hemin, and it has been recently reported that aberrant α-chains are formed in hemin-deficient reticulocytes (18). A model consistent with reports in the literature and our observations is that globin molecules, modified in the absence of hemin, inhibit chain initiation. This model suggests that hemin may play but a single role in hemoglobin synthesis; its combination with globin being both a step in the formation of hemoglobin and an act in preventing the formation of an inhibitor, the modified globin. Further experiments are in progress to characterize this system.

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